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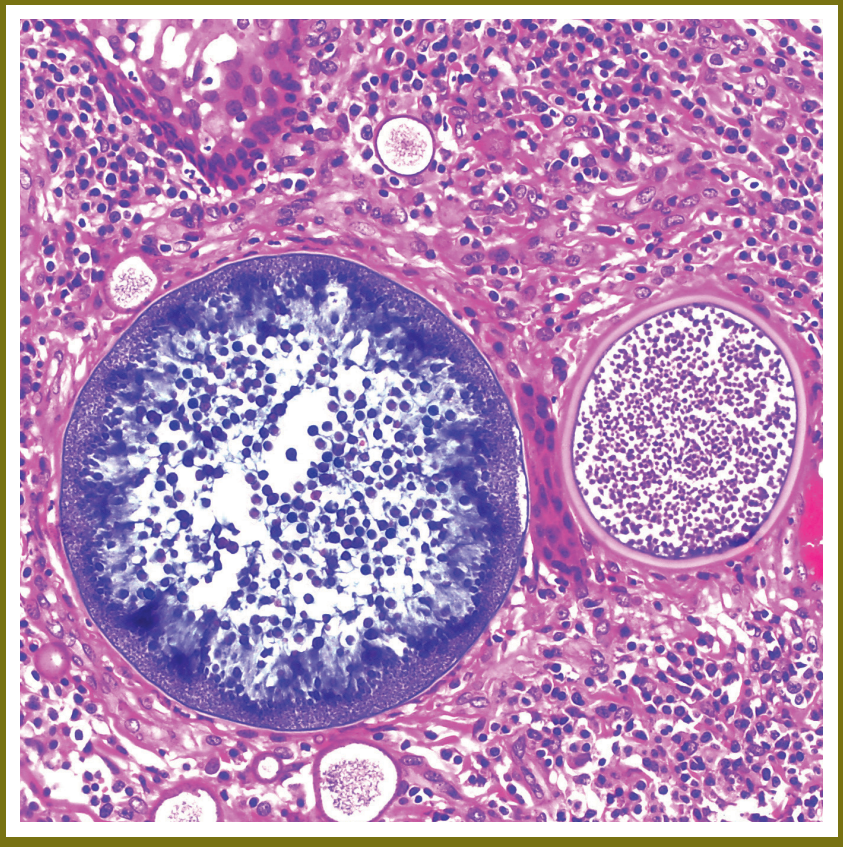

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Figura da capa: Esporângios maduros de *Rhinosporidium seeberi* em equino com rinosporidiose. HE, obj.20x. (Argenta et al., p. 2214)

Cover illustration: *Mature* Rhinosporidium seeberi *sporangia in a horse with rhinosporidiosis. HE, obj.20x.* (Argenta et al., p. 2214)

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Original Article
Animais de Produção/*Livestock Diseases*

Expression patterns of mesenchymal stem cell-specific proteins in adipose tissue-derived cells: possible immunosuppressing agent in partial allograft for restoring the urinary bladder in rabbits[1]

Saulo T.L. Pinto Filho[2]*, Micheli M. Pillat[3], Matheus P. Rosa[2], Fabíola Dalmolin[4], Henning Ulrich[3] and Ney L. Pippi[2]

ABSTRACT. Pinto Filho S.T.L., Pillat M.M., Rosa M.P., Dalmolin F., Ulrich H. & Pippi N.L. 2018. **Expression patterns of mesenchymal stem cell-specific proteins in adipose tissue-derived cells: possible immunosuppressing agent in partial allograft for restoring the urinary bladder in rabbits**. *Pesquisa Veterinária Brasileira 38(12):2183-2189*. Hospital Veterinário Universitário, Universidade Federal de Santa Maria, Av. Roraima 1000, Prédio 97, Santa Maria, RS 97105-900, Brazil. E-mail: saulovet2011@hotmail.com

Adipose tissue-derived stem cells (ADSCs) are an attractive source of mesenchymal stem cells (MSCs) for use in tissue engineering and clinical applications. This paper focuses on the characterization of ADSCs used as immunosuppressive agent in rabbits undergoing partial allograft for urine bladder restorage. For this study highlighted the characterization of the ADSCs used as immunosuppressive agents in rabbits submitted to partial allograft for restoration of the urinary vesicle, using 25 animals, six months old, New Zealand. ADSCs at the third peal were characterized by the MSC-specific CD105, CD73 and CD90 expression and by the absence of the hematopoietic marker CD45, as revealed by flow cytometry analysis. Moreover, ADSCs were efficient in preventing allograft rejection from the urinary bladder, as judged by biochemical, clinical and ultrasonography analysis. Together, these results compose characterization of protein expression profiles and immunosuppressive functionality of ADSCs in rabbits, which had undergone partial allografts of the urinary bladder, foreseeing future applications in clinical practice.

INDEX TERMS: Expression patterns, mesenchymal stem cell, proteins, adipose tissue, derived cells, immunosuppressing agent, partial allograft, restoration, urinary bladder, rabbits, ADSC, cellular therapy, flow cytometry, clinics.

RESUMO.- [**Padrão de expressão de proteínas específicas das células-tronco mesenquimais derivadas de tecido adiposo: possível agente imunossupressor em aloenxerto parcial para restauração de vesícula urinária de coelhos.**] As células mesenquimais derivadas de tecido adiposo (ADSCs) são uma fonte atraente de células-tronco mesenquimais (MSCs) para uso na engenharia de tecidos e suas aplicações clínicas. Este trabalho destacou a caracterização das ADSCs utilizadas como agentes imunossupressores em coelhos submetidos a aloenxerto parcial para restauração da vesícula urinária, sendo utilizados 25 animais, de seis meses de idade, Nova Zelândia. As ADSCs, após o terceiro repique, foram caracterizadas pela expressão específica de MSC CD105, CD73 e CD90 e pela ausência do marcador hematopoiético CD45, tal como revelado por análise de citometria de fluxo. Além disso, os ADSCs foram eficientes na prevenção da rejeição de aloenxertos da vesícula urinária, conforme avaliado por análises clínica, bioquímica e ultrassonográfica. Juntos, esses resultados compõem a caracterização dos perfis de expressão proteica e a funcionalidade imunossupressora de ADSCs em coelhos, que sofreram aloenxertos parciais da bexiga, prevendo futuras aplicações na prática clínica.

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TERMOS DE INDEXAÇÃO: Padrão de expressão, proteínas específicas, células-tronco mesenquimais, tecido adiposo, agente imunossupressor, aloenxerto parcial, restauração, vesícula urinária, coelhos, terapia celular, citometria de fluxo, clínica.

INTRODUCTION

The bladder is an organ of complex structure that can store large volumes, while simultaneously maintaining the pressure low between mictional periods. Injuries resulting from tumors, traumas, infections, inflammatory or neurological diseases affect their functional properties, leading to loss of continence and problems with urine storage, therefore damaging, sometimes in an irreversible way, the renal function. Thus, the great challenge, when one considers replacing the bladder, is to try to maintain or recreate its properties (Leite et al. 2014). Tissue engineering is a complex alternative to the functional reconstruction of the bladder. The culture of urothelial cells remains technically challenging, while muscle cells, of relatively simple culture, may not be easily obtained, mainly in cases requiring total or partial bladder replacement, such as cloacal or bladder exstrophy, and tumors. In this context, allotransplantation and stem cells may be excellent alternatives.

In this sense, Teixeira et al. (2007) studied urinary bladder allotransplantation in dogs by partial cystectomy preserving the trigone, using cyclosporine as an immunosuppressive agent. The authors concluded that urinary bladder allotransplant in dogs is viable, promoting repairing of urinary bladder capacity and other physiologic functions and resulting in partial regeneration of tissues 60 days after surgery.

At the same time, mesenchymal stem cells (MSCs) have been hailed as the great hope for the transplantation medicine, because they have been shown to be both multipotent and immunosuppressive (Technau et al. 2011, Larocca et al. 2013, Leite et al. 2014). The most common source of stem cells is the bone marrow. However, harvesting of bone marrow cells is a painful procedure with low yield of MSCs. These limitations have led to the search for alternative ways of obtaining stem cells, such as harvesting of undifferentiated mesenchymal cells from other tissues. In this context, adipose tissue-derived stem cells (ADSCs) are an attractive source of MSC for use in clinical applications. These cells have excellent plasticity and proliferative potential, undergo long-term proliferation, and are isolated in large quantities from adipose tissue using minimally invasive surgical procedures. In addition, they are able to grow by adherence to plastic or glass, making their isolation easy in the laboratory (Torres et al. 2007). Similar to other types of MSCs, it is well known that human ADSCs express the surface markers CD73, CD44, CD90 and CD105, but not the hematopoietic lineage markers CD11c, CD31, CD34, CD45, CD80 and CD86 (Dominici et al. 2006, Tárnok et al. 2010, Nery et al. 2013, Sousa et al. 2014).

Human ADSCs have been isolated from lipoaspirates and extensively characterized, however, ADSC from animal models, as such rabbits, are not well established and characterized. Rabbits have a large amount of adipose tissue and a used in basic science for different purposes for many years. Regarding tissue engineering approaches, rabbit animal models have been used for urethral, cartilage, and vocal fold reconstruction (Barretto et al. 2014).

This paper focuses on the characterization of expression profiles of clusters of differentiation (CDs) in rabbit ADSCs used as immunosuppressive agent in rabbits undergoing urinary bladder allografts. Moreover, we hypothesized that therapeutic application of rabbit ADSCs in partial allograft of urinary bladder could produce beneficial clinical results, since ADSCs could render the use of immunosuppressive drugs, as such cyclosporine, unnecessary.

MATERIALS AND METHODS

Ethics statement. Institutional review board approval of animal housing and experimental protocols was granted by the Committee of Ethics and Animal Use of the Federal University of Santa Maria, Brazil (protocol 066/2011).

Animals. Twenty-five healthy long-eared, six-months-old New Zealand White rabbits, weighing 3.73±0.88kg were obtained from central animal facility of the Federal University of Santa Maria. Twenty-four females were used for development of urinary bladder transplantation, while a single male served as donor of ADSCs. The rabbits were housed in individual cages, inside a standard laboratory environment during two weeks of adaptive feeding before starting the surgeries. Animals were identified and separated in two groups (control group - CGcy and stem cell treatment group - CGst), with 12 animals each one, according to the type of treatment. The groups were subdivided in two subdivisions with six animals each. All subgroups were evaluated following 15 and 30 days. After surgery transplantation surgery, the CGcy group was treated with cyclosporine (5mg/kg^{-1}), i.v., every 24 hours until the end of the studies. The CGst group received 1,6x10^5 ADSCs (0.8ml), processed and multiplied in our laboratory, used after the third passage. The cells were applied into the urinary wall, laterally to the suture between the graft and urinary bladder.

Clinical observations (feeding and urination behavior) were performed from the time of surgery to the end of the evaluation period for each group. Samples were collected for laboratory analysis, including blood and platelet count, BUN (Urea Nitrogen) and creatinine dosage, ALT (alanine aminotransferase), AST (aspartate aminotransferase), ALP (alkaline phosphatase) and urinalysis. Ultrasound evaluation was performed for bladder monitoring. Animals were submitted to euthanasia according to the time table of evaluation of each subgroup (15 days or 30 days).

Isolation and culture of rabbit ADSCs. For harvesting adipose tissue (AT) and processing of ADSCs, a male donor rabbit underwent the surgical procedure of lipectomy. Premedication consisted of intramuscular (i.m.) injection of a cocktail of ketamine hydrochloride (20mg/kg^{-1}), midazolam maleate (2mg/kg^{-1}) and morphine sulphate (5mg/kg-^{1}). Induction and maintenance of anaesthesia with isoflurane were done through the use of a mask. Subcutaneous AT in the interscapular adipose bag was removed under sterile conditions.

After washing with Hank's balanced salt solution (Sigma-Aldrich, St Louis, EUA) and removing AT membranes and blood vessels, ADSCs were isolated using the single collagenase digestion method. The AT were placed in 100mm^3 culture dishes, and digested for 30 min with 16ml of 0.1% type II collagenase (Sigma-Aldrich, St Louis, EUA) in a 37°C in water bath. The reaction was terminated by adding an equal volume of Dulbecco's Modified Eagle Medium (DMEM, Gibco, Grand Island/NY). Cells were filtered through a 200µm-mesh nylon screen to remove undigested tissues. The lipid layer and supernatant were discarded after centrifugation. Cells were washed with PBS, and DMEM (containing 10% foetal bovine serum (FBS, Gibco, Grand Island/NY) was added to obtain a single-cell suspension

solution. Cells were then inoculated into 75cm^2 cell culture flasks and incubated in a 37°C, 5% CO_2 cell culture incubator. Non-attached cells were discarded after 24 hours, and medium was replaced after 48 hours; after that, the culture medium was changed every other day (Zuk et al. 2002).

As a continuation of this study, a male rabbit was used to check the presence of the cells at the implantation site in the rabbits to search the Y chromosome, through SRY gene expression by PCR analysis.

Flow cytometry. Third peal cells in suspension were incubated for 20 min in PBS containing 3% FCS for blocking of nonspecific sites. Subsequently, cells were incubated with anti-CD105 PerCPCy5.5 (1:5), anti-CD90 PE (1:5), and anti-CD45 FITC (1:20) or anti-CD105 PerCPCy5.5, anti-CD90 PE, and anti-CD73 FITC (1:10) antibodies at dilutions according to the manufacturer procedures (BD bioscience). Although there were commercially validated antibodies to rabbits, they were not found in the suppliers and therefore, those of humans. After 30 min of incubation with the antibodies at room temperature, cells were washed with PBS and resuspended in 400ml PBS for data acquisition with the BD FACS flow cytometer (Becton and Dickinson Biosciences). A minimum of 30.000 cells per sample were analysed and gated for forward scatter (FSC) versus side scatter (SSC) channel signals. Analyses were performed with the FlowjoV10 software. The negative control sample consisted of cells not exposed to antibody labelling. From this sample traced to the quadrants, events observed beyond the lower left quadrant (containing the negative sample) were considered positive (Pillat et al. 2009).

Bladder transplant procedure. Anaesthetized animals were operated in pairs, with simultaneous procedures at the same operating room and each female rabbit was donor and recipient of bladder. The urine was removed, and approximately 30% of the bladder was excised at the cranial portion, leaving repairs points at the remaining portion. After the end of implant suture and bladder repletion test, ADSCs were placed as explained above.

Immediately after surgery, animals were monitored until complete restoration of consciousness and then returned to their individual cages. Tramadol hydrochloride was administered (6mg/kg^{-1}), i.m., every eight hours for three days. Cleaning of the wound was carried out with 0.9% NaCl solution once every two days. Skin sutures were removed seven days after surgery. During this time animals were clinically evaluated in order to identify any abnormalities related to the surgical procedure as well as urinary evaluation, related to the urine production and urine aspects.

Statistical analysis. Microsoft Office Excel software for Windows 7 was used to calculating average and paired t-test were used for the statistical analysis, considering significant differences at $p<0.05$.

RESULTS

In the current study, cell adherence of lipoaspirate fractions to the plastic culture flasks was observed. Fibroblastoid cell morphology was also noted after third peal in culture (Fig.1). As for size and granularity, a homogeneous cell population was observed (Fig.2A).

As a second step, the frequency of these cell populations, derived from adipose tissue of rabbits, expressing MSC marker proteins were determined by flow cytometry. It is well known that MSCs express CD90, CD105 and CD73 while not express CD45. Using the negative control sample gates were traced in the SSC versus FL3 and FL1 versus FL2 dot-plots (Fig.2B,D). Thus, flow cytometry analyses revealed that 88.0% of cells express CD105 (Fig.2C,G), 73.7% express CD90 (Fig.2E,G), 71.9% express CD73 (Fig.2F,G),

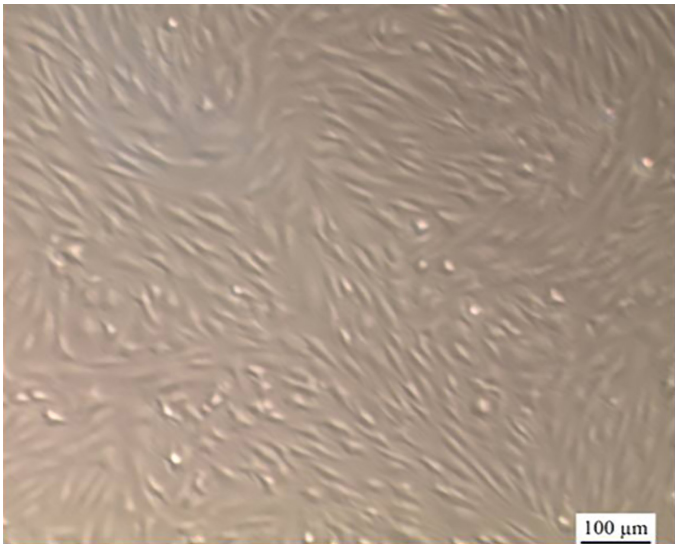

Fig.1. Morphological and cytometric characterization of mesenchymal stem cells (MSCs) derived from adipose tissue of rabbits. Fibroblastoid morphology observed after 10 days of culture. Bar = 100µm.

and 87.6% do not express CD45 (Fig.2E,G). Moreover, flow cytometry analyses revealed that when cells were triply labeled with CD105, CD45 and CD90 antibodies, 62.7% of cells were simultaneously $CD105^+CD45^-CD90^+$, i.e., 62.7% of cells express MSC marker proteins and do not express hematopoietic marker protein (Fig.2C,E). As expected, when cells were triply labeled with CD105, CD73 and CD90 antibodies, 60.5% of cells were $CD105^+CD73^+CD90^+$ (Fig.2C,F). These results demonstrate that majority cells in culture derived from adipose tissue of rabbits simultaneously express MSC marker proteins.

Both treatments, CGcy and CGst, reached satisfactory clinical results and prevented allograft rejection. All animals had satisfactory recovery, with no clinical or behavioral change as a result of treatment throughout the postoperative evaluation period. Animals were monitored daily and at no time showed alterations were noted regarding food and water intake. Signs of peritonitis, caused by suture dehiscence of bladder or rejection of the implant, did not occur in any animal until 30 days of observation. A single dose of ADSCs applied in CGst demonstrated immunosuppressive effect, avoiding clinical graft rejection during the evaluation period and did not cause any adverse effect as a result of transplantation.

The majority of laboratorial tests performed in this experiment did not reveal significant differences between times before and after surgery (Fig.3). Serum biochemical (renal and hepatic) parameters were at physiological reference standards for rabbits during the evaluation time. Clinical reference ranges for rabbits were published by Melillo (2007) and are shown as dotted line. The ALT, AST, and BUN in the blood did not change in CGst compared to CGcy considering clinical preoperative (PO) and postoperative (30D; 30 days). There was significant difference in ALP level between CGcy and CGst, however, they remained within the physiological limits (Melillo 2007).

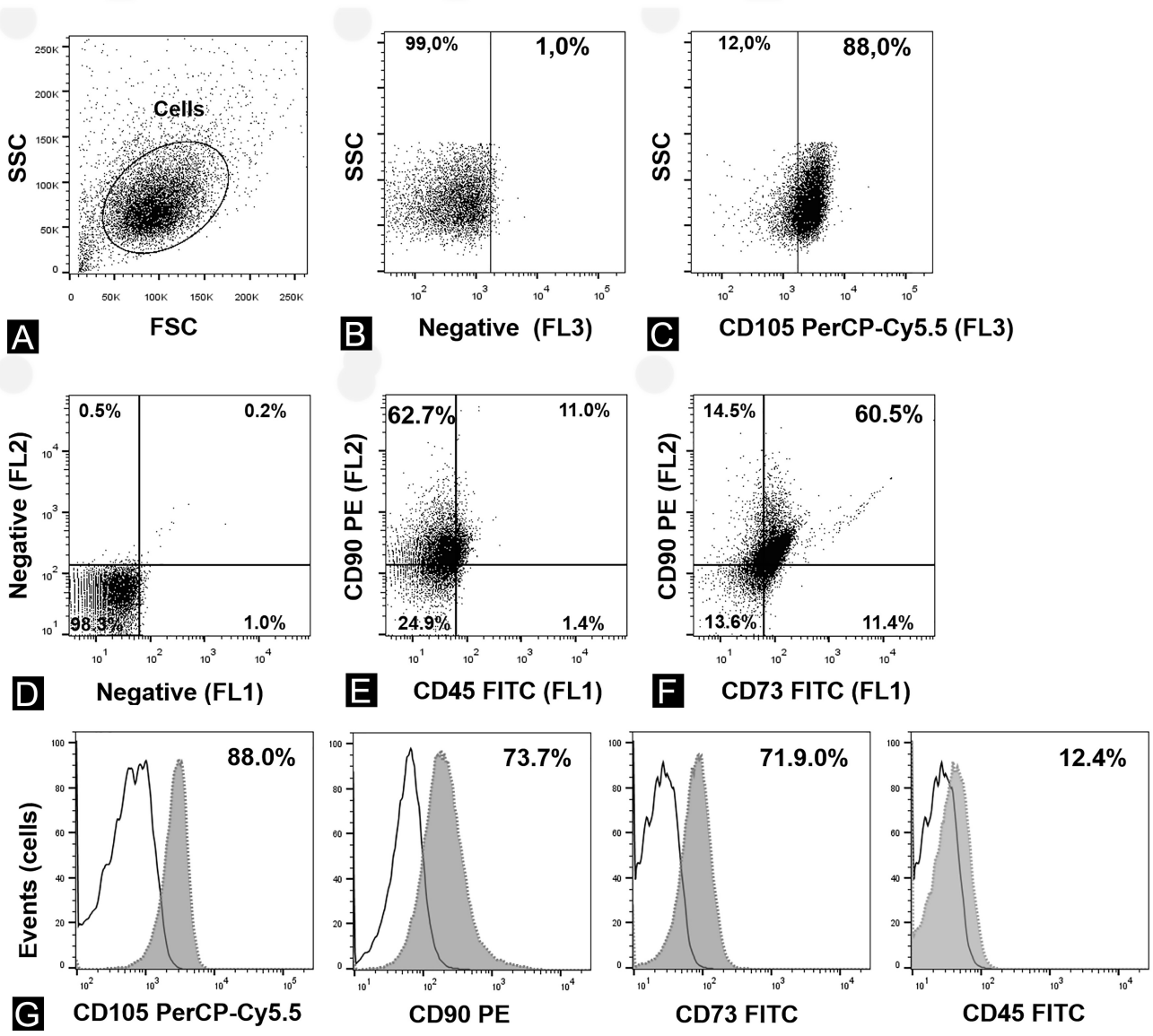

Fig.2. Phenotypic characterization of mesenchymal stem cells (MSCs) derived from adipose tissue of rabbits. (**A**) Gate selection by size (FSC, forward scatter) and granularity (SSC, side scatter) of the cells. Note the sample homogeneity with respect to these characteristics. (**B**) Using the negative control sample a gate was traced in the SSC versus FL3 dot-plot. Events observed in the right field were considered positive. The negative control sample consisted of cells not exposed to antibody labelling. (**C**) Flow cytometry dot-plot showing the percentage of cells expressing CD105 in culture derived from adipose tissue of rabbits. Anti-CD105 antibody was conjugated with PerCP-Cy5.5. (**D**) FL1 versus FL2 dot-plot showing the gate of the negative control sample in dot-plot, used for tracing the quadrants. Events observed beyond the lower left quadrant (containing the negative sample) were considered positive. (**E**) Dot-plot showing percentage of CD90+ (PE fluorophore) and CD45- (FITC fluorophore) cells derived from adipose tissue of rabbits after third passage in culture. (**F**) Dot-plot showing percentage of CD90+ (PE fluorophore) and CD73+ (FITC fluorophore) cells derived from adipose tissue of rabbits after third passage in culture. (**G**) Histograms showing individual data of frequency of CD105, CD90, CD73 and CD45 of mesenchymal stem cells (MSCs) derived from adipose tissue of rabbits.

Ultrasonography evaluation of urinary bladder after surgery of most animals (CGcy and CGst) showed similarity to the pre-operative state, considering integrity, thickness of the wall and content. Bladders in the pre- and post-operative examination revealed heterogeneous content, moderate cellularity, regular wall throughout its length, with no evidence of leakage of urine, a consistent finding of normal rabbit urinary bladder.

DISCUSSION

According to the International Society for Cellular Therapy, there are three minimum requirements for a population of cells to be classified as MSCs. The first is that MSCs are isolated from a population of mononuclear cells based on their selective to the plastic surface, when in culture. The second is that in more than 95% of the cultured cells, expression of CD105,

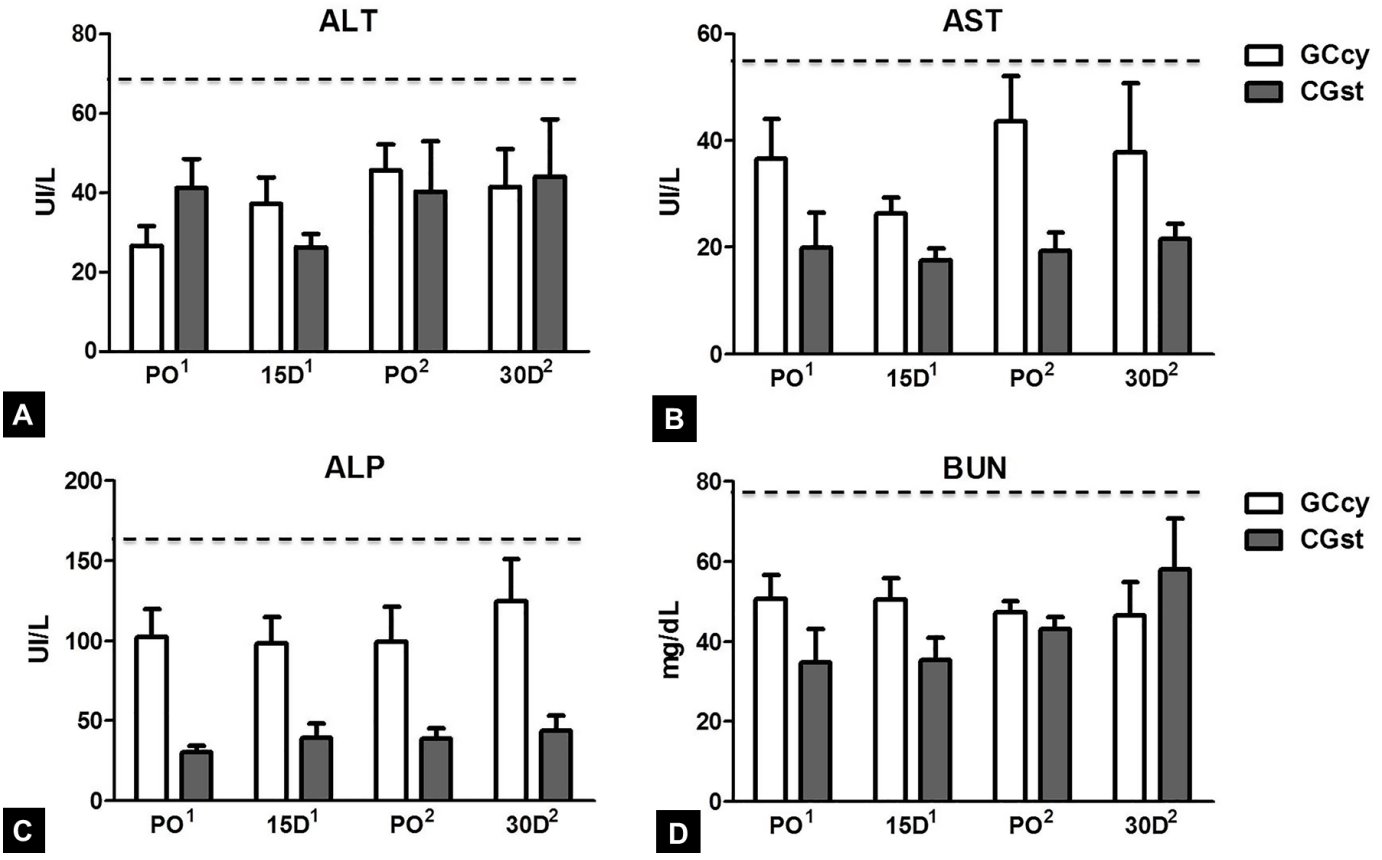

Fig.3. Clinical preoperative (PO) and postoperative (30D, 30 days) parameters of (**A**) ALT, alanine aminotransferase; (**B**) AST, aspartate aminotransferase; (**C**) ALP, alkaline phosphatase; (**D**) BUN, urea nitrogen in New Zealand White rabbits subjected to urinary bladder partial allograft. CGcy = Control Group treated with cyclosporine (5 mg/kg^{-1}) i.v., every 24 hours; CGs = group received 0.8ml ADSCs (1,6 x 10^5 cells). The cells were applied once into the urinary wall, laterally to the suture between the graft and urinary bladder. Clinical reference ranges for rabbits were published by Melillo (2007). Limits are shown as dotted lines. The data are representative for two independent experiments conducted as biological triplicates and shown as mean ± standard deviations (*P<0.05 versus CGcy, **p<0.01 versus CGcy). [1] Subgroup 15d, [2] Subgroup 30d (euthanasia).

CD73 and CD90 can be detected, while CD34, CD45, CD14 or CD11b, CD79, or CD19 and HLA-DR are not expressed. Finally, the cells need to be able to differentiate into bone, fat and cartilage (Bydlowski et al. 2009). In the current study, cell adherence to plastic culture flasks as well as fibroblastoid cell morphology identified cells used for allografts as MSCs (Torres et al. 2007, Bydlowski et al. 2009, Monteiro et al. 2010, Carvalho et al. 2013, Xu et al. 2015). Although have been identified eight surface markers for identifying MSCs, the International Society for Cellular Therapy agrees that the identification of markers CD105, CD73 and CD90, the absence of hematopoietic markers (CD45, for example), is sufficient for MSC immunophenotyping (Monteiro et al. 2010), as performed in our study. Similar with Dominici and co-workers (Dominici et al. 2006), the frequencies of MSC-specific CD105, CD73 and CD90 expressions and the absence of the hematopoietic marker CD45, revealed by our study, were more than 75% of the cultured cells. Possibly our data are a little lower than Dominici and co-workers due to differences in specificity of the antibodies, but they are sufficient to suggest the presence of MSCs.

No signs of peritonitis were observed caused by dehiscence of urinary bladder suture or rejection of the implant in any animal. Cyclosporine, used in the CGcy, is preferred by some researchers as an immunosuppressive agent in transplanted animals (Teixeira et al. 2007, Chaves et al. 2008). Pinto Filho et al. (2015) used cyclosporine to prevent rejection for 30 days after partial cystectomy surgery in rabbits and found no signs of implant rejection in all treated animals. Chaves et al. (2008) verified that cyclosporine prolonged survival of skin grafts in rabbits. Brandt et al. (2004) used azathioprine for 15 days in dogs as immunosuppressive agent in a study of bladder allograft and verified no signs of implant rejection during 18 months of evaluation. In our studies, ADSCs, applied in a single dose, demonstrated their immunosuppressive features, avoiding graft rejection during the period of clinical evaluation. It is unchallengeable that the immunomodulatory and/or immunosuppressive effect of ADSCs was consistently observed in several studies (Mizuno 2009, Casteilla et al. 2011, Lin et al. 2012, Gutiérrez-Fernández et al. 2013). According to Lin et al. (2012) ADSC's immunosuppressive activity appears to be mediated through an interleukin-6 (IL-6)-dependent inhibition of dendritic cell differentiation

and downregulation of MHC-II, CD40, and CD86 on mature dendritic cells. The same authors demonstrated *in vitro* that ADSCs do not cause alloreactivity, as they do not express MHC-II on their surfaces. However, they suppress the activity of T and B lymphocytes, an important feature for the treatment of organ allograft rejection and autoimmune disorders. In the present study, clinical observations and laboratory tests revealed that animals treated with allogeneic ADSCs did not show signs of rejection of grafts until 30 days of observation. According to Caplan (2007) immunomodulatory effects prevent recognition and expansion of T cells by blocking TNF-α and INF-γ-mediated effects, causing an increase in IL-10 levels. Although all of these immunomodulatory effects have not been well described, the available data clearly indicate that allogeneic MSCs can be used as therapeutic agents.

Postoperative clinical evaluation was realized in order to verify presence or absence of alterations after surgery. Haematuria happened 24 hours after surgery, but no on the subsequent days. As a verified by previous studies (Pinto Filho et al. 2015, 2016), it is a common finding during early postoperative of urinary bladder surgery. WBC showed no significant differences between pre- and postoperative period and between groups; during this time, leukocyte counting was in the normal range for the specie. Until 72 hours after surgery high urinary frequency was observed in some animals, sign that disappeared thereafter. Pollakiuria is a clinical sign found in inflammatory bladder diseases, due to overactivity of detrusor muscle (Teixeira et al. 2007). In this study no clinical signs of urinary tract infection in any animal happened, although some rabbits of CGcy group present bacteriuria.

Blood urea levels are indicative for renal function and can serve as an indication of glomerular filtration rate, though theoretically creatinine is more appropriate, because the quantity of creatinine present in the kidney is more constant and it is not reabsorbed by the renal tubules, such as urea. In addition, creatinine rates do not suffer variations according to the animal's diet and gastrointestinal bleeding among other parameters (Emanuelli et al. 2008). Creatinine values were maintained between 1.2-1.7mg dL^{-1}, within the normal range for this species (0.5-2.5mg dL^{-1}). Alkaline phosphatase (ALP) also showed significant differences between CGcy and CGst, in agreement with previous results (Emanuelli et al. 2008). However, CGcy following 30 days of surgery presented ALP activity levels above physiological limits. In agreement with previous studies (Özkan et al. 2012), serum ALP activity originates from bone and liver and varies with age; young individuals present ALP serum levels higher due to the rapid bone growth. This alteration could not be associated to age, because the animals in this study were adults; therefore, it could be attributed to cyclosporine use. According to Rezzani, cyclosporin stimulates the production of TGF-β in the body, which is responsible for hepatic and renal injury, stimulation of neoplastic cells, heart disease and immunosuppression (Rezzani 2006). However, according to another author, in a study using dogs, the use of cyclosporine did not determine changes in serum ALP (Morini 2005).

The bladders showed at pre- and post-operative ultrasound scans to be filled by heterogeneous content with moderate cellularity and regular wall throughout its length, with no evidence of leakage of urine, showing a consistent finding of normal rabbit bladder. In the study of Teixeira et al. (2007), the first 30 days, the wall of the urinary bladder ultrasound image showed thickening and greater echogenicity, without, however, losing the integrity, similar to results found in some animals in the current study. Abilio et al. (2004) stated in his research with dogs that ultrasound images between seven and 21 days of both groups postoperatively showed minor changes in the conformation of the urinary bladders located on the face of grafting, which however, disappeared after 28 days.

CONCLUSIONS

Based on clinical data and anatomical analysis, ADSCs prevent allograft rejection of the urinary bladder, as confirmed by clinical data and anatomic analysis.

Adipose-derived cells used for engraftment in this study were characterized by fibroblast morphology, the MSC-specific CD105, CD73 and CD90 expression and by the absence of the hematopoietic marker CD45.

Therapeutic applications can be foreseen from our pre-clinical study.

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Original Article
Animais de Produção/*Livestock Diseases*

Insulin favors acute inflammatory reaction in alloxan-diabetic tilapia during infectious aerocystitis[1]

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ABSTRACT.- Prado E.J.R., Belo M.A.A., Moraes A.C., Barbuio R., Foz E.P., Faria V.P. & Sebastião F.A. 2018. **Insulin favors acute inflammatory reaction in alloxan-diabetic tilapia during infectious aerocystitis.** *Pesquisa Veterinária Brasileira 38(12):2190-2193*. Universidade Estadual Paulista Júlio de Mesquita Filho, Faculdade de Ciências Agrárias e Veterinárias, Campus de Jaboticabal, Via de acesso Prof. Paulo Donato Castellane s/n, Jaboticabal, SP 14884-900, Brazil. E-mail: maabelo@hotmail.com

In vertebrates, the inflammatory reaction is responsible for modulating the initial nonspecific defense until specific immunity is acquired. In this context, numerous studies in mammals have demonstrated the participation of insulin in the inflammatory response, favoring cell proliferation and the migratory capacity of endothelial cells, vascular smooth muscle cells and monocytes, as well as mediating the expression of pro-thrombotic and pro-fibrotic factors. However, little is known about the effect of this peptidic hormone on the inflammatory reaction in teleostean fish. In order to evaluate the participation of insulin in the acute inflammatory response of Nile tilapia, *Oreochromis niloticus*, during aerocystitis induced by *Aeromonas hydrophila*, and 48 aloxane-diabetic tilapia were used, constituting two groups: diabetics treated with insulin and diabetics without treatment. After six, 24, and 48 hours of inflammatory stimulation, tilapia were submitted to deep anesthesia for euthanasia and necropsy, and thus, obtaining exudate and harvesting of the swim bladder for analysis of the inflammatory reaction. Based on this premise, the present study demonstrated the participation of insulin in the acute inflammatory reaction of alloxan-diabetic tilapia by favors the cellular accumulation in the exudate, the proliferative effect of fibrous tissue and neovascularization in the inflamed site. Such findings reinforce the old hypothesis that insulin plays an important role in the innate immune response during acute inflammatory reaction, being an important pro-inflammatory hormone. However, Nile tilapia proved to be a promising experimental model for studies and advances in research involving diabetes mellitus.

INDEX TERMS: Insulin, acute inflammatory reaction, alloxan-diabetic, tilapia, aerocystitis, *Oreochromis niloticus*, cichlids, diabetes, peptidic hormones, inflammation, *Aeromonas Hydrophila*, clinics.

RESUMO.- [**Insulina favorece a reação inflamatória aguda em tilápia do Nilo aloxano-diabéticas durante aerocistite infecciosa**.] Em vertebrados, a reação inflamatória é responsável por modular a defesa inicial não-específica, até que imunidade específica seja adquirida. Neste contexto, inúmeros estudos em mamíferos têm demonstrado a participação da insulina sobre a resposta inflamatória, favorecendo a proliferação celular e a capacidade migratória das células endoteliais, células do músculo liso vascular e dos monócitos, além de mediar a expressão de fatores pró-trombótico e pró-fibrótico. Porém, pouco se conhece o efeito deste hormônio peptídico sobre a reação inflamatória em peixes teleósteos. Para avaliar a participação da insulina sobre a resposta inflamatória aguda em tilápias do Nilo, *Oreochromis niloticus*, na aerocistite induzida por *Aeromonas hydrophila*, foram utilizadas 48 tilápias aloxano-diabéticas, constituindo dois grupos: dos diabéticos tratados com insulina e diabéticos sem tratamento. Após, seis, 24 e 48 horas do estimulo inflamatório, as tilápias foram

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submetidas à anestesia profunda para eutanásia e necropsia, e assim, obtenção de exsudato e colheita da bexiga natatória para analise da reação inflamatória. Partindo-se desta premissa, o presente estudo demonstrou a participação da insulina na reação inflamatória aguda infecciosa de tilápias do Nilo aloxano-diabéticas por favorecer o acúmulo positivo celular no exsudato, assim como o efeito proliferativo de tecido fibroso e a neovascularização no local inflamado. Tais achados reforçam a hipótese de que a insulina desempenha importante papel na resposta imune inata na reação inflamatória aguda, sendo um importante hormônio pró-inflamatório. Contudo, a tilápia do Nilo demonstrou ser um modelo experimental promissor para estudos e avanços em pesquisas envolvendo o diabetes mellitus.

TERMOS DE INDEXAÇÃO: Insulina, reação inflamatória aguda, tilápia, aloxano-diabéticas, aerocistite, *Oreochromis niloticus*, ciclídeos, diabetes, hormônios peptídicos, inflamação, *Aeromonas hydrophila*, clínica.

INTRODUCTION

In vertebrates, the inflammatory reaction plays an important pathophysiological role in the immune system control, responsible for modulating the initial non-specific defense until the specific immunity is acquired, resulting in the production of pro-inflammatory mediators, leukocyte recruitment, activation of complement system, alteration in the concentration of different plasma proteins, and metabolic modulations (Ashley et al. 2012, Graham et al. 2005).

Numerous studies in mammals had been demonstrated the insulin (ISN) participation in the vascular response during the inflammatory reaction by favoring cell proliferation and migration capacity through endothelial cells, as well as acting in the vascular smooth muscle cells, monocytes, and mediating the expression of thrombotic and pro-fibrotic factors (Hsueh & Quiñones 2003, Dandona et al. 2009).

The pathophysiology studies of inflammatory reaction in teleosteal fish have been the subject of researches by several authors who have tried to understand the evolution of this response during foreign body type inflammation (Sakabe et al. 2013, Belo et al. 2005, 2012, 2014), granulomatous inflammation (Manrique et al. 2014, 2015) and acute inflammation (Reque et al. 2010, Claudiano et al. 2013, Castro et al. 2014). However, little is known about the effect of INS on the inflammatory response of these animals. Based on this premise, the present study aimed to evaluate the participation of this hormone in the acute inflammatory reaction of Nilo alloxan-diabetic tilapia, submitted to aerocystitis by *Aeromonas hydrophila* infection, seeking to evaluate the potential of this experimental model for studies and advances involving diabetes mellitus.

MATERIALS AND METHODS

Nile tilapia (*Oreochromis niloticus*), mean weight 532±46.7g, belonging to the "GIFT" line and masculinized, were randomly distributed in six tanks with 1000L each to performe two treatments: diabetics treated with insulin and diabetics without treatment. This study was approved by the Committee on Ethics in Animal Use (CEUA) of the Faculty of Agrarian and Veterinary Sciences, Unesp, Jaboticabal Campus, under protocol no. 022546/11.

Induction of diabetes. The diabetic effect was induced by alloxan (Sigma®) administration through intravenous route in the caudal vessel, at a dose of 100mg.kg^{-1} of body weight, using as solvent sterile saline solution with 0.65% (Xu et al. 2004). Thus, one week after the alloxan application, tilapias with glycemic values above 150mg.dL^{-1} after 12 hours of fasting were considered diabetic following an established criterion by Xu et al. (2004). Alloxan administration was performed two weeks prior to induction of inflammatory stimulus.

Inflammatory stimulation. For the inflammatory stimulus, 1mL of the *Aeromonas hydrophila* inoculum was injected caudally to the operculum, at the level of lateral line, to reach the anterior swimming bladder, and to induce acute infectious aerocystitis. The concentration of the bacterial inoculum was 7.5x10^6 colony forming units per mL. The isolation and identification was made according to Sebastião et al. (2015).

Insulin therapy. Insulin application (Lantus®) was performed immediately after bacterial inoculation by subcutaneous route in the ventral region of the left pectoral fin, in a single dose of 10 IU.kg^{-1} of body weight.

Sampling, processing and analysis of biological material. Eight animals were sampled per group at 6, 24 and 48 hours post-infection (HPI). Deep anesthesia was performed by immersion in benzocaine, followed by necropsy to collect exudate and swimming bladder. For the evaluation of total cell accumulation, 1mL of phosphate buffer solution (PBS) was injected at 0.1 molar with pH 7.2 and containing EDTA 0.09% for the lavage and cell capture in the swim bladder. The total volume of exudate was collected with Pasteur pipette, transferred to falcon tube and kept in refrigeration. The PBS-EDTA-exudate solution was centrifuged at 167xg for five minutes in a clinical centrifuge. The supernatant was discarded, the total pellet was resuspended with the addition of 1mL (6 HPI) and 2mL (24 and 48 HPI) PBS and in an aliquot of total cell volume was counted under light microscopy in a Neubauer chamber. The number of cells found was multiplied by the dilution factor (Reque et al. 2010). For the histopathological study, the swimming bladder was removed from each animal and fixed in 4% paraformaldehyde prepared in PBS (0.1M and pH 7.2). After the procedures of fixation, dehydration, diaphanization and inclusion in paraffin, 5µm thick sections were stained with hematoxylin and eosin (HE).

Statistic. Statistical analysis was performed in a mixed model; the results were tested applying the experimental scheme by subdivided plots, comparing the treatment effect and the interactions between treatment versus time in relation to the groups. The Bartlet test (P=>0.05) and the Shapiro-Wilk test (P=>0.05) were used to establish the statistical hypothesis of homocedasticity and normality of the internally standardized residues. Tukey test (P<0.05) was used for comparisons. All statistical analyzes were processed in SAS® software (Statistical Analysis System), version 9.3.

RESULTS

In the exudative study (Fig.1), there was a greater presence of inflammatory cells in the group treated with insulin, this effect was notorious in the period of 48 HPI compared to the group without insulin therapy. In the study over time, both groups, with or without insulin therapy had high cell accumulation in the period of 24 HPI. The group without insulin therapy presented decrease in the cellular accumulation in the period of 48 HPI.

In the histopathological evaluation 6 and 24 HPI (Fig.2), alloxan-diabetic tilapia without insulin therapy showed low-proliferation of fibrous tissue and infiltration of

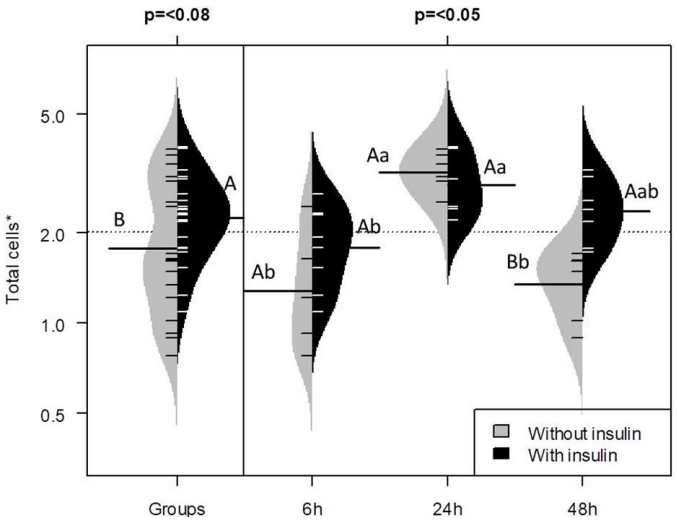

Fig.1. Total cells presented in the exudate of Nile tilapia alloxan-diabetic with acute aerocystitis induced by *Aeromonas hydrophila*. Fish were sampled six, 24 and 48 hours post-infection (HPI). *Values transformed according to Box and Cox. Comparisons realized by Tukey Test (capital letters compare treatment and lowercase compare different periods). Total number of animals observed = 48.

inflammatory cells in the swim bladder, as well as serosa thickening. On the other hand, alloxan-diabetic tilapia with insulin therapy showed high proliferation of fibrous tissue, high inflammatory cell infiltration and neovascularization with hyperemia. Acute fibrinosuppurative inflammation was observed in insulin-treated tilapia in 48 HPI. In contrast, alloxan-diabetic tilapia without insulin therapy showed decrease in the of inflammatory cell accumulation 48 HPI.

DISCUSSION

Decreased fibrous tissue proliferation and low neovascularization at the inflamed site in the diabetic Nile tilapia mimic the same effects observed in diabetic mammals. Since studies have shown that insulin promotes the formation of fibrous tissue, proliferation of endothelial cells and their migration capacity (Hsueh & Quiñones 2003, Dandona et al. 2009).

However, both groups, with or without insulin therapy, exhibit the same pattern of cellular accumulation response up to the period of 24 HPI, such result may be related to the immunomodulatory stimulus of the bacterial lipopolysaccharide (LPS) (Jimenez et al. 2008). According to Munford (2006), LPS play a major role in the activation and amplification of the inflammatory response. As the induction of the pro-inflammatory pathway results from the LPS signal transfer in a complex formed by LPS, LBP, CD14 and TLR4, which initiate a series of intracellular events involving proteins and transcription factors responsible for various genes that encode cytokines, chemokines and adhesion molecules (Cohen 2002).

In confirmation, the LPS inoculum in fish attributed the ability of macrophages to produce interleukin 1 (IL-1) and tumor necrosis factor alpha (TNF-α), which are responsible for chemotaxis, leukocyte activation and expression of endothelial adhesion molecules (Swain et al. 2008). Thus, LPS plays an important mediator in the process of leukocyte diapedesis

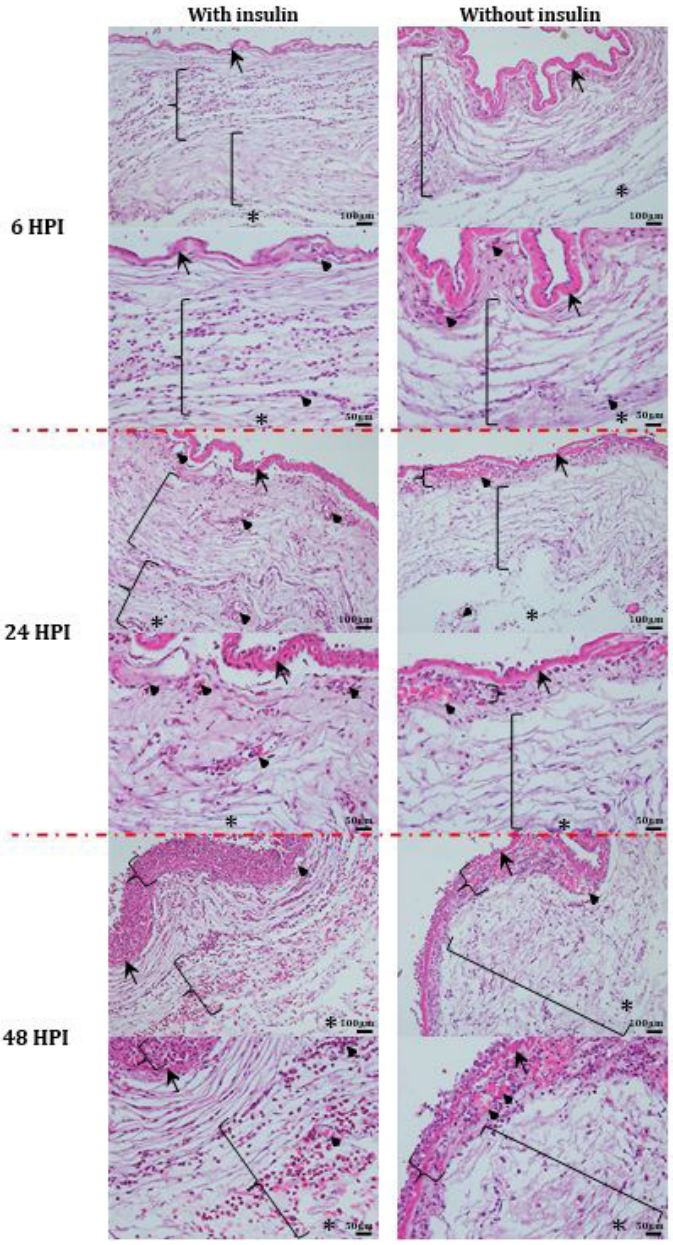

Fig.2. Photomicrographs of the swimming bladder of Nile tilapia alloxan-diabetic with acute aerocystitis induced by *Aeromonas hydrophila*. Fish were sampled six, 24 and 48 hours post-infection (HPI). Serous membrane (arrow tip), inner side of swim bladder (asterisk), fibrous tissue (bracket), blood vessel (arrow) and infiltrates of inflammatory cells (curly bracket).

in the inflammatory focus in diabetic tilapia with or without insulin therapy 24 HPI.

The pro-inflammatory effect of insulin on diabetic tilapia 48 HPI was evident, which presented a fibrinosuppurative inflammatory aspect with high cellular accumulation in the inflammatory focus. In this context, studies have shown that insulin participates in angiogenesis by favoring cell growth, the migratory capacity of endothelial cells, vascular smooth muscle cells and monocytes, mediating the expression of fibrotic factors in response to various stimuli (Clarke & Dodson 2007).

Study conducted by Dror et al. (2017) described the insulin participation in the IL-1 production by macrophages. This cytokine stimulate the expression of proteins responsible for adhesion and leukocyte diapedesis, favoring cell growth and differentiation by acting directly on innate immune cells, significantly influencing its functionality and survival, and modulating the activity of lymphocytes during adaptive immune responses (Sims & Smith 2010). To reinforce the pro-inflammatory stimulus of insulin observed in tilapias 48 HPI, in accordance with Agius & Roberts (2003) and Hodgkinson et al. (2015) macrophages are recruited in large quantity later to the inflamed site in order to maintain the parsimony between the innate and adaptive immune response.

CONCLUSIONS

The insulin hormone presented a pro-inflammatory effect in Nile tilapia alloxan-diabetic submitted to acute aerocystitis by *Aeromonas hydrophila* infection, because it favors the accumulation of inflammatory cells in the exudate and results in a proliferative effect of fibrous tissue with neovascularization in the inflamed site.

Such results associated with the success of insulin therapy in tilapias demonstrated the potential of this experimental model for studies and advances in researches involving diabetes mellitus.

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Original Article
Animais de Produção/*Livestock Diseases*

Mammary gland health of Santa Inês ewes at the drying and puerperium and evaluation of a dry-off terapy with gentamicin[1]

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ABSTRACT. Pereira P.F.V., Reway A.P., Félix A., Beutemmüller E.A., Pretto-Giordano L.G., Alfieri A.A., Lisbôa J.A.N. & Müller E.E. 2018. **Mammary gland health of Santa Inês ewes at the drying and puerperium and evaluation of a dry-off therapy with gentamicin.** *Pesquisa Veterinária Brasileira 38(12):2194-*2200. Departamento de Clínicas Veterinárias, Universidade Estadual de Londrina, Rodovia Celso Garcia Cid PR-445 Km 380, Campus Universitário, Londrina, PR 86057-970, Brazil. E-mail: pri_fajardo@yahoo.com.br

Mastitis represents an important health problem for Santa Inês breed, causing losses to the producer, due to loss of ewes or the decrease in weight gain of lambs. The aim of this work was to assess the health of the mammary gland of Santa Inês ewes at the drying and puerperium and to investigate the efficacy of a dry-off therapy with gentamicin. In this study, 64 ewes were divided in a control group (GC) and treatment group (GT), and the health of the mammary gland was assessed at the drying and puerperium. The GT ewes received 250mg of gentamicin (Gentocin® DryCow/Schering-Plough, product indicated for use in dairy cows) in each mammary half. For diagnosis, clinical examination, California Mastitis Test, somatic cell count and milk culture was performed. In the GC, of the 45 (70.3%) healthy mammary halves at the drying, 12 developed subclinical mastitis and nine clinical mastitis at the puerperium. In the GT, among 51 (79.7%) healthy mammary halves at the drying, six developed subclinical mastitis and 11 clinical mastitis at the puerperium. No association was observed between treatment and the occurrence of mastitis at puerperium. Of the 19 (29.7%) mammary halves of the GC that presented subclinical mastitis at the drying, three remained with subclinical mastitis and five developed clinical mastitis at the puerperium. In the GT, of the 13 (20.3%) mammary halves that had subclinical mastitis at the drying, four remained with subclinical mastitis and four developed clinical mastitis. No association was observed between treatment and cure or persistence of mastitis at the puerperium. The main microorganisms isolated, at the drying and puerperium, from animals with subclinical or clinical mastitis were *Staphylococcus* spp., predominantly coagulase negative *Staphylococcus* (CSN). At the puerperium, 29 cases of clinical mastitis occurred, 19 with isolation, where 10 were CNS and six *S. aureus*. *Mannheimia haemolytica* was isolated in one case of subclinical mastitis and other of clinical mastitis. News protocols and different ways of handling at drying and at puerperium must be investigated.

INDEX TERMS: Mammary gland, health, ewes Santa Inês, dry-off terapy, sheep, mastitis, etiology, clinics.

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RESUMO.- [**Sanidade da glândula mamária de ovelhas Santa Inês na secagem e no puerpério e avaliação da terapia intramamária com gentamicina na secagem.**] A mastite é um problema sanitário importante em ovelhas da raça Santa Inês, ocasionando prejuízo ao produtor em virtude do descarte de matrizes e da queda no ganho de peso dos cordeiros. O objetivo deste trabalho foi avaliar a saúde da glândula mamária de ovelhas da raça Santa Inês na secagem e no puerpério e pesquisar a eficácia da terapia intramamária com gentamicina na secagem. Sessenta e quatro ovelhas foram divididas em grupos controle (GC) e tratamento (GT), cada um contendo 32 animais, e a saúde da glândula mamária avaliada na secagem e no puerpério. As ovelhas do GT receberam 250mg de gentamicina (Gentocin® Mastite Vaca Seca/ Schering-Plough Veterinária, produto indicado pela empresa para utilização em vacas de leite) em cada metade mamária. Para o diagnóstico, foram realizados exame físico da glândula mamária, California Mastitis Test, contagem de células somáticas e cultura do leite. No GC, das 45 (70,3%) metades mamárias sadias na secagem, 12 desenvolveram mastite subclínica e nove mastite clínica no puerpério. No GT, das 51 (79,7%) metades mamárias sadias na secagem, seis desenvolveram mastite subclínica e 11 mastite clínica no puerpério. Não houve associação entre o tratamento e a ocorrência de mastite no puerpério. Das 19 (29,7%) metades mamárias do GC que apresentaram mastite subclínica na secagem, três permaneceram com mastite subclínica e cinco desenvolveram mastite clínica no puerpério. No GT, das 13 (20,3%) metades mamárias com mastite subclínica na secagem, quatro permaneceram com mastite subclínica e quatro desenvolveram mastite clínica. Não houve associação entre o tratamento e a cura ou persistência da mastite no puerpério. Os principais micro-organismos isolados, na secagem e puerpério, de animais com mastite subclínica ou clínica foram *Staphylococcus* spp., com predominância de *Staphylococcus* Coagulase Negativa (SCN). No puerpério, ocorreram 29 casos de mastite clínica, sendo 19 com isolamento, 10 com SCN e seis com *S. aureus*. *Mannheimia haemolytica* foi isolado em um caso de mastite subclínica e um caso de mastite clínica. Novos protocolos e diferentes formas de manejo na secagem e no puerpério devem ser pesquisados

TERMOS DE INDEXAÇÃO: Glândula mamária, ovelhas Santa Inês, terapia intramamária, ovinos, mastite, etiologia, antibioticoterapia, clínica.

INTRODUCTION

Mastitis is responsible for high economic losses including a decrease in milk yield, in weight gain of lambs with eventual death, ewe culling off, and treatment expenses. However, the vast majority of studies regarding intramammary infections was performed in dairy sheep herds from Europe and North America, where the raising system employed is completely different from that used in Brazil (Menzies & Ramanoon 2001, Bergonier et al. 2003). The epidemiology and etiology of mastitis in meat ewes, including Santa Ines ewes, was studied recently in Brazil (Oliveira 2007, Blagitz et al. 2008, Coutinho et al. 2008a) and in the Paraná State (Pereira et al. 2014). Moreover, studies regarding intramammary therapy at dry-off stage in meat ewes are limited. This procedure is recommended in dairy cows, goats and ewes to treat any infection acquired during lactation period and to prevent new infections during dry and subsequent lactation periods (Chaffer et al. 2003). The therapy in dry cows is one of the most effective practices for mastitis control (Spanu et al. 2011). Because of the long period between the dry-off and the following parturition, the use of this procedure in meat sheep remains controversial (Bergonier et al. 2003). It is noteworthy that a specific intramammary antimicrobial product for small ruminants is not available in Brazil.

The present study aimed to evaluate the mammary gland health of Santa Ines ewes at dry-off and puerperium period and the efficacy of intramammary dry-off therapy with gentamicin.

MATERIALS AND METHODS

All experimental procedures and animal handling were perfomed after submission and approval by the Ethics Committe and Animal Experimentation of Universidade Estadual de Londrina (CEEA-UEL, No. 94/2010).

Selection of ewes. The study was performed on a farm located at the municipality of Ibiporã city (23°16'08" S, 51°02'52" W), north region of Paraná State, from September 2010 to September 2011. From a herd of 156 Santa Ines and Texel ewes (including rams, ewes, and lambs), 64 Santa Ines ewes were selected and kept in semi-intensive raising system. During the day, the sheep were kept on star grass (*Cynodon nlemfuensis*) pasture and at night were closed in shelters. Ewe selection was based on mammary gland inspection and palpation and also examination of mammary secretions (Grunert 1993). Only ewes without physical alterations in the mammary gland and milk were used. Ewes were individually identified.

Clinical examination and study design. The selected sheep, primiparous (n=4) or multiparous (n=60), were randomly allocated at to two groups (Control and Treatment, n=32 each). At 90±5 days of lactation, ewes from Control (CG) and Treatment (TG) groups were submitted to physical exam of the udders and milk samples were collected to perform California mastitis test (CMT), somatic cell count (SCC), microbiological culture, and antibiogram. For milk sample collection, the ewes were previously kept separate from the lambs for approximately six hours. Afterwards, the milk of the females from CG and TG were completely drained by hand milking. Ewes from TG received 250mg of gentamicin (Gentocin® Mastite Dry cow, Schering-Plough Veterinaria, originally indicated for cows) in each mammary half. After dry-off, the sheep were not milked again. The ewes were kept in the routine semi-intensive raising system of the farm.

Approximately 7 days before parturition, ewes were confined in collective pens for a better monitoring. The same evaluations performed at dry-off were conducted at puerperium (7 to 10 days after parturition).

In the evaluation of the mammary gland sanitary conditions, the mammary halves were classified according to its clinical conditions, SCC and milk microbiological culture. The characterization of subclinical mastitis used 500,000 cells/mL as threshold, in accordance with Berthelot et al. (2006). Thus, at the dry-off, the mammary halves were classified as healthy or with subclinical mastitis; and at the puerperium they were classified as healthy, with subclinical mastitis or with clinical mastitis. In the mammary halves classified as healthy, the milk was normal, SCC was lower than 500,000 cell/mL, and microbiological culture was negative. In those ones classified as with subclinical mastitis, the milk sample had SCC ≥500,000 cells/mL and/or was positive in microbiological culture. The mammary halves with clinical mastitis had evident signs of inflammation (heat,

redness, tumor, pain, partial/total function loss, characterized by the absence of milk secretion) with or without bacterial isolation in microbiological culture.

Diagnostic methods. The diagnosis of clinical mastitis was performed by inspection and palpation of the udder. The mammary parenchyma was evaluated for the presence of nodules, diffuse hardness areas, and fibrosis. Moreover, the macroscopic characteristics of the milk (color, consistency, presence of lumps, pus, and blood) were evaluated, according to Grunert (1993).

Presumptive diagnosis of subclinical mastitis was performed using CMT (Schalm & Noorlander 1957), considering negative (-) and positive (score 1+, 2+, 3+) reactions.

Milk samples collected for SCC analysis were performed in vials containing bronopol. The vials were packed in boxes and sent to the Laboratório Centralizado de Análise de Leite da Associação Paranaense de Criadores de Bovinos da Raça Holandesa (Curitiba/PR), where they were analyzed by flow cytometer (SOMACOUNT 500, Bentley Instruments Inc).

Milk sample collection for microbiological culture and identification was performed according to the instructions of the National Mastitis Council (NMC 2004), preconized for bovine. Milk samples were collected after hand sanitizing, washing of the teat with water and soap, drying, discard of the first stream of milk, immersion in chlorine solution (750ppm), cleaning with alcohol (70ºGL), and drying with paper towel. The milk was collected in sterile screw cap glass vials, and transported refrigerated (4-8ºC) in isotherm container to the Laboratório de Microbiologia do Departamento de Medicina Veterinária Preventiva (DMVP-UEL). At the laboratory, samples were seeded in petri dishes with agar nutrient media (Himedia®) with 5% of ovine blood and MacConkey agar (Himedia®). They were incubated at 37±1ºC, and the readings were performed 24, 48, 72, and 96 hours later. The isolated microrganisms were identified based on morphological, Gram stain, biochemical and culture characteristics (Carter & Cole Junior 1990, Quinn et al. 1994, NMC 2004).

Staphylococcus spp. were submitted to coagulase test and identified by API-STAPH system (Bio Mérieux®, France). *Streptoccus* spp. were classified in groups by SLIDEX STREPTO-KIT (Bio Mériux®, France) and identified by API-STREP (Bio Mériux®, France).

The diagnosis of *Mannheimia haemolytica* was based on the morphological characteristics and Gram stain. For specie confirmation, the colonies were submitted to polymerase chain reaction (PCR) using primers from the intergenic region between artJ and lktC at the Laboratório de Virologia of DMVP-UEL, according to the technique previously described (Angen et al. 2009). The amplified products were sequenced and the identity was compared with sequence from public data bases.

Antibiogram was performed using disc diffusion method (Bauer et al. 1966) in Mueller Hinton agar (Himedia®) and discs (Laborclin®) impregnated with the following antimicrobial agents: Amoxicillin (AMO - 10µg), Ampicillin (AMP - 10µg), Ceftiofur (CFT - 30µg), Enrofloxacin (ENR - 5µg), Streptomycin (EST - 300µg), Gentamicin (GEN - 10µg), Neomicin (NEO - 30µg), Oxacillin (OXA - 10µg), Penicillin G (PEN - 10UI), Sulfonamide (SUF - 25µg), and Tetracicline (TET - 30µg).

Statistical analysis. Prevalence of mastitis in control and treatment groups was compared by contingency tables and qui-square test. Quantitative variables were compared by Mann-Whitney and t tests. A 5% significance level was used.

Kappa coefficient (Cohen 1960) was calculated to evaluate the agreement between SCC and microbiological culture. Agreement was classified as low (values ranging from 0.01 to 0.2), medium (0.21 to 0.4), moderate (0.41 to 0.6), substantial (0.61 to 0.8), and high (0.81 to 1.0), according to Landis & Koch (1977).

RESULTS AND DISCUSSION

Table 1 shows the results of CMT, SCC and culture of milk samples collected at dry-off and puerperium period, not considering the intramammary treatment. At dry-off, 43 (34%) of the mammary halves had CMT ≥1+, 24 (19%) had SCC ≥500.000 cells/mL, and 32 (25%) were positive in microbiological culture. At puerperium, 46 (36%) of the mammary halves had CMT ≥1+, 37 (29%) had CCS ≥500.000 cells/mL, and 33 (26%) were positive in microbiological culture. In this phase, 29 cases of clinical mastitis and 25 cases of subclinical mastitis were observed; however, in 12 cases of subclinical mastitis, as well as in one case of clinical mastitis, no microrganisms were isolated. In seven mammary halves, milk sample collection was not performed because of the severe inflammation signs and diffuse hardness of the mammary parenchyma, leading to an absence of milk production. Pereira et al. (2014) also reported clinical mastitis with diffuse hardness of the udder and absence of milk production in Santa Inês ewes. In a dairy sheep herd from Jordan, microorganisms were isolated in 39.1% of the 1,210 milk samples used in the study (Lafi 2006).

In the present study, a high number of clinical mastitis cases was observed after parturition (29/64). It was reported that in 80% of the 31 Santa Ines sheep herds studied by Oliveira (2007), the occurrence of clinical mastitis was higher during puperperium period. Similarly, an epidemiological survey in the North region of Parana State demonstrated that in 69,2% of the meat sheep flocks studied most cases of clinical mastitis occurred after parturition (Pereira et al. 2014).

Table 1. California Mastitis Test (CMT), somatic cell count (SCC) and milk culture results of 128 mammary halves of Santa Ines ewes at dry-off and puerperium period, Londrina/PR

Lactation period	No.	CMT			SCC (x10^3 cells/mL)			Microbiological culture		
		<1+	≥1+	NP*	< 500	≥500	NP*	Negative	Positive	NP**
Dry-off	128	85 (66%)	43 (34%)	0	88 (69%)	24 (19%)	16 (12%)	96 (75%)	32 (25%)	0
Puerperium	128	78 (61%)	46 (36%)	4 (3%)	72 (56%)	37 (29%)	19 (15%)	89 (69%)	32 (25%)	7 (6%)

*NP = Exam not performed due to reduced or absent milk production, **NP = Exam not performed due to absent milk production as the result of clinical mastitis.

With a threshold of 500,000 cells/mL, the kappa coeficient between SCC and milk culture results was 0.43 (P<0.001), indicating a moderate agreement between these two tests. Sensitivity and specificity of SCC was 62% and 83%, respectively. Previous studies in dairy flocks used different threshold values for SCC. A study in Slovenia with 251 dairy sheep used 250,000 cells/mL as threshold (Pengov 2001). In a study in Jordan, the threshold used was 1,000,000 cells/mL (Lafi 2006). When a threshold of 500,000 cells/mL was used the sensitivity and specificity observed was 73% and 82%, respectively (Berthelot et al. 2006).

Health conditions of the mammary gland of ewes from CG and TG at dry-off and puerperium period are shown in Table 2. An association between the treatment and the occurrence of mastitis during puerperium was not observed (P=0.261), i.e., the use of intramammary antibiotic at dry-off did not prevent the occurrence of subclinical or clinical mastitis in this study. A study with 245 dairy sheep showed that the intramammary therapy at dry-off did not affect the occurrence of intramammary infections at parturition, although the SCC significantly decreased in the treated group (Spanu et al. 2011). In contrast, a significant decreased of new cases of mastitis in the following lactation was observed after therapy at dry-off in several studies (Bergonier & Berthelot 2003, Gonzalo et al. 2004, Linage & Gonzalo 2008).

Also, there was no association between the treatment and the healing or persistency of the mastitis during puerperium (P=0.472), i.e., the use of intramammary antibiotic did not eradicate the infection and did not prevent the occurrence of subclinical or clinical mastitis. Healing rates between 65% and 98% was reported after the treatment with intramammary antibiotics (Bergonier & Berthelot 2003). A study with 85 dairy sheep in Israel reported higher healing rates in sheep treated with intramammary antibiotic at dry-off, although the rate of new infections was not different between treated and control group (Chaffer et al. 2003). A healing rate of 100% was reported after the treatment with intramammary cefalonium of sheep with subclinical mastitis at dry-off (Coutinho et al. 2008b).

Several reasons could explain why the intramammary therapy with gentamicin at dry-off fails to prevent new infections or heal previous infections. In this experiment, a high number of new infections also occurred during puerperium, most of them clinical form. The long dry period (ranging from 5 to 10 months) observed in the present study could be one explanation. In the farm studied, breeding season or reproductive management was not employed because having parturition spread all over the year was interesting to the owner. This type of management is common in several farms located in the North region of Paraná State (Pereira et al. 2014). The dry period of sheep can be as long as six months or more, and it is considered a limiting factor to the use of intramammary antibiotics at dry-off, since most of the drugs available not remain active throughout this period (Bergonier et al. 2003, Chaffer et al. 2003). A study in Spain with 229 dairy sheep with a shorter dry period (109 days in average) reported that the prevalence of intramammary infection decreased significantly from 48% at dry-off to 13% at parturition in those animals treated with an association of antibiotics at dry-off (Linage & Gonzalo 2008). However, in this same experiment, no difference in the prevalence of infections was observed in control group. The parenteral administration of tilmicosina one month before parturition did not reduce the cases of clinical mastitis, but attenuated the palpable abnormalities in the udder (Croft et al. 2000).

Table 2. Health conditions of the mammary halves at dry-off and puerperium period of 64 Santa Ines sheep from control and treatment groups, Londrina/PR

Groups		Dry-off N	Puerperium		
			Healthy	Subclinical mastitis	Clinical mastitis
Control group	Healthy	45	24 (53.3%)	12 (26.7%)	9 (20.0%)
	Subclinical mastitis	19	11 (57.9%)	3 (15.8%)	5 (26.3%)
Subtotal		64	35	15	14
Treatment group*	Healthy	51	34 (66.7%)	6 (11.8%)	11 (21.5%)
	Subclinical mastitis	13	5 (38.4%)	4 (30.8%)	4 (30.8%)
Subtotal		64	39	10	15
TOTAL		128	74	25	29

*Intramammary administration of gentamicin at dry-off.

The confinement of the ewes one week before parturition to allow a better monitoring could favor the occurrence of mastitis during puerperium. The ewes were confined in collective pens and cleaning was not daily performed. The accumulation of waste in the sheepfold and keeping healthy sheep together with sheep affected by mastitis in the same pen can favor the transmission of the contagious agent by lamb suckling, contributing for new infections. An epidemiological study in the North region of Parana State demonstrated that only 22% of the farmers performed the cleaning of the sheepfold daily and the rest of them performed the cleaning at irregular intervals, resulting in accumulation of organic waste (Pereira et al. 2014). In this same study, the intensive management system was identified as a risk factor for mastitis. Marogna et al. (2010) reported that in herds affected by chronic mastitis, the unsatisfactory hygienic conditions and the overcrowding of the sheepfolds have favored the development of microorganism and infection of the mammary gland.

Another factor to be considered as the cause of the ineffectiveness of preventive antibiotic therapy at dry-off is the decreased activity of the phagocytes. These cells play a key role in the elimination of the microorganisms that cause mastitis (Dosogne et al. 1998). Previous studies in dairy cows showed that different antibiotics indicated to be used at dry-off had a negative influence over the phagocytic activity of the somatic cells (Paape et al. 1996, Batista et al. 2006). This same effect was observed in goat milk phagocytes (Benesi et al. 2010). The effect of several intramammary antibiotics over phagocytosis was tested in vitro, and was observed that gentamicin lead to the lowest level of phagocytosis.

The effectiveness of antibiotic therapy at dry-off is controversial due to high rates of self-healing in sheep and goat, regardless the administration of intramammary antibiotic at dry-off (Fox et al. 1992). In the present study (Table 2), from 19 mammary halves with subclinical mastitis at dry-off in CG, 11 (57.9%) were healthy after parturition. Contreras et al. (2007) reported that healing rates can range

from 20% to 60% and intramammary therapy at dry-off was recommended only in herds with high prevalence of mastitis.

The microganisms isolated from the milk samples obtained from mammary halves at dry-off and puerperuim period are shown in Table 3. The most prevalent microorganism at dry-off and puerperium period in both groups was *Staphylococcus* spp. (87.7%) and the different species isolated are shown in Table 4. These results corroborate with previous studies. In 251 dairy ewes, *Staphylococcus* spp. was isolated in 99 (76.2%) of 130 milk samples with positive result in culture (Pengov 2001). The genus *Staphylococcus* is the main etiological agent of mastitis in dairy sheep; moreover, it was reported that coagulase negative staphylococci are responsible for 25% to 93% of the infections, and *S. aureus* are responsible for 3% to 37% (Bergonier et al. 2003).

Regarding coagulase positive staphylococci, were identified 16 *S. aureus*, six from clinical mastitis and 10 from subclinical mastitis, and three samples of *S. intermedius* isolated from subclinical infections. *S. aureus* is the etiological agent most commonly isolated from clinical mastitis in ewes (Kirk et al. 1996, Jones & Watkins 2000, Bergonier et al. 2003).

Species of coagulase negative staphylococci were more prevalent at dry-off and also at puerperium period, specially *S. chromogenes* and *S. xylosus*. Previous study reported that 45% of the microrganisms isolated from sheep with mastitis were coagulase negative staphylococci, and the most prevalent were *S. xylosus*, *S. chromogenes* and *S. epidermidis* (Spanu et al. 2011). A study with 3,758 milk samples showed that 87.5% of the isolated were coagulase negative staphylococci and only 0.8% were *S. aureus* (Berthelot et al. 2006). In Santa Ines ewes, 40% of the milk samples were positive for *S. aureus* and 20% for coagulase negative staphylococci (Guarana et al. 2011). In general, the most frequently coagulase negative staphylococci isolated from sheep with mastitis are: *S. epidermidis*, *S. chromogenes*, *S. xylosus*, *S. simulans* e *S. hyicus* (Pengov 2001, Bergonier et al. 2003, Spanu et al. 2011). Pereira et al. (2014) reported CNS as the most prevalent microorganism isolated form clinical mastitis cases in meat ewes.

In sheep sampled, the median of SCC of milk sample in which were isolated coagulase positive and negative staphylococci was 568,000 and 436,000, respectively; and from 20 cases of mastitis caused by coagulase negative staphylococci in the puerperium, 10 presented as clinical mastitis. In cows,

Table 3. Microrganims isolated in milk samples of Santa Ines sheep in control group and intramammary gentamicin treated group at dry-off and puperperium period, Londrina/PR

Microrganism	Control group		Treatment group		Total
	Dry-off	Puerperium	Dry-off	Puerperium	
CPS*	5	3	6	5	19 (29.7%)
CNS**	11	12	5	8	36 (56%)
Streptococcus bovis	0	0	1	0	1 (1.6%)
Streptococcus dysgalactie	0	1	0	0	1 (1.6%)
Streptococcus spp. Group D	1	0	0	0	1 (1.6%)
Streptococcus spp. Group G	0	0	1	0	1 (1.6%)
Streptococcus spp.	1	0	0	0	1 (1.6%)
Aerococcus spp.	1	0	0	0	1 (1.6%)
Klebsiella pneumoniae	0	0	0	1	1 (1.6%)
Mannheimia haemolytica	0	1	0	1	2 (3.1%)
Total of positive samples	19	17	13	15	64

*CPS = coagulase positive *Staphylococcus*, **CNS = coagulase negative *Staphylococcus*.

Table 4. Specie identification in 55 samples collected from Santa Ines sheep at dry-off and puerperium period where *Staphylococcus* was isolated, Londrina/PR

Specie	Dry-off		Puerperium		Total	
	N	%	N	%	N	%
CPS*	11	40.8	8	28.6	19	34.5
S. aureus	10	37.0	6	21.4	16	29.0
S. intermedius	1	3.8	2	7.2	3	5.5
CNS**	16	59.2	20	71.4	36	65.5
S. chromogenes	3	11.1	5	17.9	8	14.5
S. hyicus	4	14.8	2	7.2	6	11.0
S. sciuri	0		2	7.2	2	3.6
S. simulans	2	7.4	4	14.2	6	11.0
S. xylosus	4	14.8	3	10.7	7	12.7
Not identified	3	11.1	4	14.2	7	12.7
TOTAL	25	100	28	100	55	100

*CPS = coagulase positive *Staphylococcus*, **CNS = coagulase negative *Staphylococcus*

coagulase negative staphylococci are considered agents of low pathogenicity and responsible for most cases of subclinical mastitis (Contreras et al. 2007, Arsenault et al. 2008). In contrast, others authors considered coagulase negative staphylococci in ewes as responsible for the significant increase of SCC and as an important etiological agent of clinical mastitis (Fthenakis & Jones 1990, Pengov 2001).

Streptococcus spp. were isolated in five milk samples (7.8%), four at the dry-off and one in the puerperium period. This genus was the second most prevalent in a previous study (Pengov 2001). In milk sample collected from Santa Ines sheep, within 10 to 45 days after parturition, 12.1% of the isolated microrganisms were classified as *Streptococcus* spp. (Coutinho et al. 2008b). In Brazilian Northeast, 15.9% of the samples collected from Santa Ines sheep were positive for *Streptococcus* spp. (Guarana et al. 2011).

M. haemolytica was isolated from two milk samples collected during puerperium, one sample from a subclinical mastitis case (SCC of 1,342,000 cells/mL) and another from an acute clinical mastitis case, with diffuse hardness of the mammary parenchyma and reddish color milk secretion. This microrganism is considered one of the most frequent etiological agent of clinical mastitis in meat ewes. Saliva and respiratory secretions of the lambs are the main infection source of the pathogen in sheep (Kirk & Glenn 1996, Menzies & Ramanoon 2001, Omaleki et al. 2011). In Brazil, reports of the involvement of *M. haemolytica* in intramammary infections in ovine are limited. Pereira et al. (2014) isolated *M. haemolyitca* in two cases of acute mastitis in meat ewes of Paraná state. In a study of 78 animals located in Northeast region of Brazil, *M. haemolyitca* was isolated in only one sample collected from a clinical mastitis case (Santos 2008). In anothers Brazilian studies about the mastitis etiology in ovine, isolation of *M. haemolyitca* was not reported (Oliveira 2006, 2007, Blagitz et al. 2008, Coutinho et al. 2008a).

Gentamicin, antibiotic used in the present study, had sensitivity of 90.6% and 81.2% face to microrganisms isolated at dry-off and puerperium, respectively (Table 5). Other studies with Santa Ines sheep, demonstrated that gentamicin was considered one of the best drugs to eliminate the microrganisms isolated from milk samples (Domingues et al. 2006, Coutinho et al. 2008a, Guaraná et al. 2011). Previous studies reported that the microorganism isolated from Santa Ines sheep with mastitis had lower sensitivity to streptomycin, kanamycin and tetraciclin (Almeida 2007, Guarana et al. 2011). The in vitro effectiveness of the antibiotics to eliminate the etiological mastitis agent in ovine in the present study can be attributed to the sporadic use of this drug in ovine industry, unlike what is observed in dairy cattle. The efficacy of other drugs should be tested in the drying off therapy in meat ewes, as well as the appropriate time to perform them.

Table 5. Antibiotic sensitivity profile of the microrganisms isolated from ovine clinical and subclinical mastitis at dry-off and puperperium period, Londrina/PR

Antibiotic	Dry-off		Puerperium	
	Sensitivity (%)	Resistance (%)	Sensitivity (%)	Resistance (%)
Amoxacillin	32 (100%)	0	31 (96.9%)	1 (3.1%)
Ampicillin	23 (72.0%)	9 (28.0%)	27 (84.4%)	5 (15.6%)
Ceftiofur	28 (87.5%)	4 (12.5%)	30 (93.7%)	2 (6.3%)
Enrofloxacin	22 (68.7%)	10 (31.3%)	29 (90.6%)	3 (9.4%)
Streptomycin	18 (56.2%)	14 (43.8%)	22 (68.7%)	10 (31.3%)
Gentamicin	29 (90.6%)	3 (9.40%)	26 (81.2%)	6 (18.8%)
Neomicin	19 (59.4%)	13 (40.6%)	26 (81.2%)	6 (18.8%)
Oxacilin	28 (87.5%)	4 (12.5%)	26 (81.2%)	6 (18.8%)
Penicillin G	20 (62.5%)	12 (37.5%)	19 (59.4%)	13 (40.6%)
Sulfonamide	25 (78.0%)	7 (22.0%)	29 (90.6%)	3 (9.4%)
Tetraciclin	25 (78.0%)	7 (22.0%)	23 (72.0%)	9 (28.0%)

CONCLUSIONS

Considering the experimental conditions, one may conclude that intramammary administration of gentamicin at dry-off did not prevent new infections and also did not heal the previous subclinical mastitis.

Moreover, a high prevalence of mastitis was observed during puerperium, mainly clinical mastitis form.

Coagulase negative *Staphylococcus* was mostly isolated, followed by coagulase positive *Staphylococcus*, both in clinical and subclinical mastitis, corroborating with the predominance of this group of bacteria as the etiological agent of mastitis in ovine.

The low occurrence of *Mannheimia haemolytica* indicates the reduced influence of this microrganism for mastitis etiology in the flock studied.

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Original Article
Animais de Produção/*Livestock Diseases*

Physiotherapy protocol during initial postoperative period of arthroscopy in horses[1]

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ABSTRACT.- Stievani F.C., Machado T.S.L., Bezerra K.B., Silva M.M., Baccarin R.Y.A. & Silva L.C.L.C. 2018 **Physiotherapy protocol during initial postoperative period of arthroscopy in horses**. *Pesquisa Veterinária Brasileira 38(12):2201-2206.* Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Avenida Prof. Dr. Orlando Marques de Paiva 87, Cidade Universitária, São Paulo, SP 05508-270, Brazil. E-mail: fe.stievani@gmail.com

This study evaluated the effects of a physiotherapy protocol applied in joints with osteochondritis dissecans submitted to arthroscopy. Twelve horses totaling twenty joints were used and divided into two uniform groups, according to articular lesion grade. Treated Group (TG) received the physiotherapy protocol (cryotherapy, passive rage motion and controlled exercise) that initiate just after anesthetic recovery and extended for five days. Control Group (CG) remained resting in stall during the same period. Physical examination and synovial fluid analysis were used to evaluate the treatment. The synovial fluid examination consisted of physical analysis (color, aspect, and viscosity), mucin clot evaluation, Serum Amyloid A, Prostaglandin E_2 and urea concentration. Synovial samples were collected by arthrocentesis at the beginning of the surgical procedure (D1), 48 hours (D3) and 96 hours (D5) after surgery. Before arthroscopy and daily during the postoperative period joints were evaluated by physical exam: superficial temperature (°C), range of motion (degrees) and circumference (centimeters). The joint physical examination showed no significant difference between groups and neither along the days for the same group. The parameters of synovial fluid showed difference over the moments in each group but didn't have difference between groups. Color and aspect had the same patterns across moments, in CG fluid had significant change when compared D1 with D3 (color and aspect: $p<0.001$) and D5 (color: $p<0.001$; aspect: $p<0.05$) becoming mostly bloody and cloudy in D3 and D5. However in TG the difference was significant just between D1 and D3 (color and aspect: $p<0.05$), showing an improvement of synovial fluid in D5 (color and aspect: $p>0.05$). Viscosity and mucin clot evaluation showed significant change in CG between D1 and D3 (viscosity: $p<0.01$; mucin clot: $p<0.05$) and between D1 and D5 (viscosity: $p<0.01$;mucin clot: $p<0.01$). In TG no significant difference of viscosity and mucin clot was observed over the moments, showing an early improvement of synovial fluid quality. The Serum Amyloid A concentration showed an extremely significant increase in CG ($p<0.001$) when compared D1 (1217.13±664.47µg/mL) and D3 (42423.80±52309.31µg/mL). The comparison between D1 and D5 in CG, and across moments in TG, had no statistical difference. The PGE_2 eicosanoid remained statistically unchanged all over the time. Urea showed significant increase in D3 when compared to D1 ($p<0.001$) in CG, and had no variation in TG. The physiotherapy protocol minimized the inflammatory mediators and provided minor alterations in synovial fluid after arthroscopy.

INDEX TERMS: Physiotherapy, arthroscopy, biomarkers, rehabilitation, horses, surgery.

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RESUMO.- [**Protocolo fisioterápico durante o período pós-operatório inicial de cirurgia artroscópica em equinos.**] Este estudo avaliou os efeitos de um protocolo fisioterápico, aplicado em articulações com osteocondrite dissecante, submetidas à artroscopia. Foram utilizados 12 cavalos, totalizando 20 articulações, divididas em dois grupos homogêneos de acordo com a graduação da lesão articular. O grupo tratado (GT) recebeu o protocolo fisioterápico (crioterapia, movimentação passiva e exercício controlado) que se iniciou imediatamente após a recuperação anestésica e se estendeu por cinco dias. O grupo controle (GC) permaneceu em repouso na baia, pelo mesmo período. Exame físico da articulação e análise do líquido sinovial foram utilizados para avaliar o tratamento. O exame do líquido sinovial consistiu em análise física (cor, aspecto e viscosidade), avaliação do coágulo de mucina e concentrações de amiloide sérica A, prostaglandina E_2 e ureia. Amostras de líquido sinovial foram colhidas por artrocentese no início do procedimento cirúrgico (D1) e após 48 (D3) e 96 horas (D5) do procedimento cirúrgico. Antes da artroscopia e diariamente no período pós-operatório, as articulações foram avaliadas por exame físico: temperatura superficial (°C), ângulo de flexão (graus), circunferência (centímetros). A avaliação física das articulações não apresentou diferença significativa entre os grupos nem ao longo dos dias em cada grupo. Nas análises do líquido sinovial, observou-se uma variação diferente entre os momentos em cada grupo porém sem diferença significativa entre os grupos. A cor e o aspecto tiveram resultados semelhantes ao longo do tempo, no GC houve uma alteração significativa quando comparados D1 e D3 (cor e aspecto: $p<0,001$) e D1 e D5 (cor: $p<0,001$; aspecto: $p<0,05$) tornando-se sanguinolento e turvo na maioria das amostras em D3 e D5. Já no GT, houve diferença significativa apenas entre D1 e D3 (cor e aspecto: $p<0,05$), demonstrando melhora no líquido sinovial em D5 (cor e aspecto: $p>0,05$). A viscosidade e o coágulo de mucina apresentou alteração significativa no GC entre D1 e D3 (viscosidade: $p<0,01$; coágulo de mucina: $p<0,05$) e entre D1 e D5 (viscosidade e coágulo de mucina: $P<0,01$). No grupo tratado não foram observadas alterações significativas em viscosidade e coágulo de mucina, ao longo dos momentos, demonstrando uma melhora precoce na qualidade do líquido sinovial. A amiloide sérica A apresentou um aumento extremamente significante no GC ($p<0,001$) quando comparados D1 (1217,13±664,47µg/dL) e D3 (42423,80±52309,31µg/dL). Quando comparados D1 e D5 no GC e ao longo do tempo no GT não foram observadas diferenças significativas. A concentração de PGE_2 permaneceu sem alterações. As mensurações de ureia apresentaram aumento significativo em D3 quando comparado a D1 ($p<0,001$) no GC e não apresentou variação no GT. O protocolo fisioterápico minimizou os mediadores inflamatórios e proporcionou menor alteração do líquido sinovial após artroscopia.

TERMOS DE INDEXAÇÃO: Fisioterapia, artroscopia, articulação, biomarcadores, reabilitação, equinos, cirurgia.

INTRODUCTION

Degenerative joint disease is a great topic in equine medicine as in humans and it is highly associated with previous acute traumas. People who experience a capsular or ligament trauma presents 10 times more chances of developing osteoarthritis. When the trauma causes an intra-articular fracture, those chances are 20 times more (Anderson et al. 2011). The osteoarthritis physiopathogeny has being studied to permit understand the mechanisms that trigger the acute arthritis and those which perpetuate the disease, resulting in cartilage degeneration and loss of joint function (Olson et al. 2014).

Given the similarity between human and equine joint disease development and considering the athletic use and large volume joints, this specie has being a good model for studies that are intended to elucidate the articular response to injury (Kawcak et al. 2008, Grauw et al. 2009).

The osteochondritis dissecans (OCD) is a developmental orthopedic disease with great importance in sports horses. It has been defined as a focal failure of osteochondral ossification in the articular epiphyseal complex and progress in most of the times for fragmentation inside the joints (Van Weeren 2006, Denoix et al. 2013). Recently has been shown that horses with articular fragment but without pain or joint distention present increased biomarkers for cartilage degradation in the synovial fluid (Machado et al. 2012). When not treated it progress to osteoarthritis and loss of function just like humans post-traumatic arthritis progresses (Van Weeren 2006).

The use of molecular biomarkers in studies focusing on articular dynamics is increasing. Prostaglandin E_2 has been shown as a great joint disease marker, and has different levels according to the progress of the disease, as chronic, acute or with cartilage lesion already. It has been more sensitive and more specific than TNF-α and IL-1β (Bertone et al. 2001). This eicosanoid was related with the etiology of the osteoarthritis (Kirker-Head et al. 2000). Stålman et al. (2011) affirmed that there are positive correlation between the PGE_2 liberation into synovial fluid and the capsular temperature.

Serum Amyloid A (SAA) draws especial attention and is correlated to rheumatoid arthritis during the inflammatory phase when there are angiogenesis and also migration of factors that contribute to perpetuate the inflammation (Mullan et al. 2010). SAA is strongly related to cartilage matrix degradation being one of the factors responsible to induce metalloproteases (MMP) migration inside joint cavity (Connolly et al. 2012). In horses, the acute phase protein, Serum Amyloid A, has also been related to articular disease.

Synovial fluid of healthy horses had unmeasurable values of SAA while injured joints had those values varying according to the disease, and horses that had joint infection reached higher values (Jacobsen et al. 2006). Similarly, serum dosage of SAA varies according to the inflammation generated by the surgical procedure. The more invasive the procedure with large soft tissue trauma higher is SAA values (Jacobsen et al. 2009).

In humans, the physiotherapy is largely used even before articular surgery and extend for weeks during postoperative period. Cryotherapy is associated to pain reduction after arthroscopy (Fang et al. 2012). Passive range of motion was correlated with earlier improvement of joint function after shoulder arthroplasty (Denard & Lädermann 2016) and controlled exercise has been indicated since the first postoperative day (Wilcox et al. 2005). In equine medicine, physical therapies has been used to prevent sports injury and for rehabilitation. However, there are no studies evidencing the importance of using rehabilitation protocol after joint surgery.

This study objective was to test the efficacy of a physiotherapy protocol applied during the initial postoperative period of

joints with osteochondritis dissecans submitted to arthroscopic treatment.

MATERIALS AND METHODS

Ethics statement. The present work was approved by Ethic Committee in the use of animals of the School of Veterinary Medicine and Animal Science of University of São Paulo – USP (2580/2012), and was carried out in accordance with USP guidelines.

Selected joints. Seven metacarpophalangeal, seven metatarsophalangeal and six tibiotarsal joints with diagnosis of osteochondritis dissecans referred to Large Animal Surgery Service of the University of São Paulo and submitted to arthroscopic removal of the fragment and subchondral bone curettage were used for this prospective study.

Study design. During the postoperative period the horses of the control group (CG) rested in stall receiving non-steroidal anti-inflammatory drug (Phenylbutazone 4.4mg/kg, once a day, for 3 days) and antibiotics (amikacin 15mg/kg, once a day, for 5 days). On the other side, the horses of the treated group (TG) were submitted additionally to a physiotherapy protocol.

The physiotherapy protocol started right after anesthetic recovery and lasted until the fifth day of postoperative period. It consisted of cryotherapy, passive range of motion and controlled exercise. The cryotherapy was placed over the treated joint using reusable gel packs (Mercur™) for the first 72 hours. In the first day, it lasts twenty minutes, every two hours. During the other days, the frequency was every four hours. The passive range of motion started on the second day and it was performed fifteen flexions, twice a day, until the last day. The controlled exercise was hand walking for ten minutes twice a day, which started on the third day.

The studied joints were directed to TG or CG based on intra-articular damage evaluation (Silva 2014) performed during arthroscopy, and based on type of joint, metacarpophalangeal (MCP), metatarsophalangeal (MTP) or tibiotarsal (TT) aiming homogeneous groups. The score was established depending on synovial membrane (color, volume and villus number), articular cartilage surface integrity, and subchondral bone aspect and fragment characteristics (number, size and attachment). As worse the changes on those structures, higher the articular score, ranging from zero to 63 (Silva 2014). The joints had the following distribution in the groups: CG (3MCP, 4MTP and 3TT) and TC (4MCP, 3MTP and 3TT), totaling 10 joints each.

Surgical procedure was always performed during the morning and all animals were equally prepared.

Starting on preoperative period and lasting until fifth day, the joints of both groups were submitted to physical evaluation with the measurement of flexion angle using goniometry, articular circumference in centimeters and superficial temperature with thermography (Flir 440™). Physical evaluation was performed every morning before the rehabilitation protocol starts. Synovial samples was collected before arthroscopy (D1), 48hours (D3) and 96 hours after (D5).

Physical evaluation of the synovial fluid. The synovial fluid was evaluated for color, aspect and viscosity using scores from zero to four (Table 1), where zero was the best and four the worst quality for each variant.

Evaluation of mucin clot of the synovial fluid. The volume of 100μL of synovial fluid was added to 2mL of 2% acetic acid. The quality was graded from one to four, where one is a stable and well defined clot that remain on the liquid superficies after slightly shake and four is a flaccid clot that submerge on fluid even without shaking the tube (Van Pelt 1962).

Table 1. Scores for color, aspect and viscosity of the synovial fluid

Score	Physical evaluation of synovial fluid		
	Color	Aspect	Viscosity
1	Light yellow	Clear	>5cm
2	Yellow	Slightly cloudy	btw 2 e 5cm
3	Xanthochromic	Cloudy	<2cm
4	Bloody	-	-

Prostaglandin E_2 and Serum Amyloid A quantification in the synovial fluid. For both analysis the ELISA method was used. The synovial fluid was centrifuged (4000G rotation for 15 minutes at 4°C) and samples was stored at -80°C until analyses. For quantification of Serum Amyloid A de Multi-species Tridelta ™ - EUA Kit was used and for Prostaglandin E_2 was used the Cayman Chemical Company™ EUA Kit.

Synovial fluid concentration of urea. The urea concentration was measured using automatic biochemical analyzer Rendox RX Daytona.

Statistical analysis. Basal comparison for age and articular lesion score were obtained using t-student test and between categorical variables as joint type was used Chi-square test. The evaluation along the time and between the groups was performed with Kruskal-Wallis. To evaluate if the effect along the time was different between groups the Dunn test of multiple comparisons was used. The significance level was $p<0.05$ for all analyses.

RESULTS

The articular score obtained for distribution between the two homogeneous groups ranged from 4 (minor damage) to 15 (more damage). The mean of scores were 7.8 (±2.35) in the CG and 7.0 (±3.25) in the TG. There was no significant difference between groups (P=0.532). The horses were less than 10 years old and there was no difference between groups (p=0.809).

The physical evaluation of the joint, including flexion angle, circumference and superficial temperature didn't shown significant difference between groups neither when compared daily evaluation in the same group.

The synovial fluid parameters showed different presentation over the time. There was difference comparing moments for each group but there was no significant difference between groups.

Synovial fluid physical analysis

Color. In CG the color worsened from D1 (90% score 1; 10% score 2) to D3 (20% score 3; 80% score 4) with $p<0.001$ and that difference remained when D5 (20% score 2; 30% score 3 and 50% score 4) and D1 were compared. In TG the color worsened only from D1 (80% score 1; 20% score 2) to D3 (71% score 3; 29% score 4) with $p<0.05$. However in D5 (62.5% score 2; 25% score 3; 12.5% score 4) the color improved showing no difference when compared with D1 ($p>0.05$).

Aspect. The fluid aspect evolution was similar to the color. In CG it got more cloudy from D1 (90% score 1; 10% score 2) to D3 (30% score 2 and 70% score 3), with $p<0,001$, and when

compared D1 with D5 (10% score 1; 40% score 2; 50% score 3) p<0.01. However in TG the fluid became more cloudy from D1 (70% score 1; 30% score 2) to D3 (28.6% score 2; 70% score 3) with p<0.01, and improved to D5 (87.5% score 2; 12.5% score 3) showing no difference when compared with D1 (p>0.05).

Viscosity. The synovial fluid viscosity decreased only in CG when compared D1 (80% score 1 and 20% score 2) with D3 (20% score 2; 70% score 3) and D1 with D5 (10% score 1; 20% score 2 and 70% score 3) with p<0.01. For the TG there was no difference of synovial fluid viscosity across moments.

Quality of mucin clot of synovial fluid

The mucin clot variation was similar to viscosity, in CG the mucin clot quality worsened when compared D1 and D3 (p<0.05) and between D1 and D5 (p<0.01). In TG however, these changes were not observed (Fig.1) and there was no difference across moments.

Serum Amyloid A and Prostaglandin E_2

The Serum Amyloid A exhibit an extremely significant rise (p<0.001) only in CG when compared D1 (1217.13±664.47µg/mL) and D3 (42423.80±52309.31µg/mL). Between D1 and D5, and across moments in TG it remained unchanged statistically (Fig.2). The PGE_2 didn't altered significantly across moments in both groups (Fig.3)

Urea concentration in synovial fluid

The synovial fluid urea raised was extremely significant in CG at D3 (33.54±8.21mg/dL) in comparison to D1 (23.35±3.38mg/dL) with p<0.001, and in D5 returned to baseline 29.7±7.47mg/dL. In TG it remained unchanged across moments (Fig.4).

DISCUSSION

The knowledge of articular postoperative injury and the inflammatory response control is important to minimize cartilage degradation and post-traumatic osteoarthritis. The present work evaluated the effects of arthroscopy as well the protective effects of physical therapies to the joint.

We observed that arthroscopy even as a minimally invasive procedure modifies the synovial biomarkers and the synovial fluid quality in all of the operated joints, mainly on the third day, what characterize an acute injury. The establishment of an anti-inflammatory therapy was inherent to the routine of the Veterinary Hospital and even with the use of NSAIDs therapy we found synovial fluid changes in both groups.

Although in other studies the PGE2 has been associated with inflammatory process and MMP increasing into the joint (Grauw et al. 2009), we did not found any significant difference between groups and neither over the time. Our results may be associated to the use of phenylbutazone.

The Serum Amyloid A is an acute phase protein with great importance for the articular space and even in others tissues. It is responsible for induction and maintenance of the inflammation in horses and humans. This protein is believed to be one of the responsible mediators for the leukocyte and monocyte chemotaxis to the inflamed joint and it is able to activate the cytokines (TNF-α, IL-6) and MMP production. Those components perpetuate inflammation that results in articular cartilage degradation (Migita et al. 2009, Connolly et al. 2012). In this work, we observed significant increase in Serum Amyloid A (SAA) concentrations only in D3 of CG. Lower increase of this protein in TG was assigned to

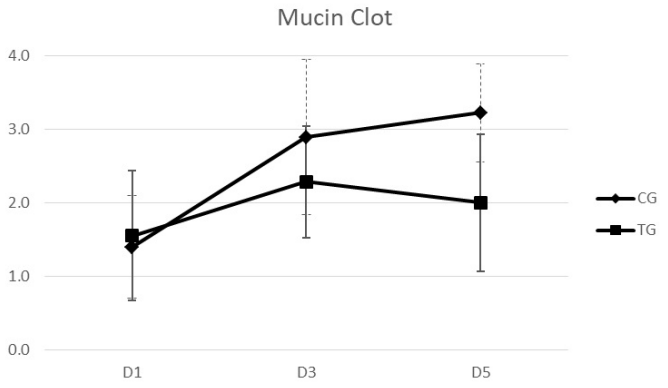

Fig.1. Mucin clot quality evaluation using a scale of 1 to 4 where one is the best quality and four is the poorest quality. D1, D3 and D5 = moments of synovial fluid analysis, before surgery, 48 hours after surgery and 96 hours after surgery respectively. CG = control group, TG = treated group.

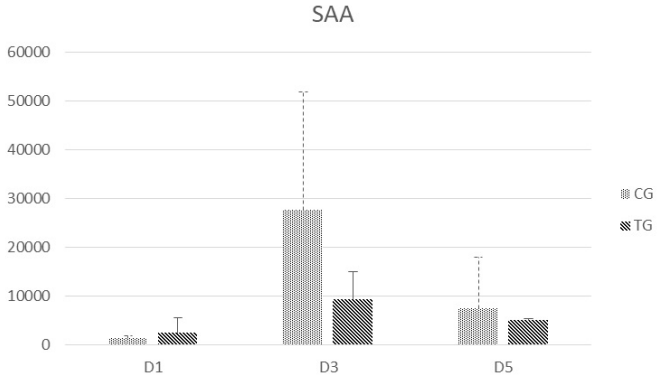

Fig.2. Serum Amyloid A means and standard deviations are represented in µg/mL. D1, D3 and D5 = moments of synovial fluid analysis, before surgery, 48 hours after surgery and 96 hours after surgery respectively. CG = control group, TG = treated group.

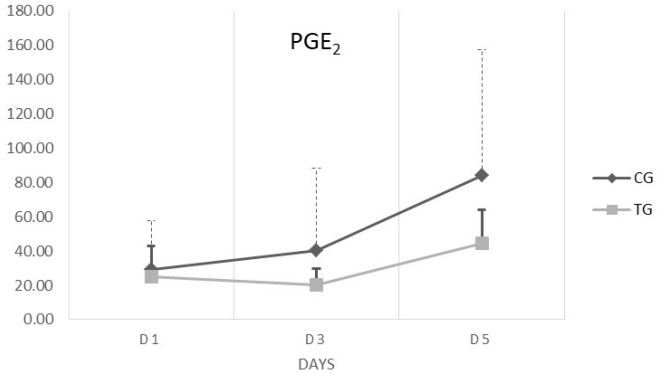

Fig.3. PGE_2 concentration means and standard deviation values are represented in pg/mL. D1, D3 and D5 = moments of synovial fluid analysis, before surgery, 48 hours after surgery and 96 hours after surgery respectively. CG = control group, TG = treated group.

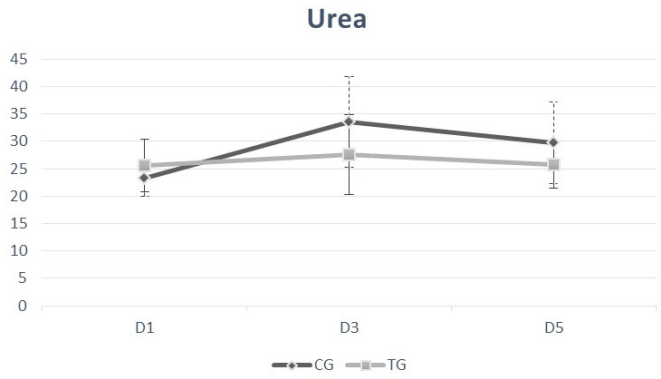

Fig.4. Urea concentration means and standard deviation are shown in µg/dL. D1, D3 and D5 = moments of synovial fluid analysis, before surgery, 48 hours after surgery and 96 hours after surgery respectively. CG = control group, TG = treated group.

physiotherapy protocol, especially to the cryotherapy which was intense in the first hours. Previous studies in humans with rheumatoid arthritis, provided that cryotherapy decreased the proinflammatory factors as TNF-α and IL-6, additionally it generated pain relief and functional improvement to treated joints (Jastrazabek et al. 2013).

SAA proved to be a good marker for acute articular inflammation. Despite to be non-significant, even in TG was possible to observe the increase in this protein concentration 48 hours after surgical procedure returning to the baseline at day 5. Our results is in consonance of Lindegaard et al. (2010) that observed the SAA dynamics, after synovitis induction, rising to the top at 48 hours and returning to baseline after 5 days.

The urea measurement is helpful as a correction factor for synovial fluid dilution, when there is an articular effusion and when it is necessary to flush the joint to recover the synovial fluid (Kraus et al. 2002). In this work the urea concentration did not decrease indicating that in both groups the joints did not had effusion. However there was significant increased urea concentration in synovial fluid of CG in D3. We believe that it is related to more intense inflammation inside those joints generating vasodilatation and increased synovial vascular permeability so increasing serum components to the joint. Oliviero et al. (2012) found that inflammatory arthritis feature higher concentration of plasmatic compounds (lipoproteins) because of the increased vascular permeability in those joints.

Considering the physical analysis of the synovial fluid is possible to found important information of joint dynamics, including the nature of disease and lesion extension. The synovial fluid is responsible for the nutrients distribution all over the joint surface and has a mechanical function. A poor synovial fluid quality reflects in the structures nutrition and protection. The bloody color was observed in 80% of the samples in CG and only 30% in TG at D3. This result is particularly important because as worse is the hemarthrosis more intense will be the cartilage degradation and greater will be the chance to progress to post-traumatic osteoarthritis (Swärd et al. 2014). The cryotherapy showed a beneficial effect in treated group with less intense bleeding, observed in D3, and faster improvement in synovial quality in D5 compared to CG.

The synovial fluid viscosity is closed related to the quality and quantity of hyaluronic acid in the fluid. Acute inflammations degrade the hyaluronic acid molecule and increase the fluid plasma decreasing its viscosity (Steel 2008). In the present work the synovial fluid viscosity changed only in the CG, with significant decrease over the time. In TG was not observed significant viscosity changes, the fluid was rapidly replaced after surgical procedure and remained with a good quality over the postoperative period.

The mucin clot results were similar to the viscosity. It is believed that the cryotherapy and range of motion promotes better fluid diffusion and better nutrition of all joint structures. The physical analysis and mucin clot of synovial fluid of this study contributes to this affirmation once in TG those parameters had more regular values and recovered faster, with D5 similar to D1.

The range of motion of joints also prevents synovial adhesion formation and limb muscular contracture (Porter 2009). Our results did not show difference of articular flexion angle. These results corroborate to the related by Kim at al. (2012) after human arthroscopy.

The circumference of the joints has been correlated to inflammation, edema formation and increased inflammatory cytokines of the synovial fluid (Szekanecz et al. 2000). Jones et al. (1993) observed enlargement of joints after eight days of carpus arthroscopy. Our results did not show difference in circumference of joints. It may be because all joints remained with bandage until the last measurement in D5 reducing edema formation and joint enlargement.

The superficial temperature did not show difference between groups or across moments. Once the thermography was performed only early in the morning, it is possible that the superficial temperature of the joints returned to normal values after the cryotherapy performed in the evening. Khoshnevis et al. (2015) found that the superficial temperature of knees and ankles submitted to cryotherapy returns to normal values 25 minutes after the end of the technique.

CONCLUSIONS

The performed physiotherapy protocol on the treated group showed beneficial effects in clinical and laboratory parameters.

The synovial fluid quality of treated group revealed minor inflammatory response, earlier recover of aspect and color and less alteration in viscosity and mucin clot.

Though the obtained results lack of significant difference between groups, the authors encourage the use cryotherapy, passive range of motion and controlled exercise during the initial postoperative period of arthroscopy once we had minimal parameters alteration over the time only in the treated group.

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Original Article
Animais de Produção/*Livestock Diseases*

Impairment on nuclear maturation rate in oocytes from cows naturally infected by bovine herpesvirus 1 (BoHV-1)[1]

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ABSTRACT.- Mendes V.R.A., Costa E.P., Queiroz V.L.D., Silva Júnior A., Alves S.V.P., Guimarães J.D. & Gomes L.L. 2018. **Impairment on nuclear maturation rate in oocytes from cows naturally infected by bovine herpesvirus 1 (BoHV-1).** *Pesquisa Veterinária Brasileira 38(12):2207-2212*. Universidade Federal de Viçosa, Avenida PH Rolfs s/n, Campus Universitário, Centro, Viçosa, MG 365700-000, Brazil. E-mail: vanessalopq@gmail.com

Bovine herpesvirus 1 (BoHV-1) is an important bovine pathogen that is responsible for causing respiratory diseases and reproductive failures. The presence of BoHV-1 in an *in vitro* embryo production system affects fertilization, maturation, and embryonic development. The objective of this study was to evaluate the developmental capacity of oocytes from naturally infected cows with no reproductive history. Moreover, this study investigated the presence of viral DNA in cumulus oophorus complexes (COCs). Experimental groups were differentiated by titrating the antibodies detected through seroneutralization assays, establishing three groups: seronegative animals (titer lower than 2), low titer (2 to 8), and animals with a titer above or equal to 16. COCs were obtained from 15 donors during 22 sessions of ultrasound-guided follicular aspiration. DNA was extracted from a pool of COCs obtained from all aspirations from the same donor as well as from whole blood and nested PCR reactions were performed. Only COCs with a compact layer of cumulus cells, an intact zona pellucida, and homogeneous cytoplasm were selected for *in vitro* culture and evaluation of nuclear maturation rate. After culturing for 24 hours, the oocytes were fixed and stained to evaluate the meiotic cell cycle stage. Oocytes that showed a chromosomal configuration in metaphase II were considered to have reached nuclear maturation. Compared with the other groups, the oocyte nuclear maturation rate in animals with a titer greater than or equal to 16 (50%) was compromised ($P< 0.05$). However, the viral titer did not influence the maturation rate of bovine oocytes in animals exhibiting low titration (62.2%) when compared with the control group (76.7%). Viral DNA was not observed in the blood samples but was detected in the COC pool from three seropositive donors. In view of the results obtained, we conclude that natural infections by the BoHV-1 virus can compromise the nuclear maturation rate in cows, depending on the titration levels of antibodies against the virus. Moreover, viral DNA could be present in COCs, contradicting the hypothesis that seropositive animals with no history of clinical symptomatology pose a negligible risk of transmitting BoHV-1 by COCs.

INDEX TERMS: Nuclear maturation rate, oocytes, bovine herpesvirus 1, BoHV-1, IBR, maturation, cattle, pathology.

RESUMO.- [**Comprometimento na taxa de maturação nuclear em ovócitos de vacas naturalmente infectadas pelo herpesvírus bovino 1 (BoHV-1).**] Herpesvírus bovino 1 (BoHV-1) é um importante patógeno bovino, responsável por causar doenças respiratórias e falhas reprodutivas. A presença do BoHV-1 em sistema de produção *in vitro* de embriões afeta a fertilização, a maturação e o desenvolvimento embrionário. O objetivo deste estudo foi avaliar a capacidade de desenvolvimento de ovócitos oriundos de vacas infectadas naturalmente sem histórico reprodutivo. Além disso, este estudo investigou a presença do DNA viral em Complexos *Cumulus Ooforus* (COCs). Os tratamentos foram definidos a partir do título

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de anticorpos detectados pelos ensaios de soroneutralização, sendo estabelecidos três grupos: animais soronegativos (título menor do que 2), título baixo (2 a 8) e animais com título maior ou igual a 16. Os COCs foram obtidos de 15 doadoras durante 22 sessões de aspiração folicular guiada por ultrassom. A extração do DNA foi realizada em um *pool* de COCs de todas as aspirações de uma mesma doadora e no sangue total para a realização das reações de *Nested*-PCR. Para avaliação da taxa de maturação nuclear, foram selecionados para o cultivo *in vitro* somente os COCs com camada compacta de células do *cumulus*, zona pelúcida íntegra e citoplasma homogêneo. Após 24 horas de cultivo, os ovócitos foram fixados e corados em lâmina para a avaliação do estádio do ciclo celular meiótico. Os ovócitos que apresentaram configuração cromossômica em metáfase II foram considerados como tendo alcançado a maturação nuclear. Verificou-se comprometimento na taxa de maturação nuclear ovocitária (P<0.05) nos animais de título maior ou igual a 16 (50%). No entanto, não houve influência do título viral na taxa de maturação de ovócitos bovinos em animais que apresentaram titulação baixa (62,2%) quando comparados com o grupo controle (76,7%). O DNA viral não foi identificado nas amostras de sangue, mas foi detectado no *pool* de COCs de três doadoras soropositivas. Diante dos resultados encontrados conclui-se que vacas infectadas naturalmente pelo vírus BoHV-1 apresentam comprometimento na taxa de maturação nuclear, dependendo do grau de titulação de anticorpos contra o vírus. Ademais, o DNA viral pode estar presente em COCs contrariando a hipótese de que animais sorologicamente positivos e sem histórico de sintomatologia clínica oferecem risco negligível de transmissão do BoHV-1 por COCs.

TERMOS DE INDEXAÇÃO: Taxa de maturação nuclear, ovócitos, vacas, herpesvírus bovino 1, BoHV-1, IBR, maturação, bovinos, patologia.

INTRODUCTION

Bovine herpesvirus 1 (BoHV-1) is the causative agent of infectious bovine rhinotracheitis (IBR). It is widespread in herds around the world (Ackermann & Engels 2006, Nandi et al. 2011, Ravishankar et al. 2013).

The infection has several clinical forms that can compromise the respiratory system and genitals of infected animals. In the context of reproduction, BoHV-1 could cause repetition of estrus at regular or irregular intervals, embryonic death or abortions (Miller & Van der Maaten 1986). The economic losses associated with the lost productivity and reproductive problems caused by the infection are extensive. An average financial expense of US $379.00 per cow is estimated (Can et al. 2016).

Brazil currently has the largest commercial cattle herd in the world, with 218.2 million animals in 2016 (IBGE 2017). National data obtained from serological surveys revealed a prevalence of BoHV-1 of 42.2% in São Paulo (Mueller et al. 1981), 52.9%, 71.3%, and 81.7% in Rio Grande do Sul (Ravazzolo et al. 1989, Vidor et al. 1995, Piovesan et al. 2013, respectively). In the state of Minas Gerais, studies conducted in 335 municipalities showed that 93.4% of the animals had positive serology for BoHV-1. In Paraná, 71.3% of the 2,018 unvaccinated herds were positive for BoHV-1 (Rocha et al. 2001).

All Alphaherpesvirinae viruses can establish latent infections, making the animals lifelong carriers and potential disseminators of the virus. Latently infected animals, after primo-infection, usually carry the viral genome in the episomal form, especially in the trigeminal and sacral ganglia. Under conditions of stress or corticotherapy, BoHV-1 can be reactivated, leading to episodes of viral re-excretion, facilitating transmission while hindering control (Kramps et al. 1996, Jones et al. 2000, Winkler et al. 2000, El Mayet et al. 2017).

Vaccination is recommended in locations where herpesvirus infection is endemic (Patel 2005). It is noteworthy that in Brazil there is no program for the control and eradication of BoHV-1, nor is there any commercial availability of marked vaccines (distinguishing vaccinated animals from those naturally infected), strategies which have allowed countries such as Austria, Denmark, Finland, Switzerland, Sweden, and Norway to achieve a virus-free status (Can et al. 2016).

The use of reproductive biotechniques such as artificial insemination and embryo transfer have reduced pathogen transmission. However, the risks of these techniques in the transmission of pathogens should not be disregarded (Thibier & Wrathall 2012). During the last few years, it has been shown that BoHV-1 can be present in the material used in the *in vitro* production system (Vanroose et al. 1999), associated with uterine tube epithelial cells and oocytes (Bielanski et al. 1993), in the follicular fluid (Weber et al. 2013), and in the spermatozoa of infected bulls (Rocha et al. 1998).

Furthermore, BoHV-1 can replicate in cumulus oophorus cells (COCs) and cause the cytopathic effect (cell lysis) characteristic of herpesviruses (Tsuboi et al. 1992, Tsuboi & Imada 1997). Moreover, the viral DNA of BoHV-1 has been detected in COCs from seropositive cows with no history of vaccination or clinical symptomatology of the disease (Oliveira 2014).

In this context, this study investigated the effects of using COCs from naturally infected cows on the oocyte nuclear maturation rate. Moreover, the presence of viral DNA was also observed in COCs of these animals.

MATERIALS AND METHODS

Ethics statement. All of the experimental procedures were conducted following the ethical principles adopted by the National Council for Animal Experimentation, and with the authorization of the Committee of Ethics in Animal Use of the Federal University of Viçosa under protocol number 112/2011.

Animals and procedures. Fifteen adult Holstein x Gir crossbred bovine females of different blood grades, were used in different phases of the estrous cycle. These animals came from herds located in Zona da Mata (State of Minas Gerais, Brazil), and had not been vaccinated against BoHV-1 to avoid an interference of vaccination with the results. None of the cows presented clinical symptoms of IBR.

Blood samples were collected in tubes without anticoagulant for the seroneutralization technique and with anticoagulant for the nested PCR technique. Both were transported at room temperature and centrifuged for five minutes at 1500G for the separation of blood serum.

For the selection of the oocyte donor cows, blood samples were collected and serum neutralization was performed in microplates according to the methodology proposed by the "Manual of Standard for Diagnostic Tests and Vaccines" (OIE 2010). Then, the animals were divided into three groups according to the title of antibodies: five animals with low title (2 to 8), seven with title greater than or

equal to 16 (Santos et al. 2014) and three serum-negative animals were used as controls.

Follicular aspiration by Ovum Pick-up (OPU). The follicular aspirations were conducted after the animals were properly restrained and intercoccygeal epidural anesthesia was used. For this procedure, an ultrasound device (DPS Mindray DP-2200VET) equipped with a micro convex transducer adjusted to an eight MHz frequency and coupled to an intravaginal guide was used. A vacuum pump (pressure of 80mmHg, corresponding to 14mL of water/min), and 40x10 (19G) needles were used. Follicles larger than two millimeters were punctured and the material collected was transported in 50mL plastic tubes (Falcon) containing 5mL of phosphate buffered saline (PBS) and 1000IU heparin at 37°C. The aspirated contents were extensively washed with PBS solution to remove blood cells.

Experimental design. The COCs were screened under a stereoscopic microscope and transferred to another plate containing TALP-Hepes medium for maintenance. Subsequently, they were categorized following the methods of Costa et al. (1997a). The oocytes destined for *in vitro* maturation were packed in 0.25mL straws in TALP-Hepes medium and packed in an embryo transporter at a controlled temperature of 38°C. The other COCs were packed in microtubes and frozen at -20°C for later detection of viral DNA by nested PCR.

Nested PCR was performed to detect the viral DNA of BoHV-1. Nested PCR for BoHV-5 was also performed, since cross-reactivity could occur through the seroneutralization.

In the laboratory, the straws containing the COCs were cut, and the contents were transferred to a culture dish on a hot plate at 38°C containing TALP-Hepes. The COCs were re-screened, washed in TALP-Hepes microdroplets and transferred to a culture dish with a pre-equilibrated TCM 199 medium supplemented with 10% serum of cow in estrus and 10µg/ml of FSH (Costa 1994). The COCs were cultured for 24 hours in an incubator at 38.5°C in an atmosphere of 5% CO2, 95% atmospheric air and 95% humidity. The nuclear maturation rate of the COCs was then evaluated.

The cultured COCs were transferred to a multiwell plate containing 300µL of TALP-Hepes medium for nuclear maturation rate evaluation. Then, the COCs were subjected to the cumulus oophorus removal procedure (Costa et al. 1997a), and were hypotonized, fixed on a slide, and stained with 2% orcein, following methods described by Costa et al. (1997b). The slides were read under an optical microscope with a magnification of 1000X in immersion. The oocytes that presented a chromosomal configuration in metaphase II were considered to have reached nuclear maturation.

Viral DNA was detected using nested PCR to check for the presence of BoHV-1. Total DNA was extracted from the COCs following the specifications of the manufacturer of the "SV Wizard Genomic" (Promega®) extraction kit. The oligonucleotide sequence and protocol for the nested PCR reactions were standardized as described by Campos et al. (2009).

Statistics. The qualitative variables were compared in contingency tables and analyzed by the chi-square test with the significance level set at 5% probability (Sampaio 2002).

RESULTS

A total of 674 COCs were recovered from 15 donors during 22 OPU sessions. Approximately 400 COCs were evaluated by nested PCR, and 165 were evaluated to determine the nuclear maturation rate. The oocytes presenting a particular chromosomal configuration at metaphase II were considered as having undergone nucleus maturation. Regarding the nuclear maturation rate, however, the number of oocytes from each treatment was enough for an appropriate interpretation. The variable "maturation rate" is dichotomous, requiring analysis by the chi-square test.

The results obtained following *in vitro* culture of oocytes are shown in Table 1. Animals that had titers above or equal to 16 demonstrated a compromised oocyte nuclear maturation rate.

Seronegative animals did not contain viral DNA in the analyzed samples. However, a positive reaction was observed by nested PCR (Fig.1) in three animals (3/12), revealing the presence of BoHV-1 DNA in the COC pool. Out of these positive animals, two had low titers against BoHV-1 (2 of 3), and one animal had a titration greater than or equal to 16 (1 of 3).

DISCUSSION

The possible interference of infectious diseases in reproductive biotechnologies is a cause for concern, especially when gametes and embryos, potential disseminators of these diseases, are affected (Perry 2005).

Brazil assumes a prominent role in using biotechnology. In 2014, Brazil was considered the world's largest producer of bovine embryos, holding 366,517 embryos, which corresponds to about 70% of the world's total (IETS 2015).

Researchers using the polymerase chain reaction (PCR) test to detect BoHV-1 DNA have concluded that the risk of transmission of BoHV-1 by COCs and follicular fluid from seropositive cows without clinical symptomatology is irrelevant (Oliveira et al. 2016), since the presence of viral DNA was not observed in any of their tested samples.

In contrast, the presence of viral DNA was reported in 4.3% (5/117) of the samples of ovarian tissue, 0.9% (1/115) of

Table 1. Nuclear maturation rate of animals with different antibody titers to BoHV-1

Treatment	Evaluated oocytes	Nuclear maturation	
		N	%
Negative titer (<2)	30	23	76.7[a]
Low titer (2-8)	45	28	62.2[a]
Titer >16	90	45	50.0[b]
TOTAL	165	96	58.2

[a, b] Percentiles with different superscribed letters indicate a significant difference (P<0.05) by chi-square analysis.

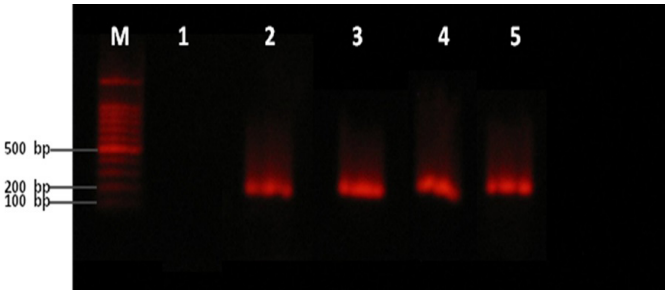

Fig.1. Visualization of PCR products on a 1% agarose gel, showing the detection of BoHV-1 DNA in COCs of seropositive cows (M = marker, 1= negative control, 2 = positive control, 3-5 = positive COC samples).

oocytes and in none of the follicular fluid samples of naturally infected seropositive cows without symptoms (Pereira et al. (2015). The same outcome was reported in this experiment from nested PCR results. The presence of BoHV-1 viral DNA was observed in pooled COCs from naturally infected seropositive animals without clinical symptoms of the disease.

Nested PCR is more sensitive than other techniques routinely used to detect BoHV-1, since it is based on two reactions derived from a DNA template. In addition, this technique is more suitable for molecular research on structures with a low viral load (Rocha et al. 1999, Moore et al. 2000, Olmos et al. 2003, Takiuchi et al. 2005).

The nested PCR method enabled the identification of the genomes of BoHV-1, BoHV-5, or both in 85.9% (213/248) of the animals, whereas the seroneutralization method identified only 44.8% (111/248), and the ELISA method identified 51.2% (127/248) (Puentes et al. 2016). Puentes et al. (2016) emphasized that the detection of latently infected animals still depends on the postmortem identification of viral DNA in the trigeminal ganglion. Thus, the non-detection of viral DNA in the blood in this study can be explained by the brief period of acute disease manifestation in naturally infected animals or by the viral latency state in the sensorial and autonomic ganglia - a typical mechanism of the subfamily Alphaherpesvirinae (Spear 2004).

Oocyte infection by BoHV-1 has been reported to compromise embryonic development in *in vitro* and *in vivo* production (Bielanski et al. 1997, Makarevich et al. 2007). However, our results indicate that the deleterious effects of the virus on the oocyte also include compromised oocyte development, even before fertilization, by impacting the oocyte nuclear maturation rate. Moreover, this effect was dependent on the serological antibody titer.

Cumulus cells play a key role in the substrate supply, transport, and production of chemical components for the oocyte, such as microRNAs (regulators of genes involved in maturation processes) during oocyte maturation (Gilchrist et al. 2016). In addition, inhibitory and meiosis-inducing factors effect maturation through the communication of cumulus cells with the oocyte mediated by gap junctions (Mahmoudi et al. 2005).

The compromised oocyte nuclear maturation rate observed in this study may be explained by the interaction of BoHV-1 with cumulus cells. The virus has glycoproteins in its envelope that are responsible for adsorption and fusion with the plasma membrane of the host cell. Then, it enters the intracellular environment and begins a lytic replicative cycle incompatible with cellular survival (Muylkens et al. 2007). Although not investigated in this study, the death of cumulus cells infected by BoHV-1, due to the cytopathic effect of the virus, could contribute to the reduction of the number of viable cells capable of supporting oocyte competence.

Despite the negative interference in the oocyte nuclear maturation rate, no morphological differences were observed in optical microscopy in COCs before and after *in vitro* culture. This agrees with observations by D'Angelo (1998), who found that both bovine oocytes matured *in vitro* with BoHV-1 and those matured in the absence of the virus had indistinguishable morphology. This finding was also verified by Gonçalves et al. (2015), who also did not observe any morphological alterations in infected oocytes.

However, despite the normal morphological appearance, cellular damage that compromises later development occurs once BoHV-1 causes a cytopathic effect on cumulus cells without interfering with their expansion (Vanroose et al. 1999). It is worth mentioning that the condition of cumulus cells is one of the main aspects used in the evaluation of COCs (Costa 1994). Therefore, the analysis of COCs for embryo production *in vitro* is virtually always based only on observations of oocytes enveloped by layers of cumulus cells.

In summary, our findings provide relevant information at the COC level, since BoHV-1 compromises the rate of oocyte nuclear maturation through an interaction with cumulus cells without causing morphological changes visible under the optical microscope. Further research is needed to clarify the mechanism by which BoHV-1 interacts with the naturally infected bovine gamete.

CONCLUSIONS

Cows naturally infected by BoHV-1 exhibit compromised nuclear oocyte maturation, depending on the antibodies titration degree against the virus.

Furthermore, viral DNA may be present in COCs, contradicting the hypothesis that serologically positive animals with no history of clinical symptomatology pose a negligible risk of transmitting BoHV-1 by COCs.

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Original Article
Animais de Produção/*Livestock Diseases*

Rhinosporidiosis in horses[1]

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ABSTRACT.- Argenta F.F., Mello L.S., Vielmo A., Pavarini S.P., Driemeier D. & Sonne L. 2018. **Rhinosporidiosis in horses.** *Pesquisa Veterinária Brasileira 38(12):2213-2216*. Setor de Patologia Veterinária, Departamento de Patologia Clínica Veterinária, Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves 9090, prédio 42505, Porto Alegre, RS 91540-000, Brazil. E-mail: lusonne@yahoo.com.br

Rhinosporidiosis is a disease caused by *Rhinosporidium seeberi*, an aquatic protist of the class Mesomycetozoa. It primarily affects the nasal mucosa and transmission is associated with contaminated water contact. This report describes seven cases of rhinosporidiosis in horses in Rio Grande do Sul covering the period of 13 years. The disease predominantly affected Crioulo and thoroughbred horses. No apparent gender predisposition occurs, and age ranged from two to 25 years, with a median of 10 years. The gross aspects were characterized by unilateral (85.7%, 6/7) or bilateral (14.3%, 1/7) polyps. These were soft to friable, whitish to pink, cauliflower-like, with an irregular, sometimes ulcerated surface, measuring 2.5 to 6.0cm in diameter. There was a severe inflammatory infiltrate of the submucosa was observed, associated with moderate proliferation of the epithelium, and numerous rounded structures were identified compatible with sporangia of *R. seeberi*. Rhinosporidiosis should be included in the differential diagnosis of other conditions affecting the respiratory tract of horses, and it is important to perform histopathology for diagnosis.

INDEX TERMS: Horses, rhinosporidiosis, rhinitis, *Rhinosporidium seeberi,* parasitoses.

RESUMO.- [**Rinosporidiose em equinos.**] A rinosporidiose é uma doença causada por *Rhinosporidium seeberi*, protista aquático da classe Mesomycetozoa. Acomete principalmente a mucosa nasal e a transmissão está associada ao contato com água contaminada. Este trabalho descreve sete casos de rinosporidiose em equinos no Rio Grande do Sul em um período de 13 anos. A doença afetou predominantemente cavalos de raça, como Crioulo e Puro Sangue Inglês, sem predisposição sexual evidente e a idade variou de dois a 25 anos, com a mediana de 10 anos. Macroscopicamente foram caracterizadas por pólipos unilaterais (85,7%; 6/7) ou bilaterais (14,3%; 1/7). Os pólipos eram macios a friáveis, esbranquiçados a róseos, com aspecto de couve flor e com superfície irregular, por vezes ulcerada, medindo 2,5 a 6,0cm de diâmetro. Havia infiltrado inflamatório piogranulomatoso acentuado na submucosa associado à moderada proliferação do epitélio e numerosas estruturas arredondadas compatíveis com esporângios de *R. seeberi*. A rinosporidiose deve ser incluída no diagnóstico diferencial de outras patologias que acometem o trato respiratório de equinos, sendo importante a realização da histopatologia para diagnóstico.

TERMOS DE INDEXAÇÃO: Cavalos, rinosporidiose, rinite, *Rhinosporidium seeberi,* parasitoses.

INTRODUCTION

Rhinosporidiosis is a disease that affects several animal species and humans (Caswell & Williams 2016) and is caused by *Rhinosporidium seeberi*, classified as aquatic protist belonging to the class Mesomycetozoa (Santos & Guedes 2016, López & Martinson 2017). The disease occurs predominantly in tropical and subtropical regions (Caswell & Williams 2016), mainly affecting the nasal mucosa and transmission is associated with contact with contaminated water. Mucosal wounds favor the entrance of the agent which induces a focal granulomatous lesion (Kennedy et al. 1995, Leeming et al. 2007).

The affected animals may be asymptomatic or present clinical signs characterized by nasal secretion, sneezing, epistaxis, breathing noise (Easley et al. 1986, Burgess et al. 2012, Caswell & Williams 2016) and obstruction of the nasal cavity occasionally (Brenseke & Saunders 2010). The diagnosis of

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rhinosporidiosis is based on observation through histological examination (Santos & Guedes 2016) and the indicated treatment is surgical excision (Arseculeratne & Mendoza 2005).

This study describes seven cases of rhinosporidiosis in horses in the state of Rio Grande do Sul, with emphasis on epidemiological and pathological findings.

MATERIALS AND METHODS

The records of histopathological examinations carried out from January 2004 to December 2016 in the Department of Veterinary Pathology of the Federal University of Rio Grande do Sul (SPV-UFRGS) were reviewed for cases of rhinosporidiosis in horses. Samples from the nasal cavity of horses from the metropolitan region of Porto Alegre and Northeastern of Rio Grande do Sul, Brazil, were fixed in 10% formalin. Epidemiological data such as age, sex and breed, as well as the date of examination, anatomical location and clinical signs were obtained from histopathological examinations. Paraffin blocks were obtained from the SPV-UFRGS files and new histological sections were stained with hematoxylin and eosin (HE) and periodic acid-Schiff (PAS).

RESULTS

From January 2004 to December 2016, the SPV-UFRGS received 832 equine biopsies, seven of which were diagnosed as rhinosporidiosis, corresponding to 0.8% of all cases. When only samples from the nasal cavity of horses were analyzed, the frequency was 31.8% (7/22). Crioulo and Thoroughbred were the affected breeds, with 71.4% (5/7) and 14.3% (1/7), respectively. An affected horse had no defined breed (14.3%, 1/7). Of the total, 57.1% (4/7) were females and 42.9% (3/7) males. Age ranged from 2 to 25 years, with median of 10 years. Clinical history was reported in four cases and sero-sanguinolent nasal secretion (3/4), epistaxis (1/4) and respiratory distress (1/4) were the clinical signs described.

Macroscopically, the lesions were characterized by polypoid mucosal thickening in the nasal cavity. The polyps were found as a solitary unilateral lesion (85.7%, 6/7) or as multiples bilateral formations (14.3%, 1/7). They were soft to friable, whitish to rosy, cauliflower-like aspect and irregular surface, sometimes ulcerated, measuring 2.5 to 6.0cm in diameter (Fig.1A).

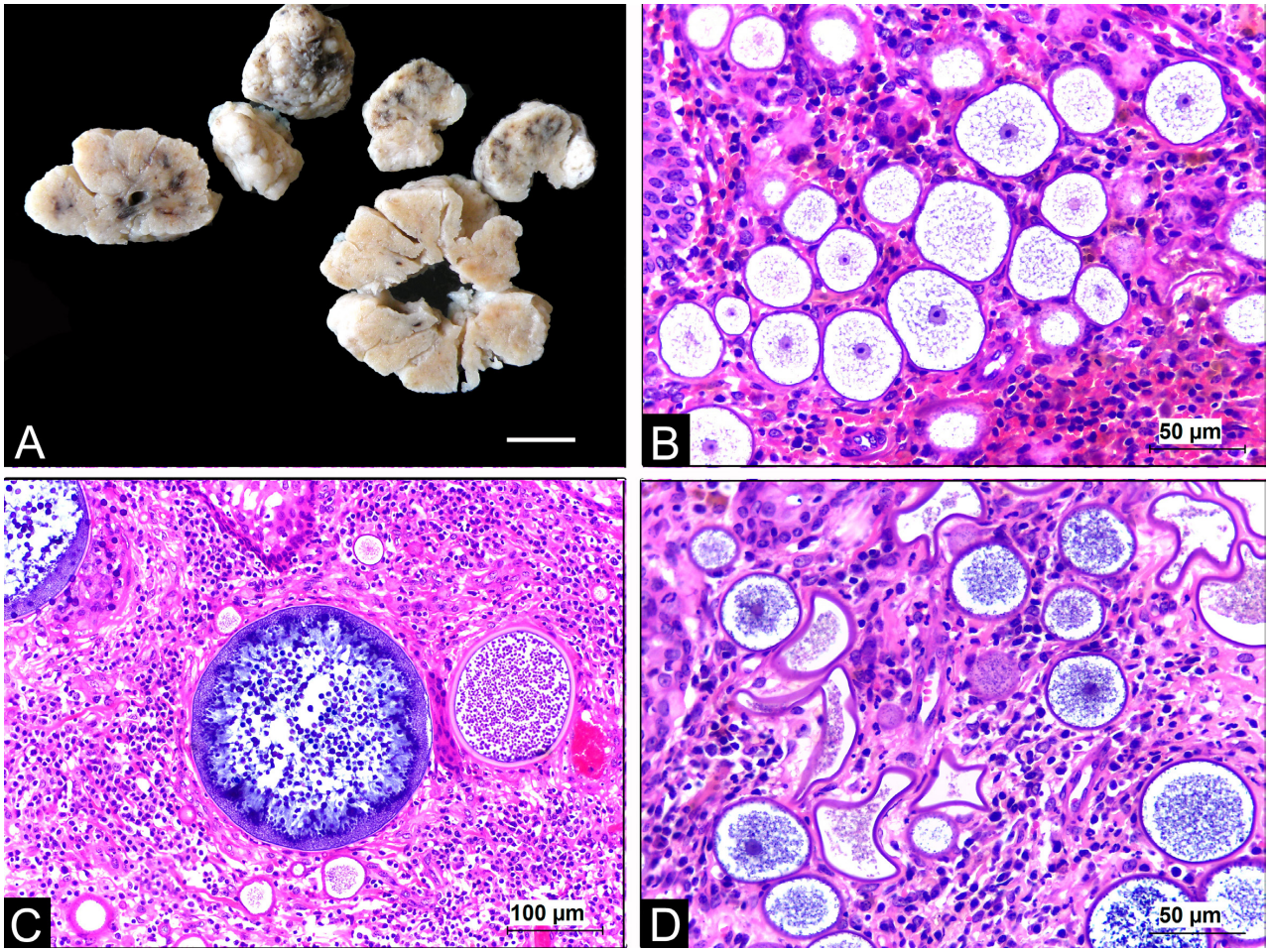

Fig.1. Rhinosporidiosis in horses. (**A**) Multiple samples of white, cauliflower-like polypoid nodule. Specimens fixed in 10% formalin. (**B**) Numerous rounded structures of different sizes, the largest measuring 55µm in diameter, unilamellar wall, central nucleus, surrounded by basophilic granular material, compatible with young *Rhinosporidium seeberi* sporangia. Inflammatory infiltrate of lymphocytes and plasma cells is also observed. HE, bar = 50µm. (**C**) Mature *Rhinosporidium seeberi* sporangia, the largest measuring 250µm in diameter and the smallest 120µm, with multiple young (periphery) and mature endospores (central) inside. HE, bar = 100µm. (**D**) Several empty and collapsed sporangia and young sporangia and inflammatory infiltrates of lymphocytes, plasm cells and macrophages. HE, bar = 50µm.

Histopathological examination revealed a polypoid structures lined by moderate epithelial proliferation associated with a marked inflammatory infiltrate of macrophages, lymphocytes and plasm cells, and occasional neutrophils. Numerous rounded structures of variable size (20 to 500µm in diameter) were identified along with inflammation and in different stages of development, compatible with *Rhinosporidium seeberi* sporangia. Young sporangia presented unilamellar wall, central nucleus, surrounded by basophilic granular material measuring from 10 to 100µm in diameter (Fig.1B). The unilamellar wall of young sporangia was visualized mainly by PAS staining. The mature ones presented bilamellar wall, with multiple endospores, measuring from 100 to 500µm (Fig.1C). Immature endospores were in the periphery of the sporangium and mature ones in the central region, which is spherical, with numerous eosinophilic bodies inside. There were occasional ruptured sporangia releasing endospores through the apical pore in the epithelium, and also multifocally in the submucosa. In addition, multiple empty and collapsed sporangia were visualized (Fig.1D). In the mucosa, multiple areas of ulceration, with deposition of amorphous fibrillar material, necrotic debris and bacterial myriads were also observed.

DISCUSSION

The seven cases were diagnosed as rhinosporidiosis based on the characteristic histological features of the disease. The diagnosis of the disease is based on the visualization of *Rhinosporidium seeberi* sporangia in histology, associated with respiratory clinical signs when present (Pereira & Meireles 2007), since the culture of the microorganism was not performed, as it cannot be isolated in artificial mediums (Jungerman & Shwartzman 1972).

In the present study, the rhinosporidiosis frequency was 0.8% of the total histopathological samples, and 38.1% when samples from the nasal cavity were analyzed. In a study of equine diseases, the frequency was 0.3% (Marcolongo-Pereira et al. 2014). In a study of tumor lesions of the nasal cavity of horses, rhinosporidiosis was the second major disease (Trotte et al. 2008), representing 27.3% of the total lesions. The low rhinosporidiosis frequency may be related to the non-reporting of cases and because not all surgically excised nasal lesions are submitted to histopathological examination (França et al. 1997).

Rhinosporidiosis affects both sexes (Londero et al. 1977), and in relation to the age group, the disease presents great variation (Londero et al. 1977, Nollet et al. 2008, Trotte et al. 2008, Burgess et al. 2012, Santos et al. 2014, Bernardo et al. 2016), as identified in the present study. The disease predominantly affected Crioulo horses, and this may be related to the higher prevalence of this breed in the state of Rio Grande do Sul (Silva & Farias 2017).

In horses, the lesion is predominantly located in the nasal mucosa and rarely in the larynx region (Nollet et al. 2008, Burgess et al. 2012, Santos et al. 2014). This high frequency of involvement of the nasal mucosa can be explained by contamination with pathogen spores in soil and water, thus the inhalation of dust and/or contact of the nasal mucosa with contaminated water inoculated via traumatic lesions would be the possible means of transmission (França et al. 1997, Caswell & Williams 2016). Therefore, one should suspect the existence of a source of infection in the place where horses are kept and of environmental characteristics, which favor the perpetuation and development of *R. seeberi*, such as flooded areas (Tiwari et al. 2015).

Macroscopic and microscopic rhinosporidiosis lesions observed in this study are similar to those described by other authors (Londero et al. 1977, Caswell & Williams 2016, López & Martinson 2017). The morphological characteristics of the different stages of *R. seeberi* found in equines are compatible with those described in literature (Chandler et al. 1980, Leeming et al. 2007, Burgess et al. 2012, Santos et al. 2014). The different stages of *R. seeberi* are easily observed in sections stained by HE; however, histochemical techniques such as PAS can be used to highlight sporangia walls (Easley et al. 1986).

The diseases of the nasal cavity of horses present similar clinical signs, mainly characterized by nasal secretion, epistaxis and respiratory distress (Nickels 1993). Differential diagnoses include rhinitis caused by *Aspergillus* spp., *Coccidioides immitis* (Caswell & Williams 2016), *Cryptococcus neoformans*, *Histoplasma* spp., *Pythium insidiosum* (Trotte et al. 2008) and *Emmonsia parva* or *E. crescens* (Caswell & Williams 2016). Other conditions affecting the nasal cavity of horses, such as congenital nasal cysts, inflammatory polyps, nasal amyloidosis, neoplasms, nasal granuloma caused by hypersensitivity and ethmoidal progressive hematoma (Pereira & Meireles 2007) should also be considered. Definitive diagnosis depends on the histopathological examination of nodular lesions in the nasal cavity of horses with the identification of the different stages of *R. seeberi* (Berrocal & Lopez 2007).

CONCLUSIONS

Rhinosporidiosis presented low frequency in horses in Rio Grande do Sul; however, when nasal cavity samples were analyzed, it was the most frequently diagnosed disease.

The disease affected predominantly Crioulo horses, with no predisposition for sex and with great age group variation.

This disease should be included in the differential diagnosis of other pathologies affecting the upper respiratory tract of horses. Histopathological examination is decisive in the diagnosis.

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Original Article
Animais de Produção/*Livestock Diseases*

Economic losses due to *Vernonia rubricaulis* poisoning in cattle [1]

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ABSTRACT.- Soares M.C., Pupin R.C., Guizelini C.C., Gaspar A.O., Gomes D.C., Brumatti R.C. & Lemos R.A.A. 2018. **Economic losses due to *Vernonia rubricaulis* poisoning in cattle.** *Pesquisa Veterinária Brasileira 38(12):2217-2223.* Faculdade de Medicina Veterinária e Zootecnia, Universidade Federal de Mato Grosso do Sul, Avenida Senador Felinto Muller 2443, Jardim Parati, Campo Grande, MS 79070-900, Brazil. E-mail: marcelocezar@outlook.com

Vernonia rubricaulis is a hepatotoxic plant found in the Pantanal biome. Under natural conditions, it is responsible for highly fatal poisonings in cattle. From January 1999 to December 2016, 33 outbreaks of *V. rubricaulis* poisoning were recorded, resulting in 1509 bovine deaths, of which 719 (47.6%) were adult females, 413 (27.4%) were adult males, 244 (16.2%) adult cattle with no information about sex and 133 (8.8%) calves. The coefficients of morbidity, mortality and lethality were respectively 2.79%, 2.77% and 99.24%. Most outbreaks occurred in properties containing up to 1,000 cattle, where the most significant economic impacts were also observed. Among the total recorded deaths, the total direct monetary loss was estimated at US$764,893.33, which represents an average of 3.05% of the total assets (US$25,090,683.51) of the herds involved in the outbreaks. The plant can cause more severe damage to properties with less than 500 cattle, and can reach 50% of the total value of the herd. In comparison to other methods, the methodology used in this study has an economic impact consistent with reality, not overestimating the losses. Toxic plants, such as *V. rubricaulis*, can cause significant economic losses in the extensive systemic livestock, and it is important decision-making and prophylactic management to avoid the occurrence of poisoning in the herds.

INDEX TERMS: Economic losses, cattle, poisoning, *Vernonia rubricaulis*, bovine diseases, economic impact, plant poisoning, toxicoses.

RESUMO.- [**Perdas econômicas causadas pela intoxicação por *Vernonia rubricaulis* em bovinos.**] *Vernonia rubricaulis* é uma planta hepatotóxica encontrada no bioma Pantanal. Em condições naturais, é responsável por intoxicações altamente fatais em bovinos. De janeiro de 1999 a dezembro de 2016, foram registrados 33 surtos de intoxicação por *V. rubricaulis* em bovinos que resultaram em 1509 mortes, sendo 719 (47,6%) fêmeas adultas, 413 (27,4%) machos adultos, 244 (16,2%) bovinos adultos sem informação sobre o sexo e 133 (8,8%) bezerros. Os coeficientes de morbidade, mortalidade e letalidade foram respectivamente de 2,79%, 2,77% e 99,24%. A maioria dos surtos ocorreu em propriedades contendo até mil bovinos, onde também foram constatados os impactos econômicos mais significativos. Do total das mortes registradas, o prejuízo monetário direto total foi calculado em US$764.893,33, o que representa em média 3,05% do total do patrimônio (US$25.090.683,51) dos rebanhos envolvidos nos surtos. A planta pode causar prejuízos mais severos em propriedades com menos de 500 bovinos, podendo chegar a 50% do total do valor do rebanho. Em comparação aos outros métodos, a metodologia utilizada neste estudo afere um impacto econômico condizente com a realidade, não superestimando os prejuízos. Plantas tóxicas, como a *V. rubricaulis*, podem causar prejuízos econômicos significativos na pecuária extensiva, sendo importantes tomadas de decisões e manejos profiláticos para evitar a ocorrência de intoxicação nos rebanhos.

TERMOS DE INDEXAÇÃO: Perdas econômicas, intoxicação, *Vernonia rubricaulis*, doenças de bovinos, impacto econômico, intoxicações por plantas, toxicoses.

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INTRODUCTION

In countries where livestock farming is based on an extensive system, there is an increase in the possibility of cattle access to toxic plants, and consequently increases the incidence of poisoning by plants (Pessoa et al. 2013). One of the main plants associated with cattle poisoning in the Pantanal region is *Vernonia rubricaulis*, a sub-bush of the Asteracea family whose development occurs in areas subject to temporary flooding, in places of brackish water or in clayey soils (Purisco & Lemos 2008, Lemos et al. 2011). The toxic principle is unknown and, under natural conditions, poisoning occurs only in cattle, while experimentally, it was toxic to sheep (Souza et al. 2015, Godoy et al. 2018). In the budding stage, *V. rubricaulis* is more toxic and more palatable, which favors poisoning (Tokarnia & Döbereiner 1982, Brum et al. 2002, Tokarnia et al. 2012, Godoy et al. 2018).

Although there are methodologies that estimate the direct and indirect damage caused by toxic plants in a particular region or country (James et al. 1992, Riet-Correa & Medeiros 2001), there are no detailed reports on the economic losses caused by these poisonings on the properties in that they occur. This approach requires the joint analysis of the economic parameters with the epidemiological data of the outbreaks, and can be used to assess mortality losses in herds due to other causes (Smith 1998, Pötter et al. 2000, Gottschall et al. 2010). The evaluation of the economic impact of health problems is important in the search for a production system that is more economically profitable and constitutes an important tool for guiding the herd management (Dijkhuizen et al. 1995, Perry et al. 2001, FAO 2016).

The objective of this study is to develop a model, based on spontaneous cases of *V. rubricaulis*, to estimate the economic losses resulting from the mortality caused in cattle by ingestion of toxic plants in extensive production systems.

MATERIALS AND METHODS

The necropsies performed in cattle from January 1999 to December 2016 at the Laboratory of Pathology Anatomy of the Federal University of Mato Grosso do Sul (LAP-UFMS) were reviewed.

The cases of poisoning were selected by *Vernonia rubricaulis* obeying the following criteria previously described by Brum et al. (2002): 1) evidence of ingestion of the plant by cattle, 2) confirmation by on-site visits by the team, 3) characteristic clinical signs, necropsy findings, and histopathology consistent with severe or massive centrolobular hepatic necrosis and multifocal bleeding.

Data relating to the total number of cattle on farms and the quantities of sick and dead cattle were collected from the reports. For economic analysis, cases from the same property and from the same period were grouped with a single outbreak.

For the epidemiological analysis, the coefficients of morbidity, mortality and lethality were calculated for each property, considering the number of animals affected and dead due to poisoning in relation to the other cattle raised under the same conditions of nutritional and sanitary management. The numeric data of diseased and dead animals corresponded to the time of sending the material.

The economic assessment was based on data on the total number of animals on the farm and on the number of animals killed, as well as on the composition of the herds and the category of affected animals, thus correcting their values to obtain a weighted average of carcass weight.

The price of the animals was calculated on the basis of the price of the kilogram (kg) of the carcass of the bull through research and analysis of the prices during the entire period of the outbreaks.

The price of adult male bovines was estimated with the average price per kg of the Boi Gordo Indicator of the Center for Advanced Studies in Applied Economics of the "Luiz de Queiroz" College of Agriculture - University of São Paulo/Stock Exchange, Commodities and Futures of the São Paulo Stock Exchange (CEPEA Esalq/BM & FBovespa) for the year 2017.

In order to calculate the average value of adult females, the difference in percentage between the values paid for fat cows and for cattle was calculated, referring to the average kilogram paid to the producer, as informed by the Indicator of the Mato Grosso South (CEASA) in 2017. All animals less than 12 months of age were considered calves and the mean value for this animal category was calculated by the difference in percentage between the values paid by CEPALE Esalq/BM & FBovespa Mato Calf Indicator Grosso do Sul in 2017 and the price of the cattle, being converted into the price of the kilogram of the carcass.

In order to estimate the monetary values and consequently the economic losses, the quotations used were converted from the real to the US dollar using the average value of the exchange rate for the year 2017 obtained from the Central Bank of Brazil.

In order to price the different animal categories of cattle, the following values were calculated:

$$\$Vma = \left(LW_{ma} * CY\right) * Pkg_{ma}$$

In which: $ Vma = the average unit monetary value of adult animals, LWma = the estimated average live weight of adult males (400kg), CY = the estimated carcass yield (50%), Pkgma = the average kilogram price of the fattened carcass paid to the producer.

$$\$Vfa = \left(LW_{fa} * CY\right) * Pkg_{fa}$$

In that: $ Vfa = average unit monetary value of adult females, LWfa = estimated average live weight of adult females (360kg), CY = estimated carcass yield (50%), Pkgfa = average price of kilograms of cow carcass paid to producer

$$\$Vaa = \left(\$Vma + \$Vfa\right)/2$$

In which: $ Vaa = unit average monetary value of adult animals where sex was not reported, $ Vma = unit average monetary value of adult males, $ Vfa = average unit monetary value of adult females.

$$\$Vca = \left(LW_{ca} * CY\right) * Pkg_{ca}$$

In which: $ Vca = the average unit value of the calves, LWca = the estimated average live weight of the calves up to 1 year of age (180kg), CY = estimated carcass yield (50%),% Pkgca = of the calf.

To calculate the other values needed for the analyzes, the following equations were used:

$$\$Vm = \left(Vma + Vfa + Vaa + Ca\right)/4$$

In which: Vm = the average unit monetary value of the herd, Vma = the unit average monetary value of the adult male animals, Vfa = the average unit monetary value of the female adult animals, Vaa = the average unit monetary value of adult animals with uninformed sex , Ca = the average monetary value of the calf.

$$\$Vth = n * \$Vm$$

In which: $ Vth = monetary value of the total herd in the property, n = total amount of the herd, $ Vm = the average unit monetary value of the herd.

$$\$TEcL = nd * \$Vu$$

In which: $ TEcL = total economic loss related to deaths, nd = total number of dead animals, $ Vu = unit commercial value of reported category.

$$\%EcL = \left(\left(\$TEcL / \$Vth\right) * 100\right)$$

In which:% EcL = percentage of estimated economic loss, $ TEcL = total economic loss related to deaths, $ Vth = monetary value of the total herd in the property.

RESULTS

All outbreaks of poisoning by *Vernonia rubricaulis* occurred in the western region of the state of Mato Grosso do Sul, Brazil (Fig.1), where the Pantanal biome is located, the largest water-covered plain in the world with chemically poor soils and limited fertility (Furlan et al. 2012).

A total of 1509 cattle were killed, of which 719 (47.6%) were adult females, 413 (27.4%) were adult males, 244 (16.2%) were adult animals in which sex was not informed and 133 (8.8%) calves from 0 to 12 months of age (Table 1). In 26 of the 33 outbreaks studied, the epidemiological information was complete, thus, the morbidity, mortality and lethality

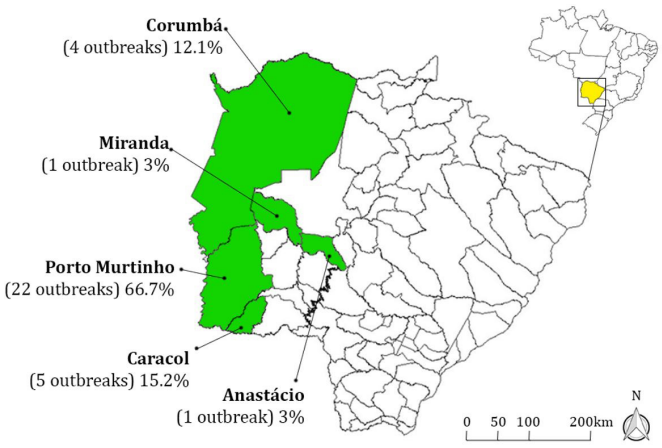

Fig.1. Geographical location of occurrences of outbreaks of poisoning by *Vernonia rubricaulis* in cattle in Mato Grosso do Sul.

Table 1. Epidemiological data on outbreaks of food poisoning by *Vernonia rubricaulis* in cattle diagnosed in the LAP/FAMEZ from 1999 to 2016

Outbreak	Year	Month	City	Age (months)	Total of cattles	Number of sick cattles	Number of dead cattles	Gender
1	1999	September	Porto Murtinho	Aa.	2300	114	114	7 M/107 F
2	1999	October	Porto Murtinho	30-36	2700	150	150	F
3	1999	October	Porto Murtinho	Aa.	4500	200	200	NI
4	1999	October	Porto Murtinho	Aa.	NI	6	6	NI
5	1999	October	Porto Murtinho	Aa.	300	17	17	NI
6	1999	October	Porto Murtinho	Aa.	2500	60	60	F
7	1999	November	Corumbá	30	1500	104	104	F
8	1999	November	Caracol	30	200	7	6	M
9	1999	December	Porto Murtinho	30	NI	8	8	NI
10	2000	February	Porto Murtinho	1 - 3	380	13	13	NI
11	2000	March	Porto Murtinho	1 - 8	3000	16	16	NI
12	2000	May	Porto Murtinho	18	121	2	2	M
13	2000	August	Miranda	60	2000	7	7	F
14	2000	November	Porto Murtinho	36	165	68	68	M
15	2000	November	Porto Murtinho	24	815	30	30	M
16	2001	May	Caracol	30	4000	120	120	F
17	2002	October	Corumbá	18	NI	NI	NI	NI
18	2002	October	Porto Murtinho	36	4000	69	69	M
19	2002	October	Caracol	18	200	13	10	NI
20	2002	November	Anastácio	2	1400	NI	4	NI
21	2002	December	Porto Murtinho	24	400	1	1	F
22	2003	May	Corumbá	1 - 3	1300	35	35	NI
23	2004	September	Porto Murtinho	48	4000	68	62	F
24	2005	November	Porto Murtinho	36	NI	NI	NI	NI
25	2006	February	Porto Murtinho	6-12	NI	NI	60	NI
26	2006	March	Porto Murtinho	24	8000	80	80	M
27	2008	October	Porto Murtinho	Aa.	2200	55	55	M
28	2009	September	Caracol	36	581	11	10	M
29	2010	November	Porto Murtinho	24	400	36	36	M
30	2011	April	Corumbá	36	1200	50	50	M
31	2012	February	Porto Murtinho	Aa.	NI	NI	3	NI
32	2013	October	Caracol	Aa.	4500	108	108	F
33	2016	March	Porto Murtinho	4-12	350	5	5	NI

NI = not reported, M = male, F = female, Aa. = adult animals (over 12 months), where the exact age was not reported.

coefficients were respectively 2.79%, 2.77% and 99.24%, respectively.

Once the epidemiological values were structured, it was possible to apply the economic formulas for each affected animal category in cases of poisoning by *V. rubricaulis* in cattle diagnosed in the LAP/FAMEZ university from 1999 to 2016, thus obtaining the mean values considered for calculating the losses generated (Table 2).

The value of the total stockholders' equity of the herds studied in the properties where the outbreaks occurred, totaled US $ 25,047,887.39, considering the average unit monetary value of US $ 472.49. The 1509 recorded deaths correspond to a loss of US $ 756,915.74, which represents 3.02% in relation to the value of the assets of the herds studied (Table 3).

The results of the analysis of the data by classes of occurrences are presented below showing the ranges of higher frequencies for the Total Value of the Herd those less than US $ 500,000.00 in which the total loss was less than US$ 20,000.00 and the percentage value of the loss in relation to the total value of the herd relative to the herd less than 10% (Fig.2-4).

Table 2. Average results of monetary values applied for each animal category present in cases of poisoning by *Vernonia rubricaulis* in cattle diagnosed in the LAP/FAMEZ from 1999 to 2016 according to the average price of 2017

Category	Average value per kilogram of carcass (US $/kg)	Average value per animal (US$)
Males adults	2.892	578.32
Female adults	2.698	485.62
Adult animals (gender was not informed)	2.795	531.97
Calves	3.267	294.08
Average unit monetary value ($Vm)		472.49

Average of the quotation of the dollar to the real, Brazilian currency (2017) of R$ 3.1826.

Table 3. Results of the economic analysis of cases of poisoning by *Vernonia rubricaulis* in cattle diagnosed in the LAP/FAMEZ from 1999 to 2016 according to the average price of 2017

Case	Total of cattles	Dead cattles	Total value of herd US$	Total loss US$	Damage %
1	2300	114	1,086,737.74	56,009.08	5.15
2	2700	150	1,275,735.61	72,842.30	5.71
3	4500	200	2,126,226.01	106,393.53	5.00
4	NI	6	-	3,191.81	-
5	300	17	141,748.40	9,043.45	6.38
6	2500	60	1,181,236.67	29,136.92	2.47
7	1500	104	708,742.00	50,503.99	7.13
8	200	6	94,498.93	3,469.92	3.67
9	NI	8	-	4,255.74	-
10	380	13	179,547.97	3,822.98	2.13
11	3000	16	1,417,484.01	4,705.21	0.33
12	121	2	57,171.85	1,156.64	2.02
13	2000	7	944,989.34	3,399.31	0.36
14	165	68	77,961.62	39,325.76	50.44
15	815	30	385,083.16	17,349.60	4.51
16	4000	120	1,889,978.68	58,273.84	3.08
18	4000	69	1,889,978.68	39,904.08	2.11
19	200	10	94,498.93	5,319.68	5.63
20	1400	4	661,492.54	1,176.30	0.18
21	400	1	188,997.87	485.62	0.26
22	1300	35	614,243.07	10,292.65	1.68
23	4000	62	1,889,978.68	30,108.15	1.59
25	NI	60	-	17,644.54	-
26	8000	80	3,779,957,35	46,265.60	1.22
27	2200	55	1,039,488.27	31,807.60	3.06
28	581	10	274,519.40	5,783.20	2.11
29	400	36	188,997.87	20,819.52	11.02
30	1200	50	566,993.60	28,916.00	5.10
31	NI	3	-	1,595.90	-
32	4500	108	2,126,226.01	52,446.45	2.47
33	350	5	165,373.13	1,470.38	0.89
TOTAL	53012	1506	25,047,887.39	756,915.74	3.02

Average of the quotation of the dollar to the real, Brazilian currency (2017) of R$ 3.1826; NI = not informed.

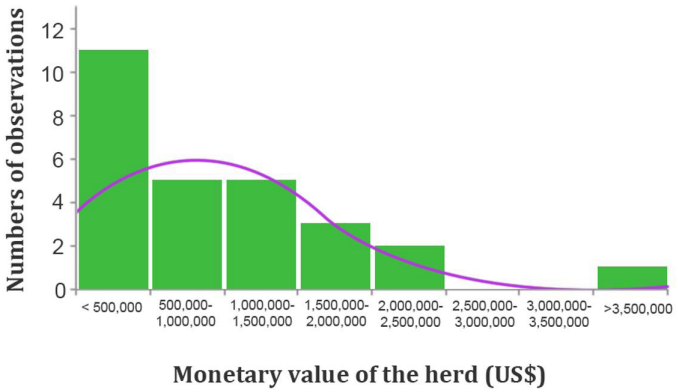

Fig.2. Number of observations referring to the frequency of outbreaks according to the total monetary value of the herds.

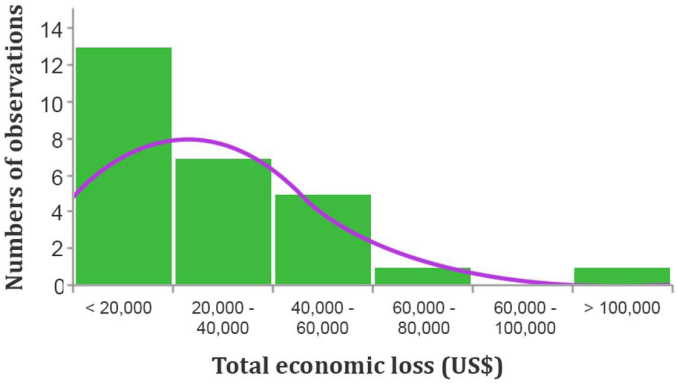

Fig.3. Number of observations referring to the frequency of outbreaks according to the monetary value of the total loss due to poisoning deaths by *Vernonia rubricaulis*.

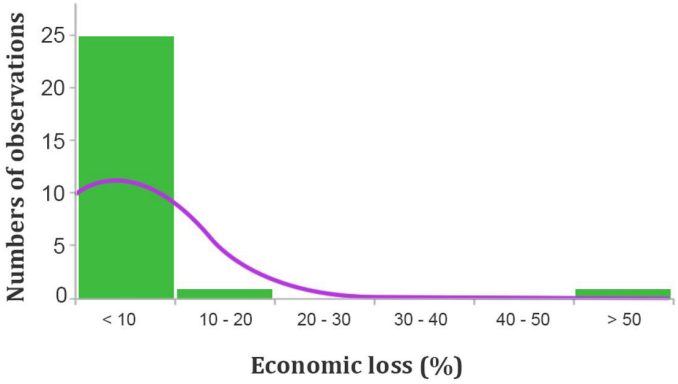

Fig.4. Number of observations of outbreaks in relation to the percentage of injury considering the total monetary value of the herd.

DISCUSSION

Poisoning by *Vernonia rubricaulis* was diagnosed in 15 of the 16 years investigated, proving to be a frequent and constant cause of economic losses for the cattle ranch in the state of Mato Grosso do Sul. All outbreaks occurred in an area of 94,067 square kilometers corresponding to five municipalities, the equivalent of 26.3% of the total area of the state, with all outbreaks analyzed in the Brazilian Pantanal region. In all outbreaks analyzed in this study, 1,509 cattle of all categories were killed, resulting in a total estimated loss of US$ 756,915.74.

So far, the reports regarding poisoning by *V. rubricaulis* (Tokarnia & Döbereiner 1982, Brum et al. 2002, Pessoa et al. 2013) describe the epidemiological, clinical and pathological aspects of the outbreaks, mentioning the total number of deaths, but do not address historical series nor estimate the economic damages caused by poisoning. The methodology adopted in the present study allows estimating the losses caused by poisoning in each property or in a set of properties, determining how much these represent on the total herd patrimony. The methodologies used in previous studies on economic losses caused by plant poisoning in production animals only evaluate the losses, without defining the methodology used (Zhao et al. 2013), or only determine the economic impact in certain regions (Nielsen 1978, Riet-Correa & Medeiros 2001), in a country (Nielsen 1988, Riet-Correa & Medeiros 2001, Pessoa et al. based on fictitious mortality rates close to 5%.

These studies are important for estimating the total losses from plant poisoning and thus directing public policies to minimize the damages caused by these poisonings. Moreover, studies aiming to determine the losses that occur in each individual property are important to guide decision making by each producer regarding the adoption of measures of control and prophylaxis of these poisonings. Of the 33 outbreaks studied, 15 occurred in 1999 and 2000, when there were large burnings in the region, which favor the budding of the plant and consequently the occurrence of poisoning, because at this stage, in addition to its toxicity, the plant is more palatable to animals (Tokarnia & Döbereiner 1982, Brum et al. 2002, Godoy et al. 2018).

In the present study, most of the outbreaks that were reported, they occurred on farms where the value of the assets found characterized medium-sized properties for the region's standards. As a result, the chances of the losses being high and more significant for the activity are higher. The two outbreaks in which the highest losses (50.44% and 11.02% of losses related to the total herd equity) were observed in herds with less than 500 herds. These occurrences can make business continuity unfeasible (Nielsen 1988). This type of observation is not detected when collection methods are used that evaluate the total data of the reported outbreaks without stratifying them by properties in which the outbreaks occurred.

Corroborating this point, the analysis of the results obtained in the classes of Total Loss and Relative Percentage Loss show, by property, that the majority of occurrences is at the level of up to 10% of estimated loss by total of equity informed, however that such percentage level may reach values of up to $ 60,000.00 for rural property. This type of analysis does not appear in general epidemiological studies on plant poisoning (Rissi et al. 2007, Souza et al. 2015), or even in specific studies of a particular plant (Carvalho et al. 2006, Carmo et al. 2011).

The mean morbidity coefficient was 2.79%, however, it is worth noting the large variation of the same from 0.25% to 41.21%. This observation, together with the geographic distribution of the outbreaks, restricted to a specific region of the state, shows that the methodologies for assessing

losses caused by plant poisoning should consider these particularities. (Riet-Correa & Medeiros 2001, Pedroso et al. 2007, Assis et al. 2010), which estimates the losses caused by plant poisoning through records of these occurrences in the diagnostic laboratories in a given region, it is not possible to calculate how much these losses represent the total number of cases referred for diagnosis and the value of these deaths in relation to the expected percentage of all cause deaths for the herd of a particular region or country.

In Brazil, it is assumed that approximately 5% of cattle die annually from various causes (Riet-Correa & Medeiros 2001, Pessoa et al. 2013). Considering that in the state of Mato Grosso do Sul, 1.4% of the cases of deaths referred for diagnosis are due to poisoning by *V. rubricaulis* (Souza et al. 2015), and that the State has 22.17 million heads (IBGE 2016), the annual death of cattle poisoned by this plant would be around 298 thousand animals, which would result in an annual loss of US $ 140,802,020.00, considering the value animal monetary unit ($ Vm) of US $ 472.49.

Comparing the total number of deaths reported in this study over a 16-year period, the estimated injury was US $ 756,915.74, or approximately US $ 47,307.23 per year, showing a difference of 99.96% less than other methodologies (Nielsen 1978, Nielsen 1988, James et al. 1992, Riet-Correa & Medeiros 2001, Assis et al. 2010, Pessoa et al. 2013, Zhao et al. 2013). Although part of this difference may be attributed to the underreporting of poisoning cases, the large variation in morbidity coefficients between outbreaks and the greater occurrence of outbreaks in certain years must also be considered.

Thus, the existence of an efficient notification system with standardized data is an indispensable tool for the elaboration of an efficient model for the evaluation of the economic losses caused by this poisoning.

Lack of higher numbers of accurate diagnosis by plant poisoning in livestock and lack of availability of more reliable data on disease outbreaks on the properties makes a more realistic estimation of the economics of cattle breeding (Nielsen 1978, Riet-Correa & Medeiros 2001). Besides the lack of data on the economic impact of plant poisoning to livestock, there is still a lack of official government programs to control and minimize the losses caused by this problem (Rissi et al. 2007).

CONCLUSIONS

Poisoning by *Vernonia rubricaulis* in cattle has mortality ratios ranging from 0.25% to 41.21%.

The outbreaks occur mainly from September to November and with an annual constancy in the state of Mato Grosso do Sul, Brazil.

The losses caused in cattle vary from 0.18% to 50.44% of the herd's total assets, and may cause serious economic impacts on rural properties.

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Original Article
Pequenos Animais/*Small Animals Diseases*

β Lapachone blocks the cell cycle and induces apoptosis in canine osteosarcoma cells[1]

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ABSTRACT.- Cruz V.S., Rodrigues F.A., Braga K.M.S., Machado P.A., Bianchi Filho C., Prado Y.C.L. & Araújo E.G. 2018. **β Lapachone blocks the cell cycle and induces apoptosis in canine osteosarcoma cells**. *Pesquisa Veterinária Brasileira 38(12):2224-2232*. Post-Graduate Program in Animal Science, Laboratório Multiusuário de Cultivo Celular, Universidade Federal de Goiás, Campus Universitário Samambaia, Avenida Esperança s/n, Goiânia, GO 74690-900, Brazil. E-mail: vanessascpimenta@yahoo.com.br

Osteosarcoma is a malignant tumor of primitive bone cells with a high incidence in dogs and humans. The need for more effective drugs with less adverse consequences has pushed the development of chemotherapeutic agents from plants and other natural sources. The aim of this study was to verify the cytotoxic effects of β-lapachone, a compound present in the sawdust of *Tabebuia* sp. (popularly known as *ipê*) wood, on canine osteosarcoma cells subcultured and treated in different concentrations (0.1μm, 0.3μm e 1.0μm) and exposure times (24h, 48h e 72h). Results were obtained through Trypan blue dye exclusion, tetrazolium reducing method, cell survival assay, Annexin V-FITC and Propidium Iodine labeling, JC-1 dye labeling and cell cycle kinetics e analysis. The group treated with 0.3μm β-lapachone presented higher decrease in cell viability (80.27%, 24h, 47.41%, 48h and 35.19%, 72h) and greater progression of cytotoxicity (19.73%, 24h, 52.59%, 48h and 64.81%, 72h). The lower IC_{50} (0.180μm) was verified in the group treated for 72 hours. Cell growth after treatment decreased as concentration and time of exposure increased, with 0.50% survival fraction at the concentration of 1.0μm. Initial apoptosis was the most frequent type of cell death in all groups, reaching bottom in the 24-hour group treated with 0.1μm (4.26%) and peaking in the 72-hour group treated with 1.0μm (85.89%). Mitochondrial depolarization demonstrated a dose-dependent phenomenon, indicating the intrinsic apoptosis. Cell growth inhibition by blocking cell cycle in the G0/G1 phase related to the exposure the time. β-lapachone is cytotoxic for canine osteosarcoma cells, induces apoptosis and promotes cell cycle arrest in G0/G1 phase.

INDEX TERMS: β Lapachone, cell cycle, induces apoptosis, canine osteosarcoma, lapachol, lineage D-17, ipê, naphthoquinone, *Tabebuia* sp., pathology.

RESUMO.- [**β Lapachona bloqueia o ciclo celular e induz apoptose em células de osteossarcoma canino**.] O osteossarcoma é o tumor maligno das células ósseas primitivas, com alta incidência em cães e humanos. A necessidade de medicamentos mais efetivos, com menor consequência adversa, tem gerado esforços para o desenvolvimento de agentes quimioterápicos compostos por plantas e outras fontes naturais. O objetivo deste estudo foi verificar os efeitos citotóxicos da β lapachona, um composto presente na serragem da madeira do ipê, sobre células de osteossarcoma canino subcultivadas e submetidas ao tratamento, de acordo com as diferentes concentrações (0,1μm; 0,3μm e 1,0μm) e tempo de exposição (24h, 48h e 72h). Os resultados foram obtidos por meio dos métodos de exclusão do corante azul de Tripan e de redução do tetrazólio, além dos ensaios de sobrevivência celular, de dupla marcação com Anexina V-FITC

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e Iodeto de Propídio, de marcação com o corante JC-1 e pela análise da cinética do ciclo celular. O grupo tratado com 0,3µm de β lapachona apresentou melhor regressão da viabilidade celular (80,27%, 24h; 47,41%, 48h e 35,19%, 72h) e maior progressão da citotoxicidade (19,73%, 24h; 52,59%, 48h e 64,81%, 72h). O menor IC_{50} (0,180µm) ocorreu no grupo tratado por 72 horas. O crescimento celular após o tratamento foi menor, de acordo com o aumento da concentração e tempo de exposição, apresentando 0,50% de fração de sobrevivência na concentração de 1,0µm. A apoptose inicial foi o tipo de morte celular mais frequente em todos os grupos, menor no grupo de 24 horas tratado com 0,1µm (4,26%) e maior no grupo de 72 horas tratado com 1,0µm (85,89%). A despolarização mitocondrial ocorreu de maneira dose dependente, indicando a ocorrência de apoptose intrínseca. A β lapachona possui efeitos citotóxicos em células de osteossarcoma canino, induz apoptose e promove o bloqueio do ciclo celular na fase G0/G1.

TERMOS DE INDEXAÇÃO: β Lapachona, ciclo celular, apoptose em células, osteossarcoma canino, lapachol, linhagem D-17, ipê, naftoquinona, *Tabebuia* sp., patologia.

INTRODUCTION

Osteosarcoma (OS) corresponds to 6% of incidental neoplasms in dogs, 80% of bone tumors and its prognosis is usually reserved (Nelson & Couto 2015, Neuwald et al. 2006, Karnik et al. 2012, Krajarng et al. 2012). In humans it represents 60% of all neoplasms that affect in the first two decades of life (Hofstaetter et al. 2013, Ouyang et al. 2013).

Therapy to induce remission of OS in dogs and humans includes the use of various substances such as cisplatin, carboplatin, ifosfamide, doxorubicin, and methotrexate (Osborne & Khanna 2012, Cinegaglia et al. 2013, Batschinski et al. 2014). Adverse effects reflect on cardiotoxicity, nephrotoxicity, ototoxicity and hepatotoxicity (Gallagher et al. 2012, Zhang et al. 2013). A significant number of human OS patients still respond poorly to intensive chemotherapy and are at risk of local recurrence or distant metastases (Ouyang et al. 2013).

Brazil has a flora rich in biological diversity and, in particular, the Brazilian cerrado biome contains tropical medicinal plants, with potential for the development of chemotherapeutic agents, with analgesic, tranquilizing, diuretic, laxative, antibiotic and antineoplastic properties (Souza & Felfili 2006). There are approximately 46 types of wood in Brazil under the designation of *ipê* (Silva et al. 2003). In the plants of the bignoniaceous family, *Tabebuia avellanedae*, *T. serratifolia*, *T. heptaphyla*, *Zeyhera digitalis* and *Z. tuberculosa,* are found naphthoquinones, in addition to other compounds such as flavonoids, lignans, monoterpenes, triterpenes, cinnamic acids, benzoic (Fonseca et al. 2003, Silva et al. 2003, Hussain et al. 2007, Silva 2009).

The derivatives of naphthoquinones present cytotoxic and genotoxic potential by the interaction with nucleophilic biomolecules, such as protein and non-protein thiols (glutathione), and by interference in the action of cellular enzymes involved in the process of cell proliferation (Klaus et al. 2010). Lapachol (LP) is a naphthoquinone with several biological properties and without serious side effects (Thomson 1997, Martin-Navarro et al. 2010). At the end of the 19th century, the conversion of lapachol into alpha-Lapachone and beta-Lapachone (βLP) by acid cyclization was described (Silva et al. 2007). βLP (3.4-dihydro-2, 2-dimethyl-2H-naphthol [1.2-b] pyran-5.6-dione), a substance used in this study, has antibacterial, antifungal and antiretroviral activity (Pardee et al. 2002, Aires et al. 2014). It is known that this compound has good results in prolonging the survival of leukemia models, blocking the multiplication of human immunodeficiency virus (HIV-1), as adjuvant therapy in proliferative retinopathy, as an anti-psoriatic agent, and has schistosomicidal activity (Pardee et al. 2002, Aires et al. 2014). The fact that it is inhibiting DNA topoisomerase I, an enzyme that plays an important role in the processes of replication and packaging of DNA, triggered studies on the antineoplastic action of βLP in tumors implants in *in vivo* mice, such as cancers of prostate, ovary and breast, as well as in human cells of *in vitro* cultivation, such as prostate and lung (Pardee et al. 2002, Kung et al. 2014).

This study aims to verify the cytotoxic effects of βLP, its action in the progression of the cell cycle and the process of cell death in the lineage of canine osteosarcoma, since this substance has antitumor activity in several types of neoplasms. The confirmation of its efficacy on the OS can allow the development of a more efficient therapeutic strategy and with less adverse effect for the patient.

MATERIALS AND METHODS

Osteogenic metastatic canine osteosarcoma (COS) cells (D-17, BCRJ 0276, Lot 000573, Passage 239) were acquired from the Rio de Janeiro Cell Bank (UFRJ, Rio de Janeiro, Brazil) originating from the ATCC (American Type Culture Collection, Manassas/VA, USA). The cells were kept in a humidified incubator at 37°C with an atmosphere of 5% of CO_2. They were cultivated in Dulbecco modified eagle medium (DMEM) plus 10% of bovine fetal serum, penicillin and streptomycin (10,000U.I./ML - 10mg/ml), amphotericin B and L glutamine (all the reagents from Cultilab, Campinas, Brazil), according to the adaptation of Yu et al. 2014.

The cells were sown in culture plates and submitted or not to treatments with βLP, acquired from Santa Cruz Biotechnology (Dallas, Texas, USA), according to the previously prepared concentrations, in the dosages of 0.1µm, 0.3µm, 1.0µm, in three different periods for each concentration, for 24h (G_{24}), 48h (G_{48}) and 72h (G_{72}). The negative control group (CG) received treatment with DMSO free of βLP in the same periods. All assays were performed with three independent experiments in triplicate.

For the Cell Viability Assay (Mosmann 1983, Peres & Curi 2005), 96-well plates with cells at the concentration of $1x10^4$ were used, and after the treatment period, the mean of each well was discarded, and the cells suspended in trypsin (Cultilab, Campinas, São Paulo, Brazil), centrifuged and resuspended. Then the cells were stained with Trypan blue dye (*Trypan Blue* - Sigma - Aldrich, St Louis, USA). The evaluation of cell viability was performed using the Luna Automated cell counter reader. The cytotoxicity was determined, and the values calculated for each group by the F test, then were compared to the F tabulated in 5% (2.2).

To calculate the concentration value that inhibits 50% of the cell viability (*inhibitory concentration* - IC_{50}), 96 wells with cells at the concentration of $1x10^4$ were used (Yu et al. 2014) At the end of the treatment period, 10µl of tetrazolium (MTT (3-(4.5-Dimethyl-2-thiazolil) -2.5-diphnyl-2H-tetrazolium) were added to each well. After incubation period of 3 hours, 50µl of Sodium Dodecyl Sulfate (SDS - Vivantis Biochemical) was added to 10% diluted in HCL/0.01N per well. The plates remained incubated for 24 hours at room temperature. The optical density was quantified

in a spectrophotometer. The IC_{50} was determined using the statistical program GraphPad Prism 6 (GraphPad Software, San Diego/CA, USA).

The cell survival assay (Cao et al. 2014, Park et al. 2014) was made in six-well plates with cells at the concentration of $1x10^6$. The mean of each well was discarded after the treatment period and the cells were suspended in trypsin, centrifuged, resuspended and cultivated in six-well plates. The mean was discarded after 10 days, the wells were washed with PBS, the cells fixed with solution containing methyl alcohol (Impex/Labinpex) and acetic acid (Synth/Labsynth, Diadema, Sao Paulo, Brazil) and, finally, stained with 500µl of Giemsa (Giemsa stain, Sigma-Aldrich, St Louis, USA). Each well was divided into nine fields for quantification of the cells in inverted microscope; the average of the fields per well and the calculation of the survival fractions was performed. The data were analyzed by the F test and compared to the F tabulated in 5% (2.2).

For the double marking assay (Park et al. 2014, Yu et al. 2014), the cells were cultivated and treated in 12-well plates at a concentration of $5x10^5$. Then, the mean of each well was discarded, the cells were suspended and centrifuged. 400µl of connection buffer and 5µl of V-FITC Annexin and 1µl of Propidium Iodine were added (Annexin V Apoptosis Detection Kit I-BD Biosciences, San Diego, USA), and the material was analyzed by the flow cytometer (Facscalibur, BD Biosciences). The cells marked only by Annexin V were classified as initial apoptosis. The cells marked by Annexin V and Propidium Iodine were classified as late apoptosis. The cells marked only by the Propidium Iodine were classified as necrosis. Cells that did not present any markings were considered viable. The data were transferred to the GraphPad Prism statistical graphics program. Data analysis, according to the classification of the column factor, estimated the individual values of viable cells, initial apoptosis, late apoptosis and necrosis. Data analysis, according to the line factor classification, compared the values found in each concentration.

In the Mitochondrial Membrane Potential Assay (Yu et al. 2014), the cells were cultivated in 12-well plates with the concentration of $5x10^5$. At the end of the treatment period, the mean of each well was discarded, the cells suspended, centrifuged, resuspended and incubated with the JC-1 dye for 15 minutes at 37°C. At the end of this period, they were washed with PBS and resuspended in a test buffer. The results were obtained through the flow cytometer and analyzed by GraphPad Prism statistical graphics program. Data analysis, according to the classification of the column factor, estimated the individual values of viable cells and apoptosis. Data analysis, according to the line factor classification, compared the values found in each concentration.

The cell cycle analysis was performed using 12-well plates with cells at the concentration of $5x10^5$. After the treatment period, the mean of each well was discarded, the cells were suspended, centrifuged and incubated for 24 hours at -20°C in ethyl alcohol at 70% stored previously at 4°C. After this period, the material was incubated in a solution containing ribonuclease a (Ribonuclease A from Bovine Pancreas, Sigma Aldrich, St Louis, USA) 0.05%, in a water bath at 37°C for 30 minutes. There was a new centrifugation, the ribonuclease was discarded, the cells were resuspended in PBS and the Propidium Iodine was added. The percentage of cells in G1, S, G2 and sub-G1 was analyzed in flow cytometer and the results were analyzed by GraphPad Prism statistical graphics program. Data analysis, according to the classification of the column factor, estimated the individual values of the cells found in each phase of the cell cycle. Data analysis, according to the line factor classification, compared the values found in each concentration.

RESULTS AND DISCUSSION

Cytotoxicity (CT) was measured using the cell viability assay by the method of exclusion of the Trypan blue dye. The G_{72} with 0.3µm treatment showed lower cellular viability (35.19%). The G_{24} with 0.3µm treatment showed higher cellular viability (80.27%). All groups presented CT, demonstrating that βLP is cytotoxic to OSC cells. At the dosage of 0.1µm there was no statistical difference between the times of exposure to the βLP. The statistical difference occurred in the groups that were treated with 0.3µm, concentration that presented the best regression of cell viability (CV) and better progression of cytotoxicity (CT), by time of exposure. Cytotoxicity rose from 19.73% in G_{24} to 52.59% in G_{48} and reached 64.81% in G_{72}. Corroborating the results of Aires et al. (2014), according to which the βLP has cytotoxic activity in several cells of human tumors.

Other to what found in the present study, the viability of OSC cells presented dose-dependent decrease when treated with α mangostin, a substance extracted from mangosteen, a tropical fruit found in Southeast Asia (Krajarng et al. 2012). Similarly, Cinegaglia et al. (2013) described that the human osteosarcoma cells (HOS) were sensitive to the dose-dependent cytotoxic effect at concentrations between 50µg to 100µg of propolis, a product collected from exudates of plants mixed with wax and saliva enzymes of bees. Also, the methoxsalen, a natural compound found in seeds of several plants, was cytotoxic to osteosarcoma cells MG63, in the dosages between 300µm and 500µm (Lu et al. 2017). On the other hand, carboplatin did not present cytotoxic activity in OSC cells in the assays performed by Stein et al. (2011) and in HOS cells in the study by Robson et al. (2007).

The concentration 0.3µm is expressively lower than the lethal concentrations used in the first studies on the βLP for different cells in cultivation, ranging between 1.0µm and 30.0µm (Docampo et al. 1979). It also differed from the report by Pardee et al. (2002), in which the βLP, in a dose of 2.0µm to 10µm, presented lethality against several tumor cells in cultivation and delayed the growth of prostate, breast and ovarian tumor cells *in vivo*.

The results of the efficacy measure of βLP indicated that the concentration required for 50% *in vitro* inhibition in G_{24} was 0.959µm, in G_{48} was 1.657µm, and in G_{72} was 0.180µm. The variation in the values of IC_{50} can be attributed to the sensitivity to the effects of the substance in the different exposure times and to the metabolism of the drug. The low concentrations found by IC_{50} in the present study may be suggestive to lower adverse effects to the patient than that of HOS cells treated with concentrations between 50µm and 100µm of baicalein, a substance used in popular treatments in China (Zhang et al. 2013).

The mean number of OSC cells, verified after the incubation period of the cell survival assay, revealed a dose-dependent decrease in groups G_{24} (5 cells/0.1µm; 3 cells/0.3µm, and 0.55 cell/1µm), G_{48} (1.33 cells/0.1µm; 0.88 cells/0.3µm, and 0.33 cell/1.0µm) and G_{72} (1.22 cells/0.1µm; 0.22 cell/0.3µm, and 0.11 cell/1.0µm). The higher the concentration and the exposure time, the lower the cell survival. It was possible to perceive that in the CG (56 cells/24 hours; 24.66 cells/48 hours and 41.22 cells/72 hours) there was cell growth compatible with the absence of treatment by βLP. The decrease in the number of cells with the period of treatment in this group is

probably due to the restriction in relation to exposure time and space for growth.

The calculation of the survival fractions was made to compare the number of cells found in the groups treated with the number of cells observed in the CG. It was observed that the cell growth after treatment was lower (0.50%) in the G_{72} with the concentration of 1.0μm and higher (13.93%) in the G_{24} with the concentration of 0.3μm. The data suggest that, after treatment with βLP, there was a little significant resumption of cell growth. The cell survival assay is important to analyze the proliferative capacity of cells after exposure to a certain antiproliferative agent (Plumb 2004).

The results obtained in relation to cell proliferation suggest possible effect of BLP in combating recurrences, as already observed in models of xenograft of breast cancer in mice treated with β Lapachone. The animals remained healthy for 200 days after the end of treatment and were considered cured (Cao et al. 2014). On the other hand, it is important to analyze this effect for longer periods in future research, because the use of cytotoxic agents and signal transduction inhibitors can promote the elimination of cancer cells in the short term, but the recurrence of residual cells may occur with drug resistance (Flick et al. 2013). In this sense, a problem reported in the case of HOS is the possibility of local recurrence with the use of conventional treatments, such as analgesics, radiation therapy, surgery and chemotherapy (Krajarng et al. 2012).

The values calculated for each group using the F test were compared to F tabulated 5% (2.2) = 19. It was concluded that there was no statistical difference for cell growth between the different concentrations and times of exposure to βLP. This may indicate that, even with low concentration and short interval of exposure time, cell growth after treatment was minimal. Similarly, Kung et al. (2014) reported a small percentage of survival of human lung cancer cells, using concentrations between 1.0μm and 5.0μm of βLP.

By double marking test with Annexin V and Propidium Iodine (Table 1) was observed that cells in initial apoptosis were marked only by Annexin V, the cells in late apoptosis were marked by Annexin V and Propidium Iodine, and the cells in necrosis were marked only by the Propidium Iodine (Fig.1). Viable cells did not present any markings. Data on the mechanism of death of the cell, promoted by β Lapachone in canine osteosarcoma cells, are presented in Tablet 1.

There was a prevalence of initial apoptosis in all concentrations of G_{48} and G_{72}. Late apoptosis was higher in the 1.0μm concentration of the G_{24}. The concentration of 1.0μm presented a higher rate of apoptosis in the three exposure times. There was no statistically significant difference between repetitions and triplicate values in each concentration (P=1.000). Initial apoptosis was the most frequent type of cell death in all groups. However, there was a statistically significant difference between the results found in the concentrations used in each treatment group ($p<0.0001$). Initial apoptosis occurred in a dose-dependent manner. It was lower in the group of 48 hours treated with 0.1μm of β Lapachone (5.22%) and higher in the group of 72 hours treated with 1.0μm of β Lapachone (85.89%).

These results indicate that the reduction of the viability of the canine osteosarcoma cells after treatment with the β Lapachone is related to apoptosis. The same mechanism of cell death by β Lapachone was reported by Pardee et al. (2002) in glioma cells and human colon cancer. Kung et al. (2014) observed that apoptosis was induced in lung cancer cells by this substance by decreasing the activation of the proliferation factors. However, there was disagreement with the study done by Park et al. (2014), where the induction of programmed necrosis, by β lapachone, was observed in cells of human hepatocellular carcinoma. For Flick et al. (2013), death of the cell by necrosis may occur with the use of β-lapachone treatment, since this compound potentializes the immune system, by means of factors associated with inflammation. Other compounds used for treatment of human osteosarcoma, such as loperamide (Regan et al. 2014), survivin (Shoeneman et al., 2012), baicalein (Zhang et al. 2013), MiR-335 (Liu et al. 2016) and mangosteen (Krajarng et al. 2012), also induce apoptosis. Doxorubicin induces autophagy in human osteosarcoma cells (Chang et al. 2014).

Table 1. Mean of the data provided by the flow cytometer on the mechanism of cell death promoted by β Lapachone in canine osteosarcoma cells

Treatment time (hours)	Concentration	Viable cells	Initial apoptosis	Late apoptosis	Necrosis
24	NC*	87.49	4.26	2.43	5.81
	0.1μM	75.35	19.02	2.02	3.42
	0.3μM	79.79	9.99	4.40	5.82
	1.0μM	3.27	29.49	49.97	14.54
48	NC*	83.66	6.36	3.73	6.58
	0.1μM	27.30	5.22	3.39	2.87
	0.3μM	56.56	25.26	9.29	8.88
	1.0μM	6.58	80.06	12.58	0.77
72	NC*	75.33	14.57	4.56	5.55
	0.1μM	86.44	5.12	4.76	3.68
	0.3μM	27.96	54.81	9.99	7.23
	1.0μM	5.08	85.89	8.93	0.09

Presentation of data on the mechanism of death of the cell promoted by β Lapachone in cells of canine osteosarcoma. *NC = Negative control.

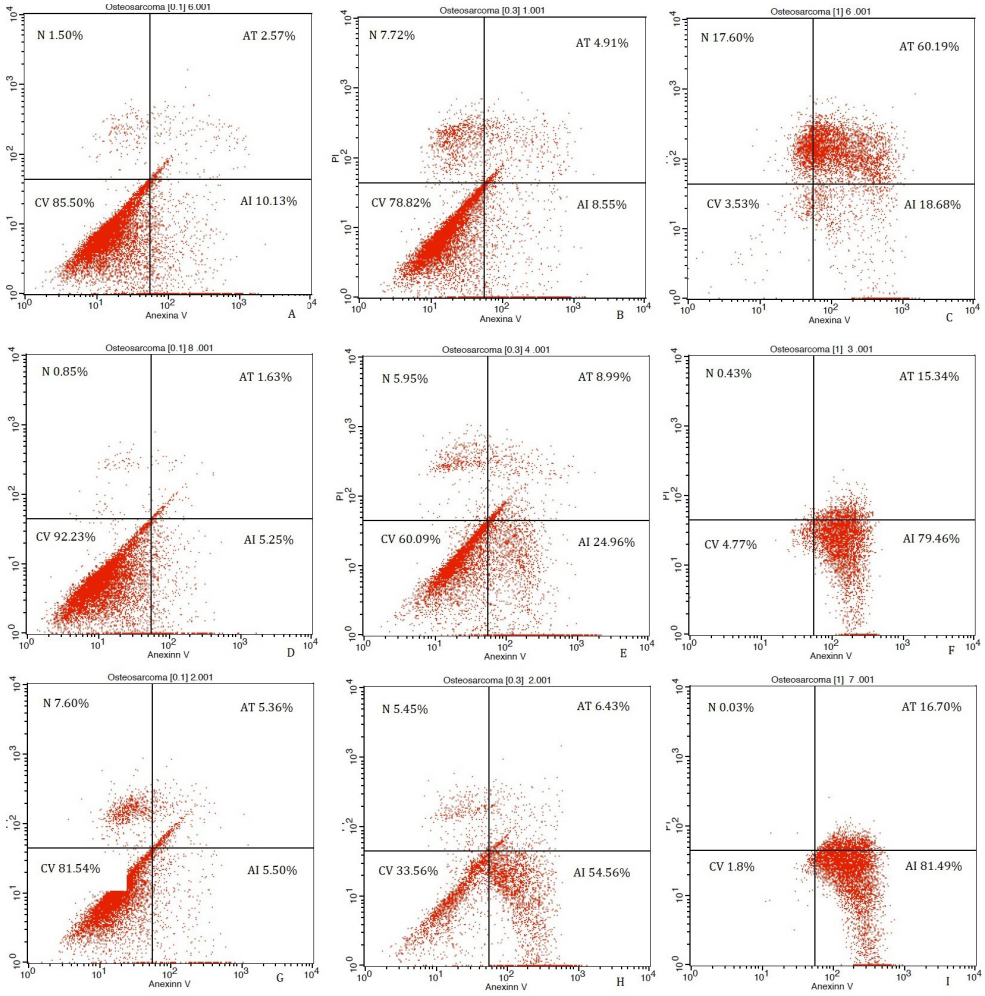

Fig.1. Representative images of samples from the analysis of the death of the cell mechanism promoted by β Lapachone in the canine osteosarcoma cells, by means of the double marking test with Annexin V (X axis) and Propidium Iodine (Y axis). (**A**) 0.1µm/24h. (**B**) 0.3µm/24h. (**C**) 1.0µm/24h. (**D**) 0.1µm/48h. (**E**) 0.3µm/48h. (**F**) 1.0µm/48h. (**G**) 0.1µm/72h. (**H**) 0.3µm/72h. (**I**) 1.0µm/72h. N = death of the cell by necrosis, AT = late apoptosis, CV = viable cells, AI = initial apoptosis. Flow Cytometer (Facscalibur, BD Biosciences).

The effect of β-lapachone on the mitochondrial membrane potential was measured for 24 hours, using the lipophilic dye JC-1 (Fig.2). Mitochondrial depolarization occurred in a dose-dependent manner. Data on mitochondrial membrane potential, promoted by β Lapachone in canine osteosarcoma cells, are presented in Table 2. Apoptosis was lower in the concentration of 0.1µm (14.86%) and higher in the concentration of 1.0µm (88.13%).

The values indicated that apoptosis triggered by β Lapachone may be related to the rupture of the integrity of the mitochondrial membrane potential. According to this result, Pardee et al. (2002) reported that β Lapachone causes release of cytochrome c, a protein associated with the internal membrane of the mitochondria, which inhibits the cell cycle. Thus, β Lapachone plays a selective antineoplastic action in mitochondrial mutations, frequent in tumor cells. Galluzzi et al. (2012) describe as intrinsic apoptosis the cell death that begins in the external mitochondrial membrane or may result from the transition of mitochondrial permeability.

Table 2. Average of the data provided by the flow cytometer on the mitochondrial membrane potential, using the lipophilic dye JC-1

Treatment time (hours)	Concentration	Viable cells	Apoptosis
24	NC*	95.15	3.47
	01µM	83.45	16.22
	0.3µM	54.91	38.80
	1.0µM	12.77	83.45

Presentation of data on the potential of mitochondrial membrane, promoted by β Lapachone in canine osteosarcoma cells. *NC = Negative control.

The values found were evaluated by the GraphPad Prism Statistical graphics program, using the bidirectional ANOVA method. There was no statistically significant difference between repetitions and triplicate values in each concentration (p=0.9583). However, there was a statistically significant

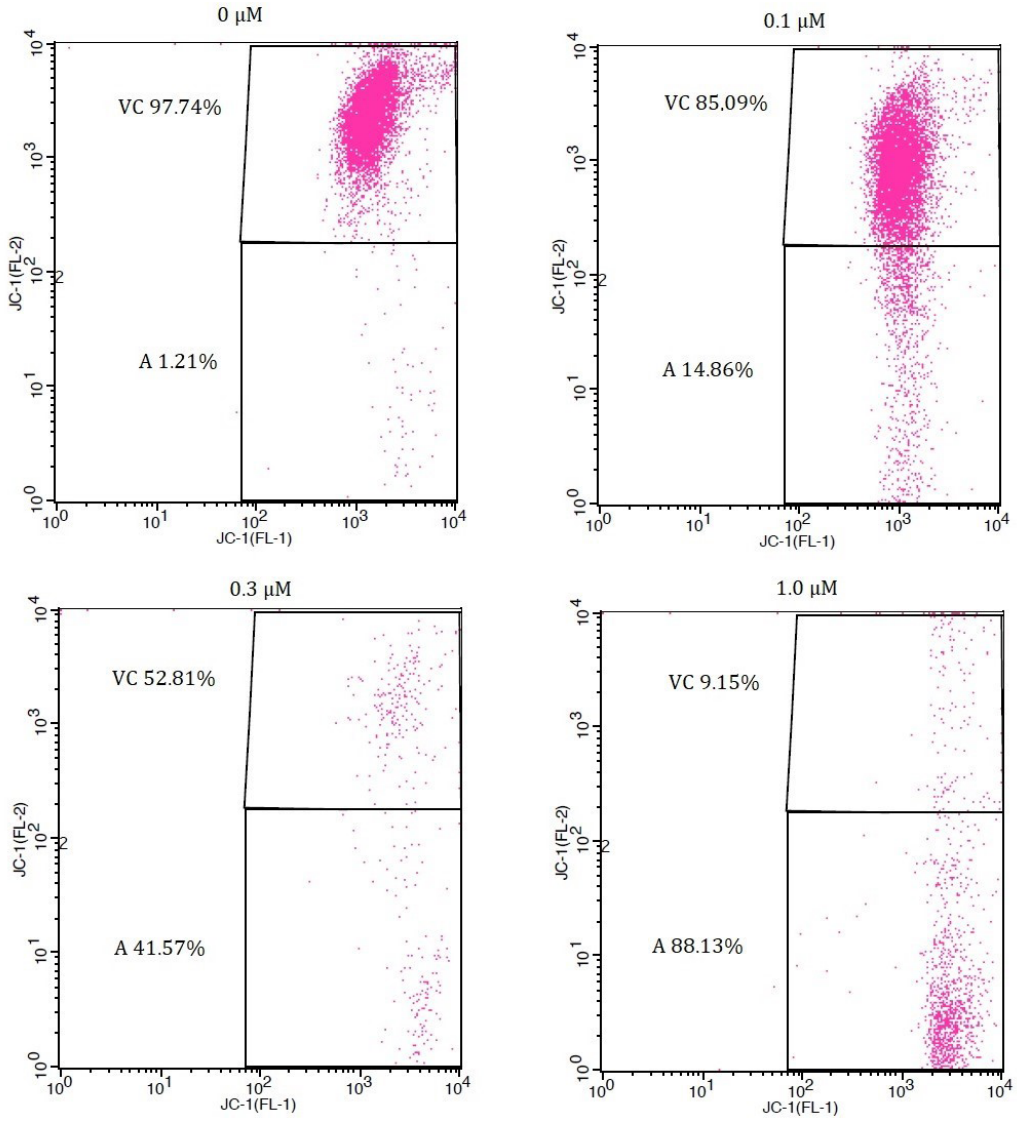

Fig.2. Analysis of the effect of different concentrations of β Lapachone in 24 hours on the mitochondrial membrane potential in the canine osteosarcoma cells, by using the lipophilic dye JC-1. There was a marked increase in apoptosis (A) and decreased cell viability (VC) in the concentration of 1.0µm. FL-1 = fluorescence channel 1; FL-2 = fluorescence channel 2. Flow Cytometer (Facscalibur, BD Biosciences).

difference between the results found in the concentrations used ($p<0.0001$). Mitochondrial depolarization was lower in the group treated with 0.1µm of β Lapachone (3.47%) and higher in the group treated with 1.0µm of β Lapachone (83.45%). The induction of the integrity loss of the mitochondrial membrane potential also occurred in cells of canine osteosarcoma treated for 3 hours, with 30µg/ml of α mangostin, substance extracted from mangosteen, tropical fruit found in Southeast Asia (Krajarng et al. 2012).

The phases of the cell cycle were characterized by the percentage of cells of canine osteosarcoma, after treatment with β lapachone, in sub-G1, G0/G1, S, and G2/M. Data on the analysis of cell cycle blockade, promoted by β Lapachone in cells of osteosarcoma, are presented in Table 3. There was a significant statistical difference ($p<0.0001$) between exposure times. In the G_{24} (Fig.3) there was similarity between the number of cells in the phase G0/G1 and S. With this exposure

Table 3. Mean of the data provided by the flow cytometer on the analysis of the cell cycle blockade of the D-17 lineage after treatment with β Lapachone

Treatment time (hours)	Concentration	Cell cycle phases			
		SUB-G1	G0/G1	S	G2/M
24	0.1µM	0.75	46.42	49.13	8.67
	0.3µM	7.35	43.46	52.11	4.43
	1.0µM	2.69	58.44	41.56	0.00
48	0.1µM	0.00	72.74	27.20	0.05
	0.3µM	0.81	65.74	33.80	0.45
	1.0µM	4.67	71.08	18.10	12.96
72	0.1µM	0.02	69.03	29.12	1.84
	0.3µM	1.45	64.99	32.01	2.98
	1.0µM	11.92	66.48	12.02	21.52

Presentation of data on the analysis of cell cycle blockade, promoted by β Lapachone in canine osteosarcoma cells.

time, the cells can synthesize the proteins needed so that each chromosome can replicate. However, this event depends on the origin and the cellular type (Pardee et al. 2002).

The difference between phases increased significantly in G_{48} and G_{72} (Fig.4), predominating in the phases G0/G1, which corresponds to the interval between mitosis and DNA duplication. As the cycle progresses to the transition G0/G1, there is the stimulus to transcription of the genes involved in the progression of the cell cycle, which encode the proteins c-Myc, p53, pRb, Ras, PKA, PKC, Bcl-2, NF-κB, CDK, cyclins and CKI. The blockade at the checkpoint, in the G1/S phase, generates mechanisms to prevent the formation of anomalous cells, perform the repair or induce apoptosis (Zörnig et al. 2001, Brooks & Gu 2006, Wu et al. 2006, Maximov & Maximov 2008). In addition, β Lapachone has as its property the ability to inhibit the topoisomerases complex, not letting them reconnect to the DNA and undoing the complex. Thus, the activation of checkpoints in the cell division process occurs, inducing the death of malignant cells (Silva et al. 2003).

There was no statistical difference between the concentrations used during the treatment of this experiment and the blockade of the cell cycle. A distinct result occurred in the HBL-100, HeLa, SW1573 and WiDr strains of human solid tumors, in which changes in the cell cycle were dependent on the concentration of β-lapachone (Rios-Luci et al. 2012). The present result also differs from that reported in HOS cells treated with baicalein, in which the distribution of cells in the G0/G1 phase increased in a dose-dependent manner (Zhang et al. 2013).

In this study, it was possible to realize that, at the dosage of 1.0μm in G_{48} and G_{72}, there were cells locked in the G2/M phase, inducing the mitotic catastrophe. For Pardee et al. (2002), chromosomal aberrations can be created indirectly by the long exposure of β Lapachone to high concentrations, and these cells are blocked in the G2/M phase. Galluzzi et al. (2012) reported that cell cycle blockade, when there is an attempt to divide cells with damaged DNA, characterizes the mitotic catastrophe.

In summary, inhibition of the growth of canine osteosarcoma cells after treatment with β Lapachone occurred by blocking the cycle in G0/G1 phase and significantly increased with exposure time. The same occurred with cells of canine osteosarcoma treated with α-mangostin (Krajarng et al. 2012). This differs from the antineoplastic agents used in the routine for the treatment of osteosarcoma, such as cisplatin, carboplatin, doxorubicin and methotrexate. These agents inhibit the synthesis of DNA in the S phase of the cell cycle (Almeida et al. 2005). Similarly, the human cells of colon cancer and hepatoma, treated by β lapachone, are also inhibiting in the S phase (Pardee et al. 2002).

This study seems to be the first to evaluate the antiproliferative effects of βLP as a treatment for OSC cells *in vitro*, demonstrating that the compound effectively presented antiproliferative activity and cytotoxic effect on the tested cell type. The dosage

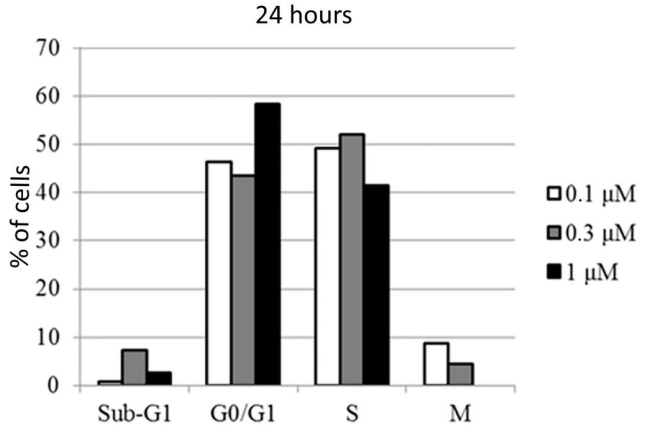

Fig.3. Graphical representation of the result of the cell cycle kinetics for G_{24}. It is noted the similarity between the number of cells in the G0/G1 and S phases. X axis = phases of the cell cycle, Y-axis = number of cells. GraphPad Prism® program.

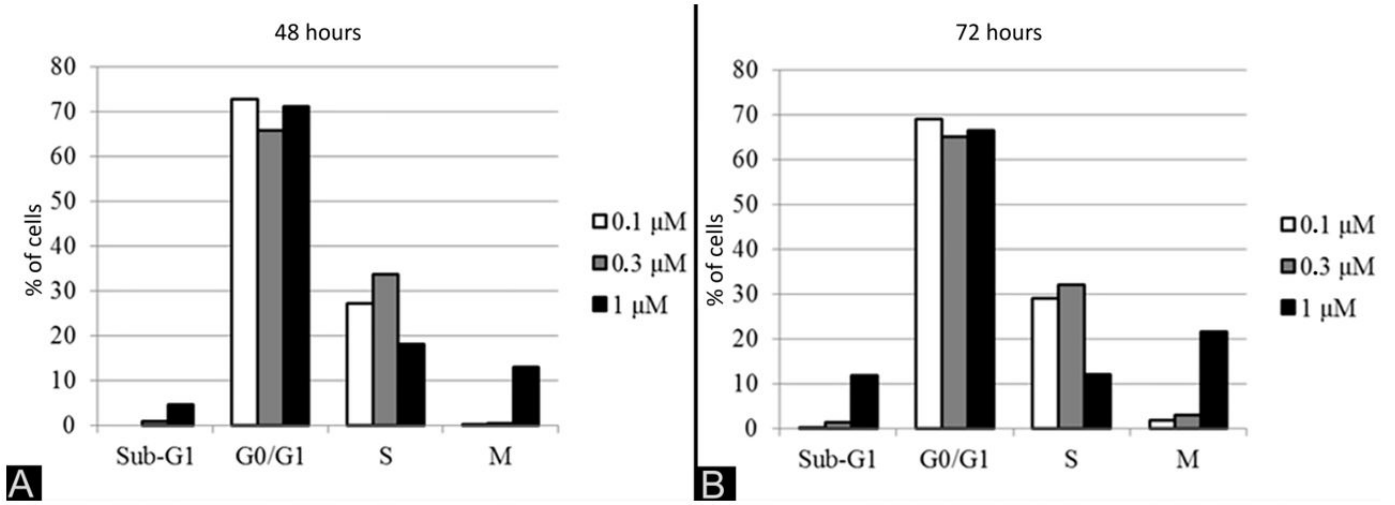

Fig.4. (**A**) Graphic representation of the result of the cell cycle kinetics for G_{48} and (**B**) G_{72}. It is noted that the inhibition of growth occurred by the blockade of the cycle in the G0/G1 phase and increased with the exposure time. X axis = phases of the cell cycle, Y axis = number of cells. GraphPad Prism® program.

of 0.3μm showed to be the most promising, suggesting that, in low concentration, it is possible to obtain the desired therapeutic action, associated with the possibility of minor adverse effects. Initial apoptosis was the most frequent type of cell death in all groups, related to the rupture of the integrity of the mitochondrial membrane potential, indicating the occurrence of intrinsic apoptosis. Inhibition of cell growth occurred by blocking the cycle in phase G0/G1. Thus, it is possible that the formation of checkpoints in the cell division process has occurred, inducing the death of the cells of canine osteosarcoma in low concentration.

Although the results of this research are very promising, *in vivo* studies are necessary to allow the validation of the possible clinical use of βLP in animals and humans. Strategies such as the observation of this treatment in xenografts of osteosarcoma cells in laboratory animals and subsequent therapeutic use in natural cases of the tumor will be important to determine the systemic and local action of the substance on neoplastic cells, as well as possible adverse effects provided.

CONCLUSION

β Lapachone has cytotoxic effects on canine osteosarcoma cells, induces apoptosis and promotes cell cycle blockage in G0/G1phase.

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Original Article
Pequenos Animais/*Small Animals Diseases*

Ceftaroline resistance in *Staphylococcus pseudintermedius* gene *mecA* carriers[1]

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ABSTRACT.- Scherer C.B., Botoni L.S., Carvalho A.U., Keller K.M. & Costa-Val A.P. 2018. **Ceftaroline resistance in *Staphylococcus pseudintermedius* gene *mecA* carriers**. *Pesquisa Veterinária Brasileira 38(12):2233-2236*. Departamento de Clínica e Cirurgia, Escola de Medicina Veterinária, Universidade Federal de Minas Gerais, Av. Antônio Carlos 6627, Cx. Postal 567, Belo Horizonte, MG 31270-901, Brazil. E-mail: adriane@ufmg.br

Infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) being a constant concern, ceftaroline fosamil has been recently approved as a new cephalosporin, active against MRSA, for use in humans; only rare cases of resistance have been reported till date. There is no report of resistance to ceftaroline in *Staphylococcus pseudintermedius*, which is the main bacterium causing dermatitis and otitis in dogs. To evaluate staphylococcal resistance to ceftaroline, 35 isolates of methicillin-resistant *S. pseudintermedius* (MRSP), carrying the *mecA* gene, from 26 dogs with folliculitis and nine dogs with external otitis, underwent disk diffusion test with cefoxitin, oxacillin, and ceftaroline. Tests with cefoxitin and oxacillin showed > 90% sensitivity in methicillin resistance detection. In the disk diffusion test, 97.14% (34/35) were resistant to cefoxitin, 94.29% (33/35) to oxacillin, and 31.43% (11/35) to ceftaroline. Of the ceftaroline-resistant strains, 27.27% (3/11) were obtained from the ears of dogs while the rest (8/11) were from the skin. The current report is the first description of MRSP resistance to ceftaroline.

INDEX TERMS: Ceftaroline, resistance, *Staphylococcus pseudintermedius*, *mecA* gene, MRSP, cefoxitin, oxacillin, dogs, bacterioses.

RESUMO.- [**Resistência à ceftarolina em *Staphylococcus pseudintermedius* portadores do gene *mecA*.**] Infecções causadas por *Staphylococcus aureus* resistente à meticilina (MRSA) são uma preocupação médica constante. A ceftarolina fosamila é uma nova cefalosporina ativa contra *Staphylococcus aureus* resistente à meticilina recentemente aprovada para uso em humanos e raros casos de resistência relatados até agora. Não há relatos de resistência à ceftarolina em *Staphylococcus pseudintermedius*, principal bactéria causadora de dermatite e otite em cães. Com o objetivo de avaliar a resistência estafilocócica à ceftarolina, 35 amostras de *S. pseudintermedius* resistentes à meticilina (MRSP), portadoras do gene *mecA*, provenientes de 26 cães com foliculite e 9 com otite externa foram submetidos ao teste de disco-difusão com cefoxitina, oxacilina e ceftarolina. Os testes realizados com cefoxitina e oxacilina mostraram mais de 90% de sensibilidade na detecção da resistência à meticilina em ambas. No teste da disco-difusão, 97,14% (1/35) foram resistentes à cefoxitina, 94,29% (3/35) à oxacilina e 31,43% (11/35) à ceftarolina. Das cepas resistentes às ceftarolina, 27,27 (3/11) foram provenientes de ouvido de cães e as demais (8/11), provenientes da pele, sendo essa primeira descrição de resistência de MRSP à ceftarolina na literatura atual.

TERMOS DE INDEXAÇÃO: Resistência, ceftarolina, *Staphylococcus pseudintermedius*, gene *mecA*, susceptibilidade, disco-difusão, MRSP, cefoxitina, oxacilina, caninos, bacterioses.

INTRODUCTION

Treatment of skin and ear infections caused by methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) is a constant concern in the veterinary community. MRSP are resistant to

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all β-lactams, including the cephalosporins (Cain 2013), which form the most prescribed class of antimicrobials worldwide, due to their broad spectrum of action and low frequency of adverse effects (Laudano 2011).

Ceftaroline fosamil, a new parenteral antibiotic used for the treatment of severe skin and soft-tissue infections, was approved for use in humans in the United States (2010), Europe (2012), and Brazil (2014) (Laudano 2011, Alm et al. 2014, Anvisa 2014). Ceftaroline fosamil exhibits a broad spectrum of activity, acting on both gram-negative and -positive bacteria, and hence considered by the Clinical and Laboratory Standards Institute (CLSI) guidelines as a new subclass of antimicrobials, cephalosporins, exhibiting activity against methicillin-resistant *Staphylococcus aureus* (MRSA) (Laudano 2011).

Staphylococci, resistant to β-lactamase-stable anti-staphylococcal penicillin, have been termed as "methicillin-resistant", even though methicillin is no longer the drug of choice for testing resistance by the disk diffusion test. According to Mimica et al. (2007), oxacillin disk-diffusion test has been the most widely used test for decades; however, oxacillin was subsequently replaced by cefoxitin, which induces the expression of the resistance-associated gene much more strongly in *S. aureus.* However, oxacillin continues to be used for the detection of methicillin-resistance in *S. pseudintermedius* (CLSI 2013, 2017).

Resistance to methicillin is associated with the presence of *mecA* gene, which is responsible for altering the sequence of penicillin-binding protein (PBP2a) present in the bacterial cell wall (Cain 2013). Historically, staphylococci had demonstrated resistance to cephalosporins and other β-lactam antibiotics due to the low affinity of these drugs to the modified PBP2a (Kosowska-Shick et al. 2010).

Evidence shows that the *mecA* gene originates from *Staphylococcus sciuri*, with a possible horizontal transfer to *S. aureus* and other staphylococcal species, including those affecting the skin and ears of dogs, such as *S. pseudintermedius* and *Staphylococcus schleiferi* (Wu et al. 1996, Bemis et al. 2006). In addition to the presence of these microorganisms in pigs and horses, there are reports of MRSA transmission between humans and dogs, (Weese & Van Duijkeren 2010), and dogs transmitting MRSP to humans (Lozano et al. 2017).

According to the guidelines of CLSI (2017), any strain containing the *mecA* gene must be reported as resistant to methicillin, given the rarity of other mechanisms of methicillin resistance (Petersen et al. 2013). Additionally, according to the guidelines of CLSI (2013), any oxacillin-resistant *S. pseudintermedius* must be considered resistant to methicillin and all β-lactams.

Unlike other β-lactams, which have low affinity for PBP2a, competitive assays demonstrated high affinity of ceftaroline to the allosteric domain of MRSA PBP2a, along with its ability to induce a conformational change potentially leading to exposure of an active antibiotic-binding site, thereby allowing a second ceftaroline molecule to bind to that site and block the activity of the protein (Kosowska-Shick et al. 2010). However, despite its veterinary importance and zoonotic potential, there is no report on the dual resistance of MRSP, carrying the *mecA* gene, to ceftaroline and methicillin in samples from dogs.

The current study aimed to: 1) compare the ceftaroline resistance in MRSP strains carrying the *mecA* gene, isolated from dogs with folliculitis and/or otitis externa, 2) to compare the sensitivity of oxacillin and cefoxitin disks in the disk-diffusion test for the diagnosis of MRSP strains, and 3) to evaluate the staphylococcal resistance to ceftaroline in the MRSP samples collected from dogs with folliculitis and/or otitis externa.

MATERIALS AND METHODS

Strain selection. Thirty-five *Staphylococcus pseudintermedius* strains were included in the study; nine were isolated from ear secretion and 26 from the skin of dogs with positive cytological evaluation for coccoid bacteria and previously treated for MRSP infection at the Department of Dermatology, Veterinary Hospital of the Federal University of Minas Gerais, from April to October 2013. Biochemical tests were performed for phenotypic identification of the members of *Staphylococcal intermedius* group (SIG), as previously described (Quinn 2011 and polymerase chain reaction (PCR) was conducted for the genotypic identification of *S. pseudintermedius* according to Sasaki et al. (2010) The protocols were approved by the Ethical Committee for Animal Usage (CEUA, protocol 246/2013). Written informed consent to allow sample collection from the dogs was obtained from the owners.

DNA extraction. Strains were cultured in Mueller-Hinton agar, and one colony from each culture was transferred to a micro tube containing 20μL of Milli-Q water. The bacterial suspensions were heated in a water bath at 100°C for 15 min and centrifuged at 60rpm, with a power of 10mA for 5min (HSIANGTAI Centrifuge, MCD-2000, New Taipei City, Taiwan). The supernatant was collected and used as the DNA sample.

Detection of *mecA* gene. All strains were analyzed by PCR for the detection of *mecA* gene using the primers F: 5′-ACTGCTATCCACCCTCAAC-3′ and R: 5′-CTGGTGAAGTTGTAATCTGG-3′, as described by Merothra et al. (2000).

Strains of *S. pseudintermedius* (MRSP 3279) and *Staphylococcus aureus* (USA 100) were used as positive controls, whereas amplification sample without template DNA was used as a negative control.

Susceptibility tests. The disk-diffusion method, recommended by the CLSI (2013, 2017), was used for phenotypic resistance testing. Each MRSP strain, confirmed by *mecA* gene detection, was suspended in 3mL of Mueller-Hinton broth and incubated at 35°C until a turbidity equivalent to 0.5 of the McFarland scale was reached (Bannoehr & Guardabassi 2012). Aliquots of the suspension were streaked on Mueller-Hinton agar plates (4-mm agar depth). Disks impregnated with 30μg ceftaroline (HardyDisk, Santa Maria/CA), 1μg oxacillin, and 30μg cefoxitin (Laboratório DME, Araçatuba, São Paulo, Brazil) were used. After 24-h incubation, the inhibition halos formed around the disks were measured and compared to the zone diameters published by CLSI (2017) for cefoxitin and ceftaroline to *S. aureus* and by CLSI (2013) for oxacillin to *S. pseudintermedius*.

Statistical analysis. Pearson's chi-squared test for equality of proportions was used for statistical analysis. The level of significance used in the statistical-test decisions was 5%. Statistical analyses were performed using the program SAS (SAS Institute Inc., Cary/NC). Cramér's V coefficient was used to evaluate the intensity of association between the studied variables.

RESULTS

All 35 samples were confirmed as MRSP by the PCR assays; however, the susceptibility results using oxacillin and cefoxitin disks varied. Of the analyzed strains, 5.71% (2/35) were

susceptible to oxacillin while 2.96% (1/35) were susceptible to cefoxitin (Table 1).

Among the tested MRSP strains, 31.43% (11/35) were resistant to ceftaroline. From the resistant strains, 27.27% (3/11) were from otitis while the rest (8/11) were from the skin. Additionally, Cramér's V value was 0.5, demonstrating a strong association among the resistance to the three antibiotics.

DISCUSSION

In this study, the disk-diffusion method with cefoxitin, a test recommended by the CLSI (2017) for *S. aureus*, was compared to that with oxacillin, a method still widely used for *Staphylococcus aureus* and considered standard for *S. pseudintermedius* by the veterinary CLSI (2013). Cefoxitin was more sensitive to the test, with 34/35 (97.14%) resistant samples, compared to oxacillin, with 33/35 (94.29%) resistant samples. All 35 strains were methicillin/oxacillin-resistant, according to the gold standard test for detection that involves PCR to investigate the presence of *mecA* gene (Velasco et al. 2005, Mimica et al. 2007). Studies comparing the cefoxitin and oxacillin disks had previously shown high specificity of both, with a greater sensitivity for cefoxitin demonstrated by Velasco et al. (2005), and an opposite result demonstrated by Mimica et al. (2007). Regarding oxacillin, the halo size, adopted to predict susceptibility, was ≤17mm, as recommended by the CLSI (2017), resulting in the identification of 2/35 (5.71%) susceptible samples. Detection of susceptible strains might also suggest that, even in the presence of *mecA* gene, resistance might not be expressed. In case of serious infection and susceptibility results in disk-diffusion test, if PCR would not be feasible, clinical laboratories can routinely adopt the determination of MIC values, a test more accurate than disk-diffusion.

Previous studies had compared oxacillin resistance, according to the disk-diffusion method, and the presence of *mecA* in staphylococcal strains collected from dogs. While Kania et al. (2004) found oxacillin-susceptible samples harboring the gene; Bemis et al. (2006, 2009) observed that the *mecA* gene was present in all oxacillin-resistant staphylococcal samples. In the studies reporting 100% resistance, diameter of the halo, considered to represent resistance, was ≤17mm, which was adopted by the CLSI from 2004 to 2008 for *S. aureus* and in 2013 to standardize the disc-diffusion test for microorganisms present in animals. In studies identifying susceptible strains, the diameter used to determine oxacillin resistance was ≤ 10 mm. In the present study, since the samples underwent disk-diffusion test after identification of the *mecA* gene, all samples were known to carry the *mecA* gene, in contrast to the studies of Bemis et al. (2006, 2009), who selected the samples based on halo sizes ≤17mm to investigate the presence of the gene. Since the size of the halo decreased to ≤10mm, in order to increase the sensitivity and efficacy of the test, the gene-carrying strains were probably discarded in the studies owing to the larger diameter.

Table 1. Resistance values of MRSP to cefoxitin, oxacillin, and ceftaroline

	Susceptible		Resistant		*p*
	n	%	*n*	%	
Cefoxitin	1	2.86	34	97.14	<0.0001
Oxacillin	2	5.71	33	94.29	< 0.0001
Ceftaroline	24	68.57	11	31.43	< 0.0001

In dogs *S. pseudintermedius* is the main causative agent of bacterial folliculitis and otitis externa. The increased incidence of these methicillin-resistant microorganisms over the last decade has reduced the efficacy of treatments using β-lactams, besides the fact that these bacteria are often resistant to multiple classes of antimicrobials (Bemis et al. 2009).

The use of ceftaroline in animals has not been described yet; therefore, there is no report of resistance in susceptibility tests using this antimicrobial in staphylococci present on the skin and mucous membranes of dogs (Sader et al. 2016). Till date, there had been no report of resistance for MRSP (Bannoehr & Guardabassi 2012) and MRSA (considering their similarities), it is necessary to find alternatives such as ceftaroline. Considering that there is no breakpoint determined for ceftaroline in *S. pseudintermedius*, and the one for *S. aureus* was adopted in this study, the current results showed that 31.43% (range: 0-3%) (11/35) of the staphylococci had resistance to ceftaroline, much higher than MRSA resistance in humans (Sader et al. 2015). The breakpoint value (if determined) may be higher for *S. pseudintermedius*, which will result in less resistant strains. The ability of ceftaroline to bind to modified PBP2a is the biggest difference with the other β-lactams (Kosowska-Shick et al. 2010), the mutation and adaptation potentials of *S. pseudintermedius* (present in dogs) and *S. aureus* are similar, since closely related strains of these bacteria have been identified (Bannoehr & Guardabassi 2012). *S. aureus* strains, resistant to ceftaroline from Thailand, have a very similar genetic background, suggesting a clonal propagation (Alm et al. 2014). The same phenomenon might have occurred in the present study, given that all the strains were collected from dogs of the same community, which might explain the high resistance rates of *S. pseudintermedius*.

Ceftaroline fosamil, approved by the FDA in 2012 despite the absence of resistance reports of >4% in MRSA, showed high resistance in MRSP, when the disc diffusion test was performed with the parameters for *S. aureus*. Since human medications are usually used in pets, and MRSP causes chronic and recurrent dermatopathies in dogs, the necessity for standardization of resistance tests against ceftaroline in *S. pseudintermedius*, still remains high.

CONCLUSIONS

There was no significant difference between the resistance values obtained for samples collected from the ear and those collected from the skin of dogs. *Staphylococcus pseudintermedius*, carrying the *mecA* gene, showed more than 90% resistance to cefoxitin and oxacillin.

The antimicrobials used in the disk-diffusion method to detect methicillin resistance showed sensitivity, with cefoxitin being more sensitive. Resistance rate of *S. pseudintermedius* to ceftaroline (33.31%) was considered high, when the breakpoint for *S. aureus* was used, given that there was no previous report of resistance in this microorganism.

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Original Article
Pequenos Animais/*Small Animals Diseases*

Malformation of the tail in Labrador Retriever dogs caused by mutation C189G in the T gene[1]

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ABSTRACT.- Silva D.M., Miguel G.G.P., Souza M.L., Cleveland H.P.K. & Ramos C.A.N. 2018. **Malformation of the tail in Labrador Retriever dogs caused by mutation C189G in the T gene.** *Pesquisa Veterinária Brasileira 38(12):2237-2240*. Faculdade de Medicina Veterinária e Zootecnia, Universidade Federal de Mato Grosso do Sul, Av. Senador Filinto Muller 2443, Vila Ipiranga, Campo Grande, MS 79070-900, Brazil. E-mail: carlos.nascimento@ufms.br

The present study reported the mutation C189G in the T gene (*Brachyury gene*) as the cause of malformation in the tail of the Labrador dog. One litter of Labradors, from a mating between a female with short tail and a male with normal tail admitted at the Veterinary Teaching Hospital of Universidade Federal de Mato Grosso do Sul, Campo Grande, Brazil, was evaluated in this study. Blood samples were collected from the female and her puppies. After DNA extraction, sequencing and PCR-RFLP were carried out. The C189G mutation was identified through both techniques only in dogs with short tail.

INDEX TERMS: Malformation, tail, Labrador Retriever, dogs, mutation C189G, T gene, genotyping, hereditary diseases.

RESUMO.- [**Malformação da cauda em cães da raça Labrador Retriever causada por mutação C189G no gene T**.] No presente trabalho relata-se a mutação C189G no gene T (*Brachyury gene)* como causa da malformação da cauda em cães da raça Labrador. Uma ninhada de labradores, provenientes do acasalamento entre uma fêmea com a cauda curta e um macho com a cauda normal, encaminhados ao Hospital Veterinário da Universidade Federal de Mato Grosso do Sul, Campo Grande, Brasil, foi avaliada nesse estudo. Amostras de sangue da cadela e filhotes foram coletadas. Após extração de DNA, sequenciamento e PCR-RFLP foram realizados. A mutação C189G foi identificada por meio de ambas as técnicas apenas nos cães com a cauda malformada.

TERMOS DE INDEXAÇÃO: Malformação, cauda, caninos, Labrador Retriever, mutação C189G, gene T, genotipagem, doenças hereditárias.

INTRODUCTION

Dogs with congenital tail malformation have been known for a long time (Pullig 1953). However, the etiology was long unknown.

Studies conducted by Haworth et al. (2001) and Hytönen et al. (2009) found association of a mutation in exon 1 of T gene (UniProtKB - Q9GL27), which encodes a transcription factor and tail malformation in many dog breeds (Cocker Spaniel, Bichon Frisé, Setter, Golden Retriever, Dachshund, Shih-tzu, Yorkshire, Fox). This mutation is the substitution of a cytosine for a guanine at nucleotide 189 of the gene (C189G), which causes a change from isoleucine to methionine at amino acid 63 of the encoded protein. The transcription factor encoded by the T gene is important for the normal embryonic development of the posterior mesoderm (Haworth et al. 2001), an embryonic tissue that will give origin to somites that will later originate the vertebrae and the tail (Hyttel et al. 2012).

Dogs with only one mutant allele (heterozygotes) have short tail or total tail absence. As homozygous animals have not been observed for the mutant allele described above, it has been proposed that when in homozygosis, mutation

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is lethal (GG genotype), causing the death of animals even during gestation or soon after birth (Haworth et al. 2001, Indrebo et al. 2008).

Although Labrador dogs were evaluated in previous studies, the presence of the mutation was not identified (Haworth et al. 2001). The present study reports the identification of the C189G mutation in Labrador dogs and its association with tail malformation.

MATERIALS AND METHODS

Animals and samples. 10 dogs from the mating between a congenital short-tailed Labrador Retriever female and a male of the same breed with normal tail were used. The female used in the mating was primiparous, and all the animals evaluated came from the city of Campo Grande, Mato Grosso do Sul, Brazil. The dogs were taken to the Veterinary Hospital of the Federal University of Mato Grosso do Sul for routine examinations and genetic counseling in May 2016. With regard to tail, pups presented the following phenotypes: 4 with normal tail, 4 with short tail, and 2 with absence of the tail.

A venous blood sample with EDTA was collected from the mother and from each of the ten pups from that crossing for further analysis.

All evaluated dogs were owned by a non-profit private breeder.

Genetic analysis. Blood samples were submitted to extraction of genomic DNA according to protocol recommended by Araújo et al. (2009). After extraction, DNA samples were evaluated for integrity and absence of PCR inhibitors by 1% agarose gel electrophoresis and PCR for constitutive gene (β-actin) (Wang et al. 2007).

Primers 5'-AGAGCCTGCAGTACCGAGTG-3' and 5'-CCGAGACTTCTCCCAGAAAA-3' developed by Hytonen et al. (2009) were used to amplify a 384bp fragment comprising part of exon 1 and intron 1 of canine T gene. Reactions were carried out in a volume of 50µl containing 10mM Tris-HCL (pH 8.3), 50µM of KCl, 1.5mM $MgCl_2$, 0.2mM of each dNTP, 1.25 U of Taq DNA polymerase (Ludwig Biotec), 11pmol of each primer, and approximately 100ηg of genomic DNA. Thermocycling occurred at 94°C for four minutes, followed by 30 cycles at 94°C for one minute, 60°C for 30 seconds and 72°C for another 30 seconds. A final step of extension at 72°C for three minutes was performed.

Amplified fragments were submitted to digestion (PCR-RFLP) with BstEII enzyme (Promega) for two hours at 60°C. The digestion product was visualized in UV transilluminator after 3% agarose gel electrophoresis. Based on *in silico* restriction analysis performed with the aid of *NEBcutter* software (Vincze et al. 2003), the expected fragments were 189bp and 195bp in normal individuals, homozygous genotype (CC), and 195bp, 158bp and 31bp in heterozygous individuals (CG).

In addition, amplified DNA fragments from 4 animals, 1 normal and 3 with tail alteration were purified with CleanSweep PCR Purification (Thermo Fisher Scientific) according to manufacturer's recommendations and submitted to sequencing in both directions, with primers described above in ABI-3130 sequencer (Apllied Byosistems).

The sequences were analyzed with the aid of the BLASTn (Altschul et al. 1990), MEGA 7 (Kumar et al. 2016) and CodonCode Aligner software (CodonCode Corporation).

RESULTS AND DISCUSSION

PCR-RFLP analysis revealed restriction patterns compatible with *in silico* analysis. All dogs with tail malformation presented the same pattern (3 fragments, 2 visible bands), which was different from that presented by dogs with normal tail (2 fragments, 1 visible band) (Fig.1). Another assay, also

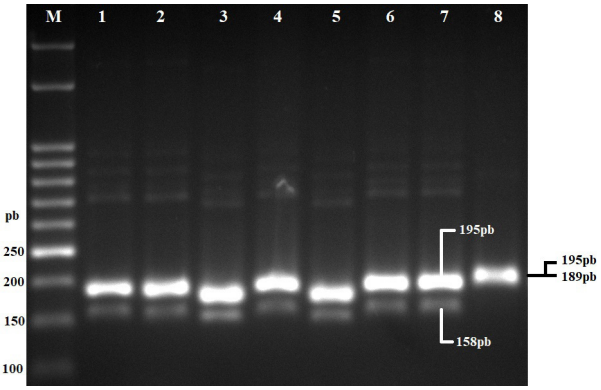

Fig.1. PCR-RFLP for detection of C189G mutation. Marker of base pairs (M), affected dogs - short tail (1-7) and animal with normal tail (8).

based on PCR-RFLP, was successfully used to identify the same mutation in the T gene of dogs, but using a polyacrylamide gel electrophoresis system (Gruszczynska & Czapla 2011). The performance of the polyacrylamide gel electrophoresis could improve the resolution of bands relative to DNA fragments in the present study. However, practicality would be reduced, since this system is more laborious. In the present study, even though it was not possible to visualize all the DNA fragments generated after digestion, it was possible to easily distinguish genotypes using agarose gel electrophoresis.

In the analysis of sequenced samples, heterozygosity C (cytosine) G (guanine) was observed in nucleotide 189 of exon 1 only in dogs with tail malformation. The location of the mutation was based on the T gene mRNA sequence available in the Genbank (accession number: AJ245513).

At the *in silico* analysis, alteration of amino acid 63 from isoleucine to methionine was also observed. All DNA sequences obtained in the present study were deposited in Genbank under accession numbers: MF495488 (short tail), MF495489 (absence of tail), MF495490 (absence of tail) and MF495491 (normal tail).

Although only one genotype was found for the present condition (CG for animals with tail malformation), a phenotypic variation was observed in affected dogs, which is the tail size. Some animals presented short tail (approximately 3-4 vertebrae), and others showed absence of tail (approximately 1-2 vertebrae) (Fig.2). Similar phenotypes were also observed by Haworth et al. (2001) in dogs. Although an explanation for this is not yet known in dogs, Buckingham et al. (2013), studying congenital tail size variation in cats, found evidence of haploinsufficiency caused by multiple mutations in the T gene. C.1199delC, c.1169delC and c.998delT mutations were associated with different levels of gene expression, which could explain the different phenotypes among dogs with tail malformation (Buckingham et al. 2013).

The hereditary character of the mutation can be evidenced by the heredogram analysis (Fig.3) of the litter studied. It was possible to verify that the mutation has an autosomal dominant inheritance pattern. However, no dominant homozygous (GG) genotype was found in the present study, reinforcing the observations that when in homozygosis, the mutated T gene causes fetal death (Haworth et al. 2001). Hytonen et al. (2009)

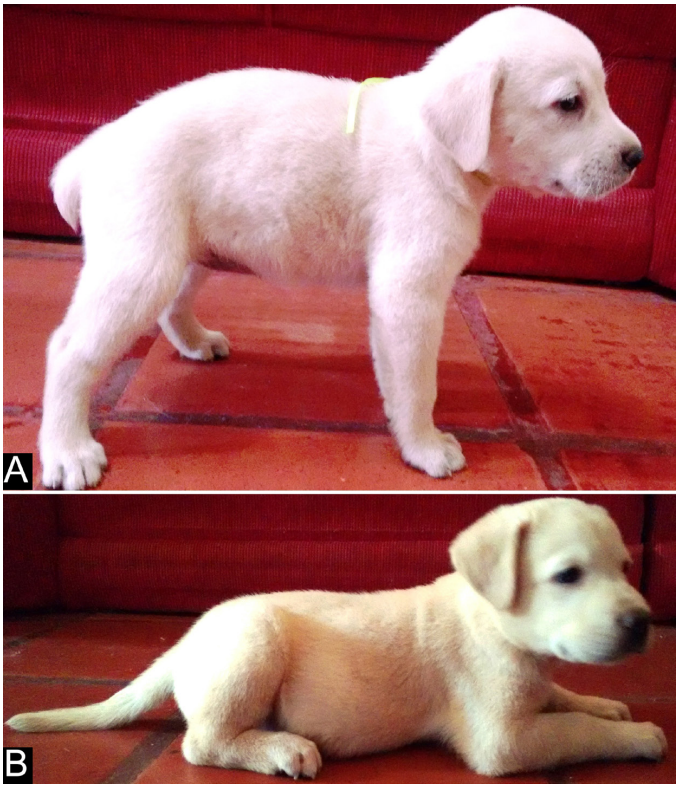

Fig.2. (**A**) Dog carrying mutation C189G in the T gene. (**B**) Dog from the same litter without C189G mutation.

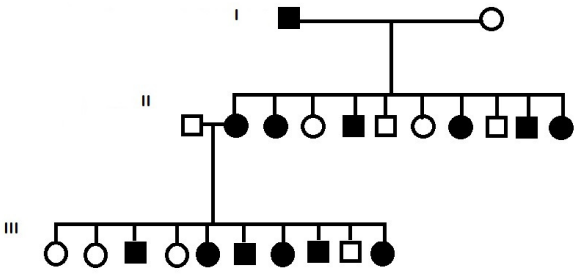

Fig.3. Heredogram showing the inheritance pattern of the C189G mutation in the T gene of Labrador Retriever. Crossbreeding between maternal grandparents (I), litter of the affected dog and its crossing with normal male (II) and litter analyzed (III).

observed that litters from crosses between normal-tailed animals (CC genotype) were 29% larger than litters from crossbreeding between malformed tail animals (CG genotype). This result is compatible with the expected reduction of 25% in offspring from crosses involving lethal alleles.

Mutations in the T gene have been associated with tail malformation in other species such as mice and cats (Wilson et al. 1995, Buckingham et al. 2013). And in some of them, other changes are also observed, such as urinary and fecal incontinence in cats (Robinson 1993). However, in dogs, to date, only tail malformation has been observed in heterozygous animals (CG) (Indrebo et al. 2008). Although in some canine breeds the "tail malformation" phenotype was not associated with the C189G mutation (Boston Terrier, English Bulldog, King Charles Spaniel, Miniature Schnauzer, Parson Russell Terrier, and Rottweiler), the large number of breeds affected suggests an ancient mutation (Hytonen et al. 2009), being present in ancestral dogs before the formation of many breeds. However, interracial crossbreeding may also have contributed to the mutation diffusion, since CG heterozygous animals appear to have no libido or reproductive performance alterations. Another factor that may have contributed to mutation diffusion was the use in the reproduction of dogs without tail, when esthetic caudectomy was still permitted. In this period, it is likely that many animals with tail agenesis have been used as reproducers, since tail absence was desirable in some breeds, contributing to the mutation diffusion.

Currently, the practice of caudectomy (surgical tail removal) is a prohibited procedure in several countries of the world such as those of the European Union and Brazil (Haworth et al. 2001, CFMV 2013). In some European Union countries, genetic tests to identify the C189G mutation in the T gene are used to verify if the absence of tail in some breeds is of congenital origin or the animals underwent irregular surgical procedure. PCR-RFLP used in the present study was a simple and accurate technique to identify this mutation and could be used as evidence to identify illegal caudectomy practice.

Due to the scarcity of information about the C189G mutation in the T gene of dogs, there is no further information regarding its association with other morphological or even physiological characters, and therefore needs to be investigated.

CONCLUSION

The present study confirms the occurrence of C189G mutation in the T gene of Labrador Retriever dogs and its association with tail malformation.

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Original Article
Pequenos Animais/*Small Animals Diseases*

Malignant perineal tumors in dogs: the contribution of computed tomography for staging and surgical planning[1]

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ABSTRACT.- Lorigados C.A.B, Fonseca Pinto A.C.C.B., Matera J.M. & Modena D.F.A. 2018. **Malignant perineal tumors in dogs: the contribution of computed tomography for staging and surgical planning.** *Pesquisa Veterinária Brasileira 38(12):2241-2245*. Departamento de Cirurgia, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Avenida Prof. Dr. Orlando Marques de Paiva 87, Cidade Universitária, São Paulo, SP 05508-270, Brazil. E-mail: clorigados@usp.br

The contribution of computed tomography for staging and surgical planning of malignant perineal tumors in dogs is discussed. Five dogs diagnosed with malignant perineal neoplasms underwent to computed tomography (CT) examination. The CT image enabled investigation of cleavage planes between neoplastic lesions and adjacent structures such as the rectum, anus, vagina, urethra and perineal muscles. Accurate assessment regional lymph nodes and adjacent bone structures was also possible. All tumors evaluated in this region presented heterogeneous appearance in pre and postcontrast CT images, but only the anal sac adenocarcinomas presented lymphadenopathy. Computed tomography proved to be a valuable tool for tumor staging and determination of lesion extension and invasion of adjacent tissues, providing significant contributions to clinical and surgical therapeutic planning.

INDEX TERMS: Malignant perineal tumor, computed tomography, surgical planning, dogs, image diagnosis.

RESUMO.- [Tumores perineais malignos em cães: contribuição da tomografia computadorizada para estadiamento e planejamento cirúrgico.] A contribuição da tomografia computadorizada para estadiamento e planejamento cirúrgico de tumores perineais malignos em cães é discutida. Cinco cães diagnosticados com neoplasias perineais malignas foram submetidos ao exame de tomografia computadorizada (CT). A imagem por TC permitiu a investigação de planos de clivagem entre as lesões neoplásicas e estruturas adjacentes, como o reto, o ânus, a vagina, a uretra e os músculos perineais. A avaliação precisa dos linfonodos regionais e estruturas ósseas adjacentes também foi possível. Todos os tumores avaliados nesta região apresentaram aspecto heterogêneo nas imagens de TC pré e pós-contraste, mas apenas os adenocarcinomas de saco anal apresentaram linfonodopatia. A tomografia computadorizada mostrou ser uma ferramenta valiosa para o estadiamento da neoplasia, determinação da extensão da lesão e invasão de tecidos adjacentes, proporcionando contribuições significativas para o planejamento terapêutico clínico e cirúrgico.

TERMOS DE INDEXAÇÃO: Tumor perineal maligno, tomografia computadorizada, planejamento cirúrgico, caninos, diagnóstico por imagem.

INTRODUCTION

The perineal region is potential location for the development of neoplasms in dogs. The majority of the malignant tumors affecting this region arise from these glandular tissues being circumanal and anal sac adenocarcinomas the most common ones (Turek & Withrow 2013). Circumanal glands are sebaceous glands located around the anus and anal sacs are cutaneous diverticula lined with serous and sebaceous glands and located on either side of the ventrolateral aspect

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of the anus, between the internal and external sphincters (Dyce et al. 2002).

Circumanal adenocarcinomas affect neutered and intact male and female dogs and do not seem to be hormone dependent. Higher predisposition has been reported in large breed males aged 11 years on average. Circumanal adenocarcinomas are associated with low metastatic rates, approximately 15% (Vail et al. 1990).

As regards apocrine gland adenocarcinoma of the anal sac, some authors suggest higher predisposition in older female dogs (Goldschmidt & Zoltowski 1981, Ross et al. 1991), while others report no gender predisposition (Williams et al. 2003, Polton et al. 2006, Polton & Brearley 2007). Spaniels, particularly English Cocker Spaniels, seem to be at higher risk of anal sac apocrine gland adenocarcinoma development (Polton et al. 2006), with mean age at diagnosis ranging from 9 to 11 years (Goldschmidt & Zoltowski 1981, Ross et al. 1991, Williams et al. 2003). Anal sac apocrine gland adenocarcinomas are invasive tumors with highly variable rates of metastasis formation (36% to 72%), although metastasis formation rates of approximately 50% are reported in most studies (Ross et al. 1991, Williams et al. 2003, Emms 2005, Polton & Brearley 2007). Regional lymph nodes, including sacral, internal iliac and medial iliac lymph nodes, are the most common metastatic sites (Williams et al. 2003, Emms 2005, Polton et al. 2006, Polton & Brearley 2007). Distant metastases are rare and tend to occur in the most advanced stages of the disease. The liver, spleen, kidneys, pancreas, adrenal glands, heart, lungs, mediastinum and bones may be affected (Ross et al. 1991, Turek et al. 2003, Williams et al. 2003, Brisson et al. 2004, Emms 2005, Polton et al. 2006, Polton & Brearley 2007).

Lymphomas, soft tissue sarcomas, squamous cell carcinomas, melanomas and mast cell tumors (MCTs) are other examples of malignant neoplasms that may affect the perineal region (Turek et al. 2003, Turek & Withrow 2013).

Advanced diagnostic imaging modalities are routinely used for cancer patient staging in human medicine. In contrast, in veterinary medicine, thoracic radiography and abdominal ultrasonography (US) are still the most commonly tools used due to wider availability and lower cost of these methods. However, with the growing availability of computed tomography (CT) scanners, this diagnostic imaging modality has being increasingly used for cancer staging purposes in veterinary patients, particularly for lymph node assessment (Rossi et al. 2011, Grosso et al. 2017).

This study set out to investigate the contribution of CT for staging and surgical planning in dogs affected with malignant perineal tumors, bearing in mind the invasive and metastatic nature of some malignant tumors that affect this region.

MATERIALS AND METHODS

Five dogs with cytologically and/or histologically confirmed diagnosis of perineal malignant tumors that underwent to CT examination from January 2013 to April 2015 were included in this study. Following initial physical examination, chest radiography and transabdominal ultrasonography examinations were performed in order to search for distant metastasis. Perineal and caudal abdominal CT scanning was then performed to assess the tumor and the regional lymph nodes as an effort to collect useful data for surgical planning. A single row detector helicoidal CT scanner[1] was used and pre and postcontrast transverse images cross-sectional images were acquired with 2 to 3mm slice thickness and same increment. Postcontrast images were acquired following intravenous bolus administration of 1.5ml/kg of a 300mgI/ml non-ionic iodinated contrast agent solution[2]. Pre and postcontrast neoplasm attenuation were evaluated. Tumor longest one-dimensional diameter was determined using the RECIST method; volumetric measurements were also obtained. The presence of cleavage planes between neoplastic lesions and adjacent structures and the involvement of regional lymph nodes and bone structures (i.e. caudal lumbar vertebrae, sacrum and hip bone) were investigated.

RESULTS

Signalment and clinical features of the five patients evaluated over the course of the study period are presented in Table 1. Dyschezia was the most prominent clinical manifestation in four out of five dogs. Neoplasms presented as firm and sessile masses on clinical examination; ulceration was noted in two cases. Cytological and/or histological examination revealed well-differentiated anal sac adenocarcinomas (Cases 1, 2 and 4), hemangiosarcoma (Case 3) and MCT (Case 5).

No radiographic changes in lungs, mediastinum or pleural spaces were noted. Enlarged, hypoechoic medial iliac lymph nodes (two cases; Dogs 1 and 4) were the only sonographic abnormal findings. Computed tomographic assessment revealed slightly heterogeneous soft tissue attenuation and moderate postcontrast enhancement in adenocarcinomas and MCT. Heterogeneous postcontrast enhancement were noted in anal sac adenocarcinomas and hemangiosarcoma (Table 2). With the exception of the MCT, no cleavage planes were detected between neoplasms and the rectal wall (Fig.1B and 1D) or local muscles (e.g. internal obturator, external anal sphincter, coccygeal muscle, levator ani, ventral, lateral and medial sacrocaudal muscles). Cleavage planes were also lacking between the neoplasm and the dorsal vaginal wall and the urethra in one female (Case 4) and one male (Case 1) respectively. Significantly enlarged medial iliac (Fig.2A), internal iliac (Fig.2B) and sacral (Fig.1A and 1C) lymph nodes were noted in two dogs (Dogs 1 and 4). One dog (Dog 4) also had enlarged caudal mesenteric and aortic lumbar lymph nodes (Fig.3). Enlarged lymph nodes presented mainly round shape; heterogeneous particularly peripheral enhancement. No changes were noted in the bones evaluated.

The owners of Dogs 1 and 4 declined surgical procedure because of the presence of metastasis in pelvic and abdominal lymph nodes and the possibility of resection of part of the anal sphincter, which may lead to fecal incontinence. In Dogs 2 and 3 it was possible to resect the tumor, preserving the anal sphincter, but without safety margin. In the dog with MCT it was possible to remove the tumor completely, with adequate surgical margin.

DISCUSSION

The surgery is used as the primary treatment option for dogs presenting perineal tumors. Studies with dogs affected by perineal neoplasia report longer survival time in patients

[1] Model XPRESS/G6, Toshiba.

[2] Omnipaque 300®. GE Healthcare do Brasil, Comércio e Serviços para Equipamentos Médicohospitalares LTDA.

Table 1. Patient signalment, affected site and tumor type

Dog	Breed, gender, age	Affected site	Cytology/Hystology
1	Cocker, male, 12 years	Dorsolateral to anus, rightsided	Anal sac adenocarcinoma
2	Poodle, female, 14 years	Dorsolateral to anus, leftsided	Anal sac adenocarcinoma
3	Labrador, female, 12 years	Ventrolateral to anus, rightsided	Hemangiosarcoma
4	Cocker, female, 12 years	Lateral to anus, rightsided	Anal sac adenocarcinoma
5	Brazilian terrier, male, 12 years	Perianal, rightsided	Mast cell tumor

Table 2. Tomographic features, tomographic measurements and lymph node involvement

Dog	Attenuation	Post-contrast enhancement	One-dimensional measurement (RECIST/cm)[a]	Volumetric measurement (cm^3)	Lymphadenomegaly
1	Slightly heterogeneous	Moderate/slightly heterogeneous	8.08	143.964	Medial iliac, internal iliac and sacral
2	Slightly heterogeneous	Moderate/heterogeneous	5.16	75.235	No
3	Heterogeneous	Intense/heterogeneous	7.51	87.484	No
4	Slightly heterogeneous	Intense/heterogeneous	9.31	220.908	Medial iliac, internal iliac, sacral, aortic lumbar and caudal mesenteric
5	Slightly heterogeneous	Moderate/heterogenous	5.17	78.347	No

[a] RECIST = Response evaluation criteria in solid tumors.

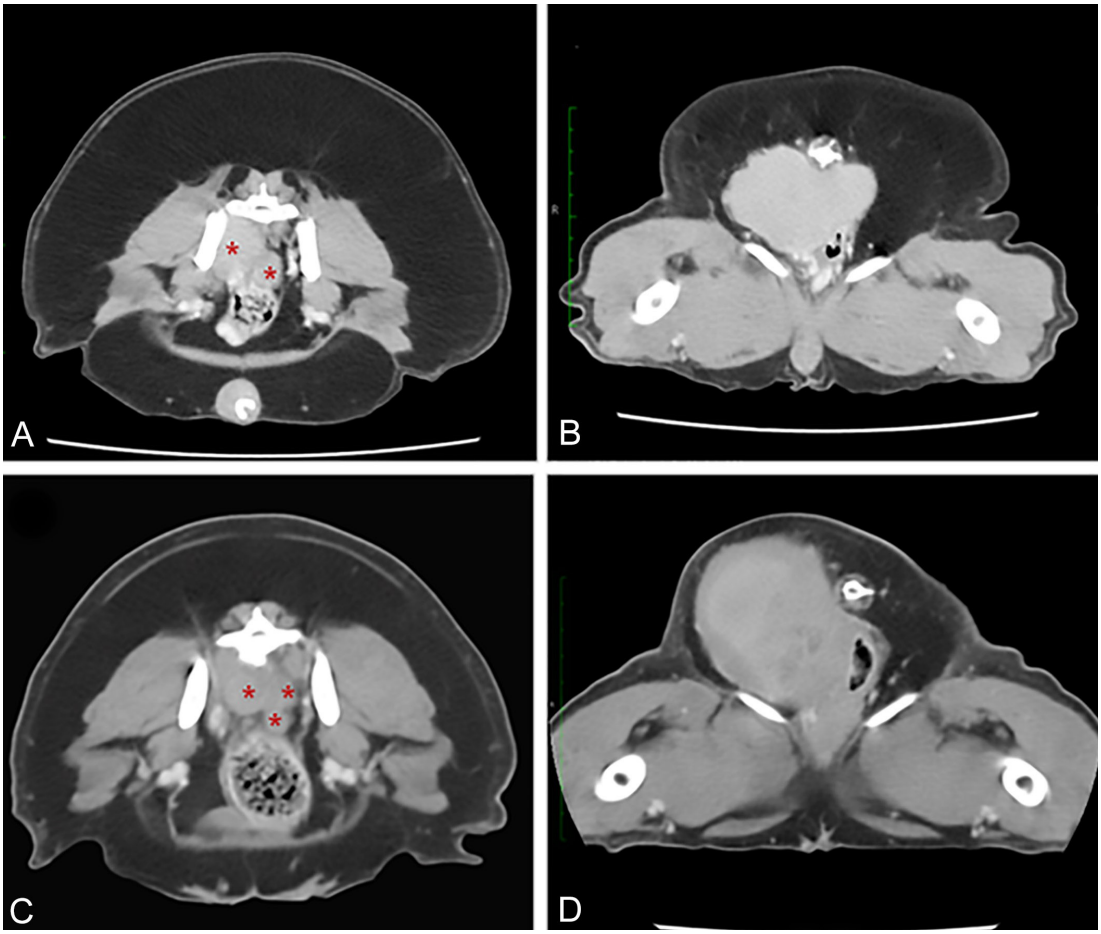

Fig.1. Post-contrast transverse CT images of dogs with anal sac adenocarcinoma in (**A**,**B**) Case 1 and (**C**,**D**) Case 4. (**A**,**C**) Enlarged sacral lymph nodes with rounded shape were observed (*). (**B**,**D**) No cleavage plane was detected between neoplasms and the rectal wall.

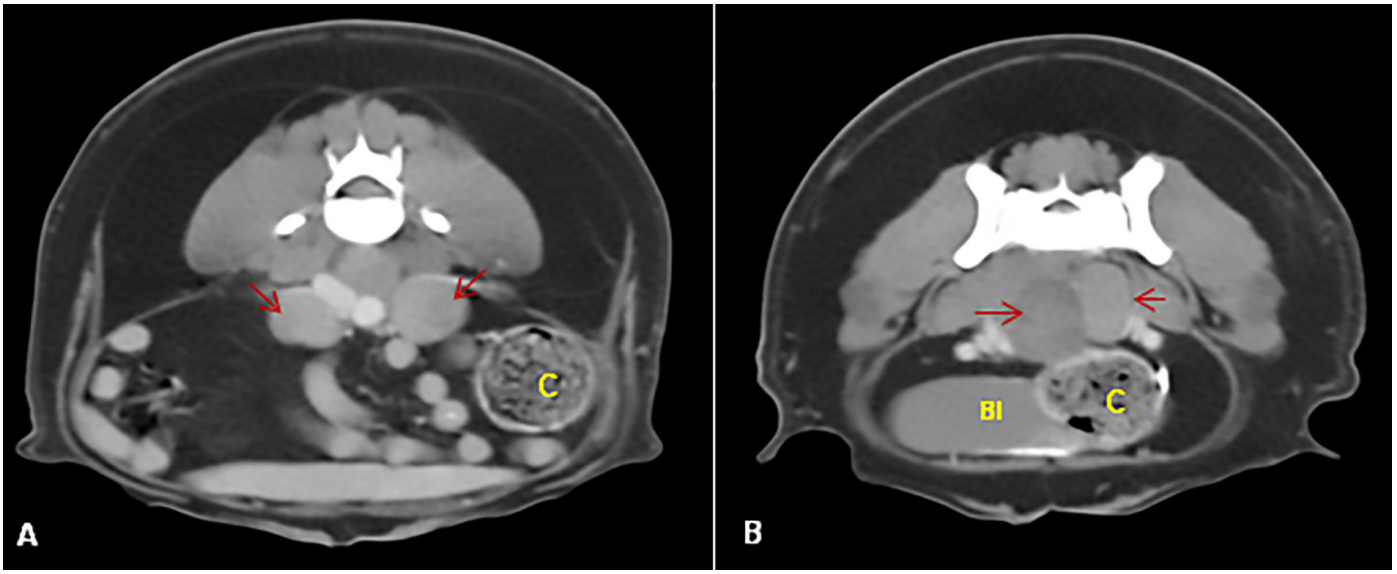

Fig.2. Post-contrast transverse CT images of a dog with anal sac adenocarcinoma (Case 4). (**A**) Enlarged medial iliac and (**B**) internal iliac lymph nodes are observed (arrows). C = colon, Bl = bladder.

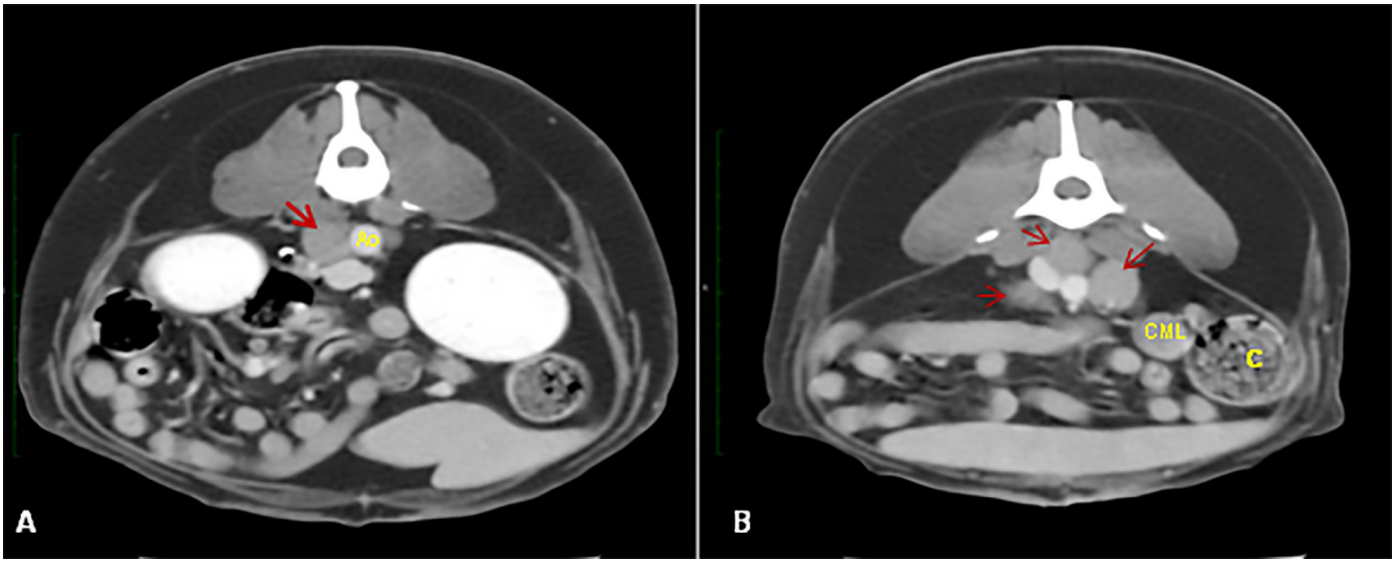

Fig.3. Post-contrast transverse CT images of a patient with anal sac adenocarcinoma (Case 4). (**A**) Enlarged aortic lumbar lymph nodes (arrow) and (**B**) caudal mesenteric (CLM) lymph nodes with enlarged medial iliac and internal iliac lymph nodes (arrows). Ao = aorta, C = colon.

submitted to multimodal therapeutic approaches combining surgical resection, chemo and radiotherapy as indicated (Vail et al. 1990, Bennett et al. 2002, Dyce et al. 2002, Polton & Brearley 2007, Palladino et al. 2016, Barnes & Demetriou 2017). Surgical margins are a significant factor in tumor resection. Cleavage planes between neoplastic lesions and different important perineal structures were lacking in patients in this sample. For the anal sac carcinomas, a segmental resection of the anal sphincter is required when the neoplasia reaches enormous proportions. The resections involving more than 180° of the sphincter circumference may be associated with partial or complete fecal incontinence. When the sphincter can be preserved, the surgical margins may not be adequate, increasing the probability of local recurrence. Hence, initial treatment with chemotherapy was recommended to promote tumor volume reduction prior to surgery. Chemotherapy was also indicated as palliative treatment for patients with signs of metastatic dissemination to regional lymph nodes; in such cases, owners were informed of the guarded to poor prognosis. Metastatic lymph nodes can be removed by open ventral midline coeliotomy. Some lymph nodes are straightforward to excise for experienced surgeons, whilst others, mainly the sacral lymph nodes, are difficult to remove by this access (Barnes & Demetriou 2017) and a pubic osteotomy is necessary.

Ultrasonography is thought to be a valuable imaging modality for abdominal lymph node evaluation. However, location of major sites of metastasis, such as the sacral and internal iliac lymph nodes (Vail et al. 1990, Dyce et al. 2002, Turek & Withrow 2013), makes sonographic assessment difficult. Recently, CT and magnetic resonance imaging (MRI) have been shown be more sensitive than ultrasound in identifying lymphadenopathy in dogs with adenocarcinoma of the anal sac (Anderson et al. 2015, Palladino et al. 2016). Also, CT provided complementary assessment of bones in the search for metastasis which, although uncommon, may still occur (Turek & Withrow 2013).

CONCLUSIONS

All tumors evaluated in this region presented heterogeneous appearance in pre and post-contrast CT images, but only the anal sac adenocarcinomas presented lymphadenopathy.

Inherent characteristics of computed tomography, volume contour mapping, detection of cleavage planes between neoplastic lesions and adjacent structures and tumor staging data, make it a suitable method in order to offer a better clinical and surgical management of patients with perineal tumor.

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Original Article
Pequenos Animais/*Small Animals Diseases*

Using allogeneic cortical graft preserved in glycerin as spacer in the advancement of tibial tuberosity in 34 dogs[1]

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ABSTRACT. - Gomes Junior D.C., Oriá A.P., Vieira J.V.R., Barbosa S.F., Estrela-Lima A. & Dórea Neto F.A. 2018. **Using allogeneic cortical graft preserved in glycerin as spacer in the advancement of tibial tuberosity in 34 dogs**. *Pesquisa Veterinária Brasileira 38(12):2246-2253*. Escola de Medicina Veterinária e Zootecnia, Universidade Federal da Bahia, Av. Adhemar de Barros 500, Ondina, Salvador, BA 40170-110, Brazil. E-mail: aestrela@ufba.br

Cranial cruciate ligament is the main responsible for knee stability by preventing cranial tibial displacement regarding the femur. Deficiency in this ligament (CCLD) may cause subluxation of the tibia and dysfunction of the pelvic member due to overloading. Tibial osteotomies are among the more current surgical techniques for treating CCLD in dogs and they proportionate the dynamic stability by means of modifying bone geometry and the distribution of forces acting on the articulation. The objective of this work is to describe the use of the allogeneic cortical bone graft conserved in glycerin as a spacer on the tibial tuberosity advancement (TTA) for treating the CCLD. In order to do that, 34 dogs submitted to TTA surgery correction were evaluated, being 23 males (67.35%) and 11 females (32.35%). Surgical procedures happened from May 2011 to October 2015. Regarding the surgical procedure after osteotomy of the tibial tuberosity, a disk of allogeneic cortical disk, sawn wedge-hapsed, conserved in glycerin, proportions of 2x1mm was applied as spacer, enabling TTA. Advancements from 3 to 12 mm were executed, depending on the need of the patient. For animals with patella dislocation, trochleoplasty and TTA were executed in order to correct the deviation. The mean ± SD age of animals was 6.67±3.58 and weight was 15.16±12.97 kg. Mongrel dogs, Poodles and Yorkshire terriers were the most affected ones. From the 36 evaluated knees, 11 (30.56%) were associated with some traumatic process and in 25 (69.44%) there was no relation with previous trauma. From those wounds, 20 (55.56%) happened in the right limb and 16 (44.44%) in the left limb and two animals had CCLD bilaterally. Animals had continuous support, discreet drawer movement and negative tibial compression 15 days after surgery. At 30 days, 26 cases (72.22%) had firm support (FS); at 45 days, 24 cases (66 test at 7 and 67%) had FS and eight cases (22.22%) without claudication (WC). During subsequent radiographic evaluations the progressive incorporation of the graft and osteotomy union were observed. In this study, most of the diagnosed CCLD occurred in males diverging from results obtained by other authors that found greater frequency in females. Support without claudication it was observed in most of the cases of implants at 60 days. We concluded that the conserved allogeneic cortical bone graft was able to promote bone union in TTA of dogs with CCLD. None of the animals had signs of contamination, infection of the surgical wound or rejection related with the presence of the graft, demonstrated by the complete graft-bone incorporation observed early at 45 days in some animals. The glycerin was a good conservation medium for those fragments intended for grafting because, besides being of low cost, it kept bone fragments free of contamination, reducing antigenicity and preserving the

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functions of osteoinduction and osteoconduction. The possibility of molding the graft to the animal need is a characteristic favorable to executing the modified technique that could be molded according to the size of the animal, allowing perfect adaptation to the osteotomized local in different breeds. Intercurrences commonly observed in TTA with patellar dislocation, meniscal lesions, tibial crest fracture and displacement were not found in the animals of this study, probably due to the better distribution of forces between the pass screw in TT and the TTA plate confirming that it has good adaptation to the technique conferring to the modified TTA advantages regarding the conventional TTA.

INDEX TERMS: Allogeny, cortical graft, glycerin, tibial tuberosity, dogs, cranial cruciate ligament deficiency, TTA, bone union, osteoinduction, surgery.

RESUMO.- [**Uso de enxerto cortical alogênico preservado em glicerina como espaçador no avanço da tuberosidade tibial em 34 cães.**] O ligamento cruzado cranial é o principal responsável pela estabilidade do joelho, impedindo o deslocamento da tíbia cranial em relação ao fêmur. A deficiência neste ligamento (CCLD) pode causar subluxação da tíbia e disfunção do membro pélvico devido à sobrecarga. As osteotomias tibiais estão entre as técnicas cirúrgicas mais atuais para o tratamento de CCLD em cães e proporcionam a estabilidade dinâmica por meio da modificação da geometria óssea da distribuição das forças que atuam sobre a articulação. O objetivo desse estudo é descrever o uso do enxerto ósseo cortical alogênico conservado em glicerina como espaçador no avanço da tuberosidade tibial (TTA) para o tratamento do CCLD. Para isso, 34 cães submetidos à cirurgia de TTA foram avaliados, sendo 23 machos (67,35%) e 11 fêmeas (32,35%). Os procedimentos curúrgicos aconteceram entre maio de 2011 e outubro de 2015. Com relação ao procedimento cirúrgico após a osteotomia da tuberosidade tibial, um disco alogênico cortical, em forma de cunha serrada, conservado em glicerina com proporções de 2 x 1mm foi aplicado como espaçador possibilitando a TTA. Avanços de 3 a 12mm foram executados, dependendo da necessidade do paciente. Para animais com luxação da patela, realizou-se a trocleoplastia e a TTA para a correção do desvio. A idade média dos animais foi de 6,67±3,58 anos e pesos médios de 15,16±12,97kg. Cães sem raça definida, Poodles e Yorkshire Terriers foram os mais afetados. Dos 36 joelhos avaliados, 11 (30,56%) foram associados a algum processo traumático e em 25 (69,44%) não havia nenhuma relação com um trauma prévio. Dos ferimentos, 20 (55,56%) aconteceram no membro direito e 16 (44,44%) no esquerdo, sendo que dois animais apresentavam CCLD bilateralmente. Os animais tiveram suporte contínuo, discreto movimento de gaveta e compressão tibial negativa 15 dias após a cirurgia. Aos 30 dias, 26 casos tinham suporte firme (FS); aos 45 dias, 24 casos tinham FS e oito casos sem claudicação (WC). Durante avaliações radiográficas subsequentes, observou-se a incorporação progressiva da união do enxerto e da osteotomia. Neste estudo, a maior parte do CCLD diagnosticado ocorreu em machos, divergindo dos resultados obtidos por outros autores que encontraram maior frequência em fêmeas. Suporte sem claudicação foi observado na maioria dos casos de implantes aos 60 dias. Foi concluído que o enxerto ósseo cortical alogênico conservado foi capaz de promover a união óssea na TTA de cães com CCLD. Nenhum dos animais apresentou sinais de contaminação, infecção da ferida cirúrgica ou rejeição relacionada à presença do enxerto, demonstrada pela incorporação completa do enxerto ósseo observada precocemente aos 45 dias em alguns animais. A glicerina foi um bom meio de conservação para os fragmentos destinados à enxertia porque, além do menor custo, manteve os fragmentos ósseos livres de contaminação, reduzindo a antigenicidade e preservando as funções de osteoindução e osteocondução. A possibilidade de moldagem do enxerto à necessidade do animal é uma característica favorável à execução da técnica modificada que pode ser moldada de acordo com o tamanho do animal, possibilitando perfeita adaptação ao local osteotomizado em diferentes raças. Intercorrências comumente observadas na TTA com luxação patelar, lesões meniscais, fratura da crista tibial e deslocamento não foram encontradas nos animais deste estudo, provavelmente devido à melhor distribuição de forças entre a passagem do parafuso no TT e a placa do TTA, confirmando que tem boa adaptação à técnica conferindo às vantagens da TTA modificada em relação à TTA convencional.

TERMOS DE INDEXAÇÃO: Enxerto cortical, alogenia, glicerina, tuberosidade tibial, caninos, ligamento cruzado cranial, ATT, união óssea, osteoindução, cirurgia.

INTRODUCTION

Deficiency of the cranial cruciate ligament (CCLD) is associated to a previous trauma or ligament degeneration, causing subluxation of the tibia and dysfunction of the pelvic member due to overloading. Due to the frequent demand of surgeries for correction CCLD, several techniques were elaborated, modified and adapted with the objective of improving results and reducing costs (Silva 2012, Castilho et al. 2014).

In tibial tuberosity advancement (TTA) procedures, the use of titanium cages are necessary for the maintenance and filling of the space created by osteotomy and TTA. Such implants are made in pre-determined sizes of 3, 6, 9 and 12mm, and with a wide variety of lengths. This method has been used with good results; however, complications such as infection, tibial tuberosity fracture and meniscal lesions are observed (Costa et al. 2017, Dyall & Schmokel 2017).

Several studies have used materials alternative to the titanium cage to provide biocompatible implants that would be absorbed and replaced by the host bone. Among these, Silva (2012) used hydroxyapatite and polycaprolactan, observing bone integration after six months of evaluation. Von Lande et al. (2012) compared radiographic scarring in 48 dogs using bovine xenograft and in 34 dogs as a spongy autogenous bone graft to fill the gap resulting from TTA. Similarly, Marques et al. (2017) evaluated the time for bone consolidation with spongy grafts in titanium cages. Medeiros

(2011) used castor bean polymer spacers in clinical cases. Castilho et al. (2014) fabricated calcium phosphate cement spacers by 3D prototyping for the treatment of cranial cruciate ligament rupture (CCLR) in dogs and concluded that the design, fabrication and application of the proposed implant was successful.

The use of bone grafts is a relevant and well established procedure in both human and veterinary orthopedic surgery. Traditionally, large bone defects are filled with autogenous bone grafts for faster bone formation, or with preserved allogenic graft segments for greater biomechanical resistance. The allogeneic cortical graft have interesting characteristics as high biological value, providing temporary mechanical structure, serving as a framework for new bone growth (osteoconduction) while maintaining the ability to induce osteogenesis (osteoinduction), besides being absorbed and slowly replaced by the host's own tissue (Ragetly & Griffon 2011).

Conserved cortical grafts are easy to acquire, maintain and are indicated for the filling of major defects, which makes it possible to use them as spacers in TTA. For that it is necessary to modify the main TTA technique for graft fixation and osteotomy stabilization.

The objective of this work was to evaluate and follow up animals that used both the dog allogenic bone graft, glycerin-preserved, as a spacer in replacement of the titanium cages in TTA surgeries, and the modification of the conventional technique.

MATERIALS AND METHODS

Thirty-four dogs submitted to TTA surgery for CCLD correction were evaluated, using bone graft of dog conserved in glycerol at 98% as spacer. Surgical procedures happened from May 2011 to October 2015, in two veterinary hospitals.

As an inclusion criterion, animals should have, besides history compatible with CCLD, positive drawer and tibial compression tests. Results will be descriptively presented by percentage and mean.

After physical test, radiographic preoperative evaluations of affected knees on mid-lateral and cranial-caudal projections were performed in order to measure TTA and to evaluate the degree of joint degeneration. X-rays of the tibia in medial-lateral projection for calculating the angle of the tibial plateau were executed according to methodology established by other author (Slocum & Devine 1983).

For surgical procedure, animals were intramuscularly pre-medicated with morphine sulfate (Dimorf® 1%, 0.5mg/kg, Cristália, São Paulo, Brazil), acepromazine hydrochloride (Acepram® 0.2%, 0.02-0.05mg/kg, Vetnil, São Paulo, Brazil) or chlorpromazine hydrochloride (Longactil® 0.5%, 0.5mg/kg, Cristália, São Paulo, Brazil) depending on the results from clinical evaluation.

After lumbosacral and affected pelvic limb trichotomies the anesthetic induction was executed, intravenously, using propofol (Propovan® 1%, 5mg/kg - dose dependent, Cristália, São Paulo, Brazil). Maintenance with isoflurane (Vetflurano®, Virbac, São Paulo, Brazil)was executed in oxygen at 100%, in semi-closed system. For epidural anesthesia, lidocaine hydrochloride with vasoconstrictor (Xylestesin® 2%, 0.25mg/kg, Syntec, São Paulo, Brazil) was used. Additionally were also used, by intravenous route, cephalothin (Cefalotina Sódica® 20%, 30mg/kg, Eli Lilly, Pernambuco, Brazil)and meloxicam (Maxicam® 2%, 0.2mg/kg, Ouro Fino, São Paulo, Brazil)

For TTA execution, joints were medially addressed according to the routine technique (Piermattei et al. 2006). Medial meniscectomies were performed in all knees and the fragments from the ruptured cranial cruciate ligament were removed. Animals having patella dislocation were submitted to chondroplasia for deepening the trochlear groove (Fig.1A). Knees were irrigated with physiological solution, patella was repositioned and the joint capsule sutured with Wolf's point or simply interrupted with polyamide thread. The prolongation in the distal direction was extended in order to approach the tibial tuberosity (TT) and the proximal medial face of the tibia. Then, the length and width of TT were measured, determining the positioning of the osteotomy (Fig.1B). Osteotomy was performed with the help of oscillating saw and TT was advanced with help of a lever.

For TTA a disk of varied dimensions was used, depending on the patient's need (3-12mm of diameter), wedge shaped sawed, measuring in proportion of 2x1mm of metacarpal or dog ulna preserved in glycerin (Fig.1C). The graft was hydrated with physiological solution, applied at the osteotomy place and fixed with titanium or stainless steel bolt (1.5, 2.0, 2.7 or 3.5mm) trespassing the TT toward the tibia (Fig.1D). In animals having medial patellar dislocation, TT was transposed laterally (Fig.1E). Immediately thereafter, a titanium plate for TTA (Cão Médica Comércio de Materiais Cirúrgicos Veterinários Ltda, São Paulo, Brazil) was molded and applied on the medial surface of the tibia with the help of titanium or stainless steel bolts (Fig.1F).

Immediately thereafter, the plate and the osteotomy were recovered by the union of adjacent tissues with stitches in Sultan using polyamide yarn (Nylon®, 3-0 a 0, Shalon, Goiás, Brazil), followed by subcutaneous suture with simple continuous standard using polyglactin yarn (Vicryl®, 3-0 a 0, Ethicon, São Paulo, Brazil) and dermorrhaphy with simples stitches using polyamide yarn.

During post operative amoxicillin with potassium clavulanate 20mg/kg/VO/BID (Agemoxi CL®, Agener União, São Paulo, Brazil) or cephalexin 30mg/kg/VO/BID (Rilexine®, Virbac, São Paulo, Brazil) were prescribed during 10 days; meloxicam 0.1mg/kg/VO/SID(Meloxivet®, Duprat, Rio de Janeiro, Brazil) during five days; dipyrone 25mg/kg/VO/TID (Generic drug) during 7 days and tramadol hydrochloride 3mg/kg/VO/TID (Cronidor®, Agener União, São Paulo, Brazil) during 5 days. Space restriction and exercise limitation were recommended during the first weeks of post-operative. All animals remained with splint during the first 15 days.

Clinical and radiographic evaluations were performed postoperatively between the 7th and the 10th day, when stitches were removed, and repeated at 15, 30, 45 and 60 days of surgery. During clinical evaluations were observed the degree of claudication, return to function and limb support, being classified in: without support (WS), when the animal had no support from limb; continuous support (CS), when the animal had support from the limb, however it had difficulties walking; firm support (FS), when the animal had support from the without major difficulties during gait; and no claudication (WC), when it had no degree of claudication. During radiographic evaluations were observed the positioning of implants, graft fusion and the retraction of the space between tibia and TT.

RESULTS

Individual information can be seen in Table 1. A total of 36 knees in 34 dogs were evaluated, being 23 males (67.35%) and 11 females (32.35%), their ages between six and 10 years old and five of them (14.71%), between 11 and 14 years old. The mean ± SD of animals ages was 6.67±3.58 years and of weight 15.16±12.97 kg.

From dogs included in the study were found: one (2.94%) representatives of breeds Lhasa Apso, Maltese, Pit-Bull, Golden

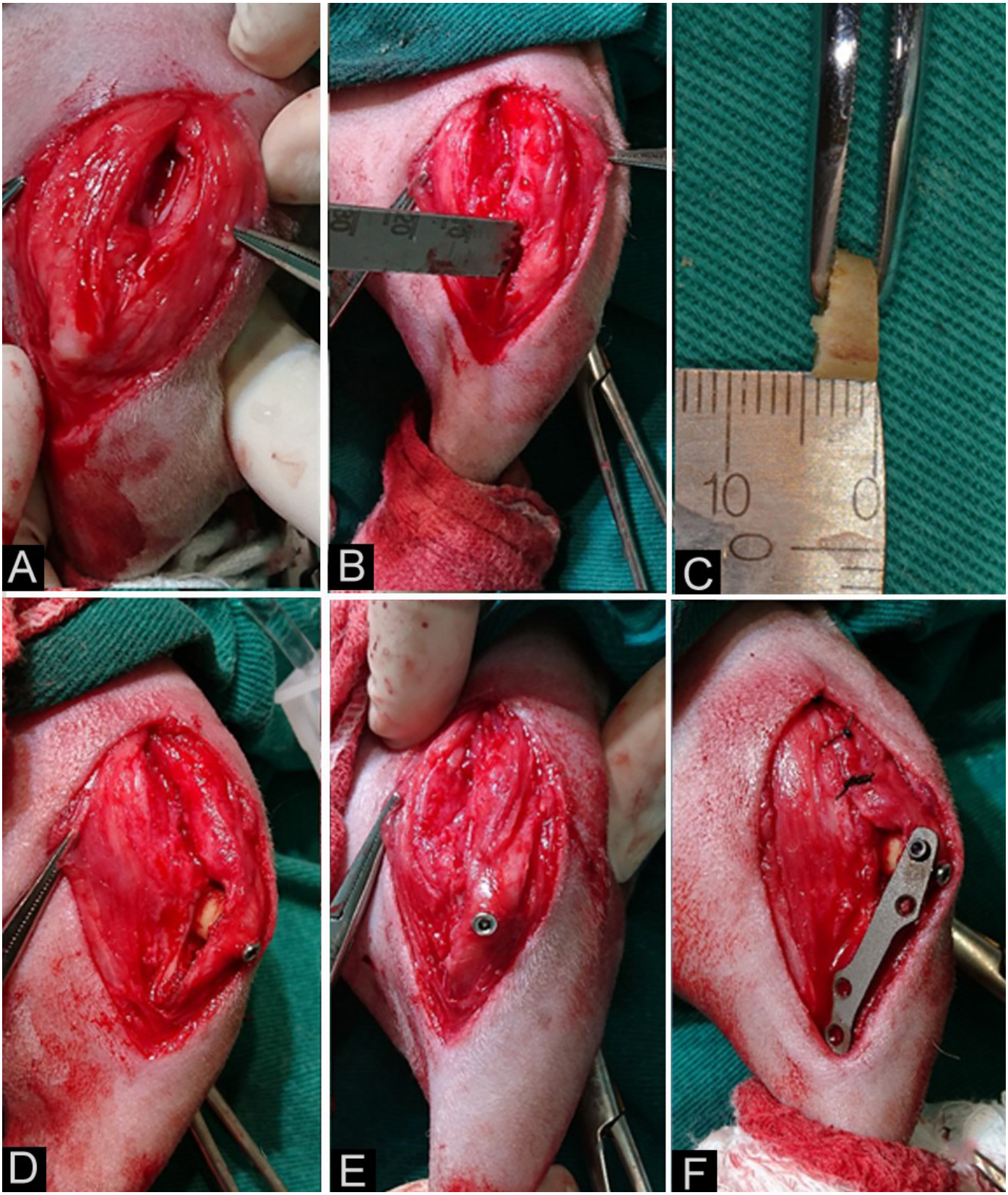

Fig.1. Images of tibial tuberosity advancement (TTA) using bone graft. (**A**) Deepening of the trochlear groove, (**B**) tibial tuberosity (TT) osteotomy with the help of oscillating saw, (**C**) preparation of the wedge shaped sawed graft, (**D**) application of graft and fixation of bolt trespassing TT, (**E**) cranial aspect of the bolt applied on TT, (**F**) application of TTA plate on the medial surface of the tibia.

Table 1. Animals submitted to tibial tuberosity advancement (TTA), cranial cruciate ligament (CCLD) traumatic or not and clinical presentation time

Case no.	Sex	Breed	Age (years)	Weight (Kg)	PL	Knee Affected	Cause	TL
1	F	Mixed	13	6.0	N	Right	T	01
2	M	Poodle	6	6.3	Y	Right	NT	01
3	M	Poodle	7	5.4	Y	Right	NT	04
4	M	Mixed	4	16.9	N	Right	NT	02
5	M	English Bulldog	2	23.5	N	Left	NT	02
6	M	Poodle	11	10.3	Y	Left	T	01
7	M	Labrador Retriever	9	46.8	N	Left	NT	01
8	F	Poodle	6	5.3	Y	Right	NT	01
9	M	Pitbull	2	37.5	N	Left	NT	03
10	M	Brazilian Mastiff	8	51.0	N	Right	NT	01
11	M	Yorkshire Terrier	2	4.5	Y	Right	T	01
12	F	Golden Retrievier	4	30.9	N	Left	NT	01
13	F	Pinscher	8	6.5	Y	Left	NT	01
14	F	Brazilian Mastiff	6	39.2	N	Right	NT	04
15	M	Mixed	4	16.5	N	Right	NT	03
16	F	English Bulldog	3	23.5	N	Left	NT	01
17	M	Mixed	5	29.0	N	Right	NT	01
18*	F	Poodle	9	5.5	Y	Left	NT	02
18*	F	Poodle	9	5.5	Y	Right	NT	03
19	M	Lhasa Apso	5	6.2	Y	Right	T	05
20	M	Yorkshire Terrier	3	3.4	N	Right	NT	09
21	F	Yorkshire Terrier	10	4.2	Y	Left	NT	04
22	M	Mixed	10	29.7	N	Left	NT	07
23	M	Pinscher	8	8.1	Y	Left	NT	03
24	M	Mixed	8	8.3	Y	Left	NT	17
25	F	Yorkshire Terrier	5	5.8	Y	Right	NT	08
26*	F	Mixed	2	19.3	N	Left	T	03
26*	F	Mixed	2	19.3	N	Right	T	02
27	M	Poodle	7	14.7	N	Left	T	01
28	M	Yorkshire Terrier	3	5.8	Y	Left	T	01
29	M	Mixed	13	10.3	N	Right	NT	04
30	M	Mixed	9	9.1	N	Right	T	04
31	F	Yorkshire Terrier	13	4.7	Y	Left	NT	02
32	M	Mixed	14	14.7	Y	Right	T	03
33	M	Poodle	5	4.6	Y	Right	NT	04
34	M	Maltese	5	7.7	Y	Right	T	02

Retriever, and Labrador Retriever, two (5.88%) representatives of breeds Brazilian Mastiff, English Bulldog and Pinscher. Mixed dogs were the most affected ones (10 animals, 29.41%) followed by Poodles (seven animals, 20.59%) and Yorkshires (six animals, 17.65%).

Regarding the weight, it ranged from 3.4 until 51 kg, from those, 11 animals (35.33%) in the range between 3.4 and 6kg, seven animals (20.57%) between 6.2 and 9.1 kg, nine animals (26.45%) between 10.3 and 23.5kg and six animals (17.65%) between 29 and 51 kg.

From the 36 evaluated knees, 11 (30.56%) were associated with some traumatic process and in 25 (69.44%) there was no relation with previous trauma. From those wounds, 20 (55.56%) happened in the right limb and 16 (44.44%) in the left limb, from those previously mentioned, two animals had CCLD bilaterally, represented by Cases 18 and 26. Time between lesion occurrences until clinical conduction ranged between one and 17 weeks. In most of the cases, 31 (86.11%) of them, this period did not exceed four weeks. In animals of breeds Poodle, Yorkshire, Pinscher and Maltese, 18 knees (50%) had patella luxation at clinical evaluation.

During the 15 initial days, animals were kept with splint and had CS of limbs. After 30 days of the surgical procedure, from the 36 operated knees, 26 (72.22%) had FS, three (8.33%) CS (Cases 29, 30 and 33), five (13.89%) WS (Cases 3, 5, 10, 18 and 31) and two (5.56%) WC (Cases 19 and 34). At 45 days, 24 (66.67%) had FS, 1 (2.78%) CS (Case 33), three (8.33%) WS (Cases 3, 5, 10 and 17) and eight (22.22%) WC. At 60 days two knees (5.56%) had FS (Cases 10 and 28), two (5.56%) WS (Cases 3 and 33) and 33 (88.89%) WC.

Cases 2, 3, 4, 5, 6, 17, 28 and 33 had plates removed due to exposition of implants, loose implants or patient discomfort. In Case 3 trauma was reported at 90 days with exposition of implants. Those were removed, obtaining FS after one week and WC after 40 days of the removal of implants. In Case 28, a surgical intervention was performed for removal of plate at 45 days.

In Case 33, at 15 days of postoperative, after removal of splint, a small exposition of the plate in the portion inserted on the tibial tuberosity was observed. A dermorrhaphy was performed with polyamide yarn 3.0 with simple interrupted stitches that were removed after 10 days. The same patient remained with CS during the whole evaluating period and, still, there was marked instability. At 90 days, another procedure was performed to removal of grafts and stabilization using the technique of fabelo-tibial suture.

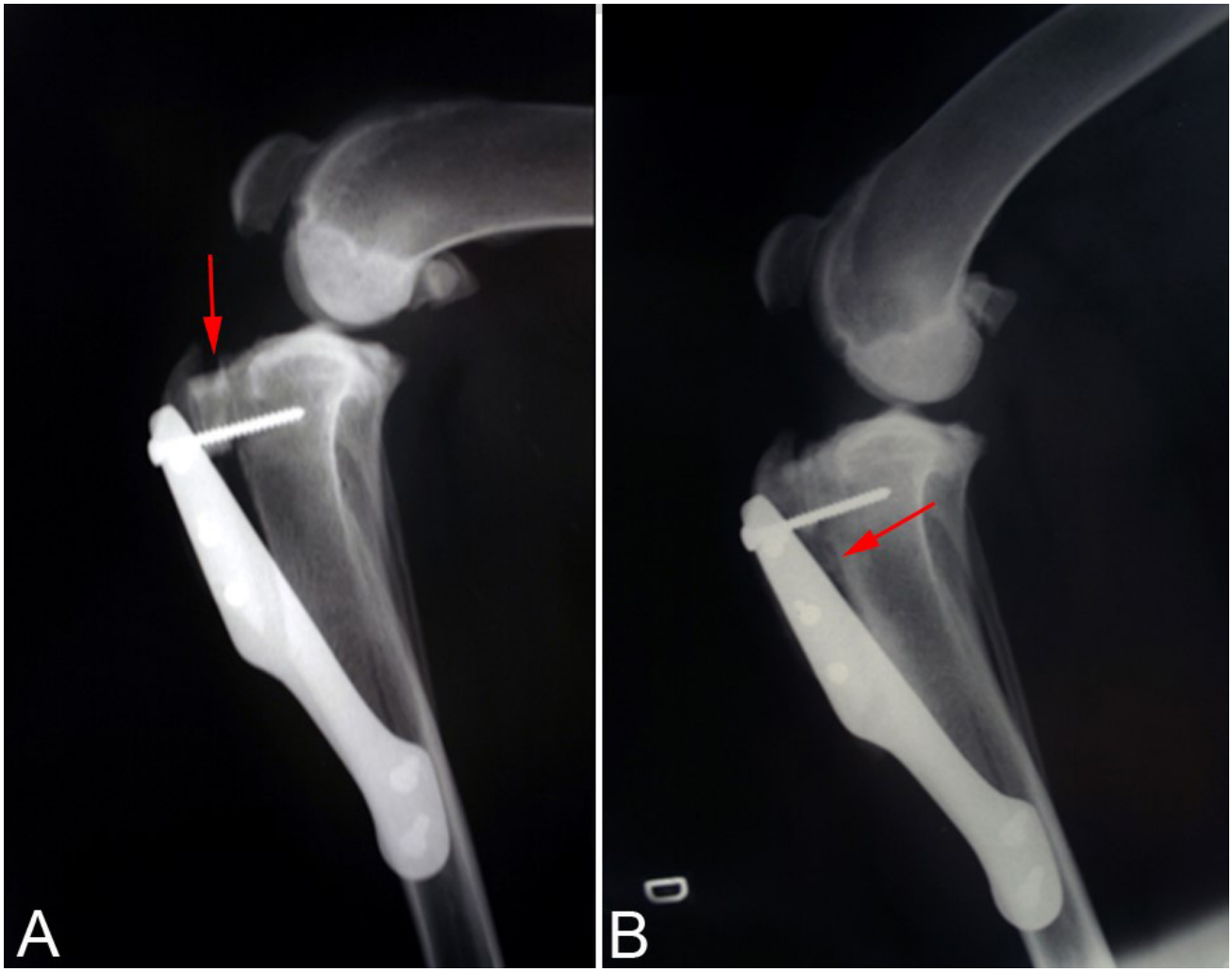

Fig.2. Radiographic images of the stifle joint of a dog at (**A**) 30 and (**B**) 60 days after tibial tuberosity advancement (TTA) procedure. (**A**) Evident margins of the bone graft between the tuberosity of the tibia and the body of the tibia and discrete bone proliferation on the proximal surface of both (arrow). (**B**) Less evident graft margins and identification of periosteal proliferation near the cranial surface of the tibia filling the space between the plaque, the graft and the tibia, without signs of infectious process or rejection, indicating an adequate process of bone consolidation (arrow).

No late evaluations of the other cases were possible, however, it was reported by phone contact that the animals marched without alterations.

Regarding radiographic evaluations, at 30 days was evidenced the beginning of the process of graft incorporation with the production of new bone on the interfaces between TT, tibia and graft. After 45 and 60 days it was noticed the filling of the gap over the graft, enabling reduction of graft density, incorporation and remodeling of the new bone, distal union of crest towards the graft and, in some cases, indication of total closure of the advanced space (Fig.2).

DISCUSSION

Characterizing the population of the dogs of this study, it is possible to perceive that most of the diagnosed CCLD occurred in males (67.35%), diverging from results obtained by (Bach et al. 2015) that found greater frequency in females (68.75%). Predominance was observed in wounds in Mixed dogs (29.41%), followed by breeds Poodle and Yorkshire Terrier (38.24%), suggesting that such discovery is due to concomitant dislocations of the patella and overweight as already described by other authors (Duval et al. 1999, Guerrero et al. 2007, Drygas et al. 2010).

In most of the cases of implants (88.89%) at 60 days, it was observed support without claudication. The animal represented by Case 33 kept claudication in continuous support at the end of the 60 days of follow-up, that can be associated to loose implants or positioning of the crest bold that, when positioned over the patellar ligament may generate claudication. It cannot be excluded the possibility of individual sensibility of the animal, not reacting well to the technique. However, due to excessive instability in this knee it was necessary to use the additional technique of fabelo-tibial suture, most probably due to the partial wound on the caudal cruciate ligament or on the medial collateral that was not perceived during the initial surgical act.

Patella dislocations were no hindrance for performing TTA with bone graft, on the contrary, the possibility of transposing TT and the application of the bolt pierced by TT, the graft and the tibia, facilitated the reorganization and alignment of the muscular apparatus of the quadriceps without increasing operative time (Duval et al. 1999, Guerrero et al. 2007, Drygas et al. 2010).

Glycerin was a good conservation medium for those fragments intended for grafting because, besides being of low cost, it kept bone fragments free of contamination, reducing antigenicity and preserving the functions of osteoinduction and osteoconduction, as described by other authors (Cavassani et al. 2001, Ziliotto et al. 2003). Furthermore, none of the animals had signs of contamination, infection of the surgical wound or rejection related with the presence of the graft, demonstrated by the complete graft-bone incorporation observed early at 45 days in some animals.

During radiographic evaluations of the 30 days, it is possible to see the beginning of the graft incorporation into the bone tissue and, in subsequent evaluations, it is possible to verify absorption and remodeling of the graft, besides retraction of the tibia advancement gap. Similarly, other author (Silva 2012) when using the hydroxyapatite block evidenced, at 30 days of postoperative, fast bone consolidation of the tibial tuberosity and bone formation over the ceramics in proximal region. However, it is known that calcium phosphate ceramics with hydroxyapatite are very slowly absorbed, causing a very time-consuming process of remodeling.

Compared to what happened in TTA with bone graft in this study, in the surgeries of member preservation in which it was also used allogeneic cortical bone graft conserved in glycerin at 98%, it was found that in 30 days there was onset of graft resorption and bone bridges formation, that became more evident at 90 days. In 120 days the graft resorption was almost complete, having formation of bone callus and bone remodelation without perception of clinical, radiographic or histopathological signs of implant rejection (Ziliotto et al. 2003).

Cages used in TTA had sizes of 6, 9 and 12mm of width (Hoffmann et al. 2006), bringing a limitation to the technique to fit perfectly in different sizes of dogs. The possibility of molding the graft to the animal need is a characteristic favorable to executing the modified technique, corroborating results of the castor polymers (*Ricinus communis*) in the making of the spacer for the TTA technique who observed that it was a biocompatible material that could be molded according to the size of the animal, allowing perfect adaptation to the osteotomized local in different breeds (Medeiros 2011).

Using the technique of TTA modified with bioceramic spacer, constituted of brushite, tricalcium phosphate and monetite, 3D printed, of porous morphology and highly permeable was observed that the material used had good acceptance without presenting adverse reactions, promoting the recovery of the motor function. X-rays showed that there were signs of graft reabsorption and bone remodeling around it, with significant union of osteotomized interfaces, demonstrating that the graft promoted better biocompatibility and osteoconductivity when compared with the standard titanium cage, used in the conventional TTA technique (Castilho et al. 2014).

The main postoperative complications observed in TTA on the medium and long term are: Patellar dislocation, meniscal lesions, tibial crest fracture and displacement (Hoffmann et al. 2006, Kim et al. 2008). Tibial crest fracture may be attributed to fails during osteotomy and improper positioning of the plate or of the cage (Nutt et al. 2015). Intercurrences commonly observed in TTA such as patellar dislocation, meniscal injury, tibial tuberosity fracture and infection (Costa et al. 2017, Dyall & Schmokel 2017) were not observed in the animals of this study, probably due to the better distribution of forces between the pass screw in TT and the TTA plate. Another reason could be attributed to the plate being fixed with bolts in TT and not with a clip as in the original technique.

No intercurrences that could be attributed to the graft were observed, corroborating that it has good adaptation to the technique conferring to the modified TTA advantages regarding the conventional TTA.

CONCLUSION

The preserved allogeneic cortical graft was able to promote adequate advancement of tibial tuberosity (TT) and bone union in all cases and proved to be feasible to be used in this kind of procedure in the absence of titanium cages.

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Original Article
Animais Selvagens/*WildLife Medicine*

Pathological and molecular findings of avian avulavirus type 1 outbreak in pigeons (*Columba livia*) of southern Brazil[1]

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ABSTRACT.- Souza S.O., Fredo G., Dupont P.M., Leite-Filho R.V., Teifke J.P., Pavarini S.P., Canal C.W. & Driemeier D. 2018. **Pathological and molecular findings of avian avulavírus type 1 outbreak in pigeons (*Columba livia*) of southern Brazil.** *Pesquisa Veterinária Brasileira 38(12):2254-2261.* Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves 9090, Porto Alegre, RS 95320-000, Brasil. E-mail: davetpat@ufrgs.br

The Newcastle disease, caused by avian avulavirus type 1 strains (APMV-1) is an important avian disease involved into high rates of mortality and economic losses. Several outbreaks have been reported over the last 30 years in Columbiformes in different parts of the world, caused by a adapted variant strain of AAvV-1, called pigeon paramyxovirus type 1 (PPMV-1). A high mortality associated with an outbreak was analyzed in free-living pigeons (*Columba livia*) in a public square in Porto Alegre in Southern Brazil. A total of 24 pigeons moribund or freshly dead, within five weeks interval were submitted to necropsy, histopathological, immunohistochemical (anti-Newcastle), and RT-PCR followed by sequencing of the amplification products analysis. They presented neurological signs, non-suppurative encephalitis and encephalomyelitis, and mononuclear inflammatory infiltrate in different organs. Immunohistochemical analysis in nine pigeons tissue showed that anti-Newcastle was expressed in brain, kidney, liver and pancreas. The RT-PCR test for the M protein of Newcastle disease virus was positive in six pigeons. The differential diagnosis of *Influenza*, West Nile, *Mycoplasma gallisepticum* and *Mycoplasma synoviae* in all pigeons presented negative results. The sequence of amino acids in the cleavage site region of the F protein was ^{112}RRQKRF^{117} classifying the strain as virulent. The phylogenetic analysis classified this virus strain into Class II and VI genotype.

INDEX TERMS: Molecular findings, avulavírus type 1, *Columba livia,* pigeons, PPMV-1, Newcastle disease, encephalomyelitis, columbiformes, immunohistochemistry, pathology.

RESUMO.- [**Achados patológicos e moleculares da infecção por avulavirus aviário tipo 1 em pombos (*Columba livia*) do sul do Brasil.**] A doença de Newcastle, causada por cepas de avulavirus aviário tipo 1 (AAvV-1), é uma doença de aves importante por causar altos índices de mortalidade e perdas econômicas. Vários surtos têm sido relatados ao longo de 30 anos em aves da ordem Columbiformes, em diferentes partes do mundo, causados por uma cepa variante específica de AAvV-1, denominada *Pigeon paramyxovirus* tipo 1 (PPMV-1). Foi analisado um surto de mortalidade em pombos domésticos (*Columba livia*), provenientes de uma praça pública em Porto Alegre, no Sul do Brasil. Vinte e quatro aves moribundas ou mortas foram submetidas, no intervalo de cinco semanas, ao exame de necropsia, exame histopatológico, imuno-histoquímico anti-Newcastle, RT-PCR e sequenciamento. Apresentaram sinais neurológicos, encefalite e encefalomielite não supurativas, além de infiltrado inflamatório mononuclear em diversos órgãos. Nove aves demonstraram exame imuno-histoquímico positivo em órgãos como cérebro, rim, fígado e pâncreas. Seis aves foram positivas no exame de RT-PCR para a proteína M do vírus da Doença de Newcastle. Nos exames de diagnósticos diferenciais de *Influenza*, West Nile, *Mycoplasma gallisepticum* e *Mycoplasma synoviae*,

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todas as aves testadas foram negativas. A sequência dos aminoácidos na região do sítio de clivagem da proteína foi ^{112}RRQKRF^{117}, classificando a cepa como virulenta. De acordo com a análise filogenética o vírus identificado foi classificado como pertencente à classe II e ao genótipo VI.

TERMOS DE INDEXAÇÃO: Avulavirus aviário tipo 1, *Columba livia,* pombos, PPMV-1, Doença de Newcastle, encefalomielite, Columbiformes, patologia.

INTRODUCTION

Avian avulavirus can be divided into nineteen species: AAvV-1 to AAvV-19 (ICTV 2015, 2017), and several avian species, including domestic and wild, can be affected (Alexander 2000a, Clavijo et al. 2000). Virulent strains of avian avulavirus type 1 (AAvV-1) causes Newcastle disease (ND), which is considered one of the most important avian diseases involved in high rates of mortality and economic losses, making the disease a notifiable condition to the World Organization for Animal Health (OIE 2012). In the past, Newcastle disease virus (NDV) was classified into at least three major pathotypes (lentogen, mesogen and velogen) based on the severity of the disease in chickens. Tests such as the mean time of death in eggs, the intravenous pathogenicity test and variations of these tests were used (Alexander & Senne 2008), but, by international agreement, a definitive evaluation of virus virulence is based on the intracerebral test pathogenicity (ICPI). The current OIE definition also recognizes advances in understanding the molecular basis of pathogenicity and allows confirmation of virus virulence, but not the lack of virulence, by in vitro tests that determine the amino acid sequence at the cleavage site of the F0 protein (OIE 2012).

Several outbreaks in Columbiformes have been reported over the past 30 years in many parts of the world caused by an adapted variant AAvV-1 denominated pigeon paramyxovirus 1 (PPMV-1) (Alexander 2011), which have been described in countries of the South America (Zanetti et al. 2001). These panzootic strains belong to genotype VI, which presents 9 subgenotypes (Dimitrov et al. 2016), and has been previously described in high mortality outbreaks worldwide in different species of birds (Alexander et al. 1985, 1997). Pigeons must be considered seriously as a potential source of NDV infection and disease for commercial poultry flocks (Kommers et al. 2002), and may be subclinically infected, spreading the virus for a considerable period of time without clinical signs (Carrasco et al. 2008, Catroxo et al. 2011). These may consist of apathy, anorexia, weight loss, prostration, diarrhea, polyuria, conjunctivitis, periocular edema, ruffled feathers, sneezing, dyspnea, incoordination, lack of balance, tremors, dehydration, proventricular dilatation, crop emptying problems, leukopenia and death. Some other symptomatic and asymptomatic birds had sudden death (Clavijo et al. 2000, Catroxo et al. 2012). Brazil has the status of free of pathogenic NDV in commercial poultry, and in the suspect of the disease the notification to the official veterinary service in the country is mandatory (Brasil 2007, Orsi et al. 2010).

In this paper, we describe the clinical signs, pathological and molecular findings of the first reported outbreak of an avian avulavirus type 1 infection in free-living pigeons (*Columba livia*) in Southern Brazil in the summer of 2014.

MATERIALS AND METHODS

Samples. The mortality losses of free-living pigeons (*Columba livia*) from a town square in the center of the city of Porto Alegre (30°1'59"S, 51°13'48"W) Rio Grande do Sul State, Brazil, were analyzed and evaluated. Necropsies were performed on 24 freshly dead pigeons that had exhibited neurological signs. Gross examinations were performed over five weeks (one pigeon in the first week, six pigeons in the following week, one pigeon in the third week, six pigeons in the fourth week, and ten pigeons in the fifth week). Tissue samples from all birds, but with the exception of spinal cord that was collected only from 17 birds, were collected for histological analyses, fixed in 10% neutral-buffered formalin, processed in a routine manner and stained with hematoxylin and eosin (HE) for histological examination. Dry cloacal and oropharyngeal swabs were collected, added with 1mL of Phosphate Buffer Saline (PBS) pH 7.2 was added, mixed and stored at -80°C until use. Tissue samples of 5.0g (trachea, kidney, lung and brain) were grounded with 2.0g autoclaved white sand in 5mL of PBS (pH 7.4), centrifuged at 10,000 rpm for 10 min and 100µL of the supernatant was store at -80°C for RNA extraction. Both tissue samples and swabs were analysed by PCR. For each bird, a full diagnostic assay was performed, such as histology, immunohistochemistry and PCR.

Immunohistochemistry. Immunohistochemistry (IHC) assay was performed in paraffin-embedded tissues of all birds, namely brain, liver, kidney and pancreas using the peroxidase method and using the primary antibody directly conjugated to a label. The tissues tested were chosen because they presented histological changes in most birds. The endogenous peroxidase activity was unmasked with hydrogen peroxide and methanol (1:9) for 20 minutes. To block the nonspecific sites the slides were incubated in 3% fat-free dry milk (Molico, Nestle ', Brazil) for 30 minutes. For antigen retrieval, the sections were heated in 10 mM citrate buffer (pH 6.0) for 5 min at 100°C using a microwave oven. After antigen retrieval, the sections were incubated overnight in a humidified chamber at room temperature with a primary antibody (Rabbit anti-Newcastle, BIOSS®, bs-4814R-HRP/ Polyclonal antibody, HRP conjugated) used at 1:500 dilution in phosphate-buffered saline (PBS). The sections were revealed by 3-amino-9-ethylcarbazole (AEC, DakoCytomation®, Inc. North America, Carpinteria, California, USA) counterstained with Mayer's hematoxylin for approximately 10 sec, coverslips were mounted using an aqueous mounting medium (S1964, DakoCytomation®), and slides were examined using light microscopy. Positive (brain and liver tissues from a pigeon RT-PCR positive for NDV) and negative controls were incubated with antibody diluent (catalogue no. S3022, DakoCytomation®).

RNA extraction and reverse transcription PCR (RT-PCR). RNA was extracted from 250µL of each sample using TRIzol® LS Reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions and eluted in 50µL of ultrapure water. The freeze-dried attenuated live vaccine Avinew® (Merial Animal Health Limited, Lyon, France) was used as a positive control and ultrapure water was used as a negative control. In order to detect NCDV, a previously described RT-PCR based on M protein gene amplified 231 bp (Seal et al. 1995). The cDNA was synthesized with SuperScript® III Reverse Transcriptase Kit (Life Technologies) in a total volume of 20µL, following the manufacturer's recommendations. The PCR was conducted in a total volume of 25µL. The amplification products were separated by gel electrophoresis in 2.0% agarose using 0,1 µg/mL of Blue Green Loading Dye I (LGC Biotecnologia, Cotia/SP, Brazil) in Tris-acetate buffer-EDTA (1x concentrated) (Sambrook et al. 1989). Visualization was done under UV light which

was compared with amplification products with molecular weight standards scale of 100 bp (Fermentas, USA).

Primer design for complete fusion protein (F) gene amplification. Twenty seven APMV-1 complete genome sequences were selected in GenBank® (http://www.ncbi.nlm.nih.gov/genbank/) in order to design primers for sequencing of complete F gene. The sequences were aligned using Molecular Evolutionary Genetics Analysis version 6 (MEGA6) (Tamura et al. 2013). The complete genome sequence of the strain AV 3224/96 (GenBank accession number GQ.429292.1) were selected for primer design due to the high identity with our samples and align using MEGA 6 with a complete fusion protein sequence of the isolate Amazon/Missouri/31378/1996 (GenBank acession number JN.942032.1). The primers were based on the fusion protein region of this sequence. Specific primers were selected to amplify the complete sequence of 1855 base pairs (bp) using Vector NTI Advance® Software (Life Technologies).

Amplification of the F protein gene by RT-PCR. The complete F protein gene was amplified using the 4 primers pairs (Table 1). The cDNA was synthesized with SuperScript® III ReverseTranscriptase Kit (Life Technologies) using Exo-Resistant Random Primer (Life Technologies), following the manufacturer's recommendations. Each primer pair was performed in a single PCR reaction and was conducted in a total volume of 25µL containing: 1× PCR buffer, 1mM of $MgCl_2$, 0.5mM of dNTP mix, 0.24mM of each forward and reverse primer and 1 unit of Platinum® Taq DNA Polymerase (Life Technologies), under the following conditions: an initial denaturation at 94°C for 3 min, 35 cycles of 45s for denaturation at 94°C, 45s of primer annealing at 52°C, 45s for extension at 72°C and a 7 min final extension at 72°C. The amplification products were separated by gel electrophoresis in 2.0% agarose using 0,1µg/mL of Blue Green Loading Dye I (LGC Biotecnologia, Cotia, SP, Brazil) in Tris-acetate buffer-EDTA (1x concentrated) (Sambrook et al. 1989). Visualization was done under UV light which was compared with amplification products with molecular weight standards scale of 100bp (Fermentas, USA).

Sequencing and phylogenetic analysis. The PCR products generated by M and F protein were purified using the NucleoSpin Extract II Kit (Macherey-Nagel, Düren, Germany), and both DNA strands were sequenced with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) using a BigDye Terminator v.3.1 cycle sequencing kit (Applied Biosystems). The gene sequences of the present study were assembled using SeqMan (DNASTAR Lasergene® 11, Madison, USA). Nucleotide sequence editing, analysis, prediction of amino acid sequences and alignments were performed by using the software MEGA 6. For construction of F protein phylogenetic tree, reference sequences representing reconized genotypes in class II and I were retrieved from Kim et al. (2008) and GenBank® (http://www.ncbi.nlm.nih.gov/ genbank/) and aligned with BioEdit version 7.1.3 software using CLUSTAL W. Molecular Evolutionary Genetics Analysis version 6 (MEGA6) (Tamura et al. 2013) was used for phylogeny inference according to the Maximum Likelihood criterion and the General Time Reversible model. The robustness of the hypothesis was tested with 1000 non-parametric bootstrap analyses.

Table 1. Primers selected to sequence the F protein gene

Primer	Sequence (5'-3')	Target
NC Fusion-1F	5'CTATCTAATTAGAAAAAACACGGGTAGAAG3'	F
NC Fusion-1R	5'TGAGTTAGGGCAGGGGAAGT3'	F
NC Fusion-2F	5'GCAACAGTTTGTCAATGACCAA3'	F
NC Fusion-2R	5''TGTATTGCCGCTCAGACAAGA3'	F
NC Fusion-3F	5'AGGTAGTGACACAAGTCGGCTCTG3'	F
NC Fusion-3R	5'AACGATATAGGTAATGAGAGCAGATGT3'	F
NC Fusion-4F	5'ATCGTGACAGGCAACCTTGATATATC3'	F
NC Fusion-4R	5'CCGTTCTACCCGTGTATTGCT3'	F

Additional exams. Out of 24 pigeons examined for NDV, six were also tested, using PCR, for Influenza virus, West Nile virus, *Mycoplasma gallisepticum,* and *Mycoplasma synoviae*. PCR tests for *Mycoplasma gallisepticum* and *Mycoplasma synoviae* were performed with NewGene MGAmp and NewGene MSAmp, respectively.

RESULTS

Clinical and epidemiological features

A mortality surge in free-living pigeons (*Columba livia*) from a public square in the city of Porto Alegre in Southern Brazil in November of 2014 was analyzed. The death of these birds was notified to the official Brazilian veterinary service. Clinical signs observed included tremors of the head, stiff neck, lack of balance, incoordination, paresis, paralysis, drooped wings, and regurgitation (Fig.1A). The approximate number of dead pigeons observed was 120. However, the exact number of birds involved was not obtainable as the birds are free-living and the authors only had access to a limited portion of their habitat. The data provide epidemiological information including the time between the onset of clinical signs and death (an average of 24-48h). There were no reports that other bird species had died in this square. Only adult free-living pigeons were affected, all of them have a regular body condition and both males and females were analyzed. The birds had access to a fountain and fed on what they found on the ground.

Gross findings

Gross findings included multifocal to coalescing subdural hemorrhages in all segments in the spinal cord (8/24) (Fig.1B). The proventriculus and small intestine serous showed marked hyperemia. Both the spleen and liver were markedly enlarged, and hyperemic. The pancreas showed a mildly increase in size and a multifocal whitish color. In a focally extensive area of the dorsal cervical region, bright areas of hemorrhage extended into the subcutis. No other significant gross abnormalities were noted.

Microscopic findings

The histopathological alterations in organs were as follows, kidney (22/24), liver (20/24), pancreas (19/24), brain (18/24), spleen (13/24), spinal cord (8/17), testis (5/11), and oviduct (2/13) (Table 2). A histopathologic examination revealed non-suppurative encephalitis and encephalomyelitis involving all compartments of the central nervous system, with prominent perivascular lymphoplasmacytic cuffing (14/24), microgliosis (12/24) (Fig.2A), neuronophagia (8/24), neuropil vacuolization (4/24), lymphoplasmacytic meningitis (4/24) and Gitter cells (3/24). In the spinal cord perivascular lymphoplasmacytic cuffing (7/17), microgliosis (5/17)(Fig.2B), neuronophagia (3/17), vascular proliferation (3/17), hyperemia in the dura mater (3/17), Gitter cells (2/17), lymphoplasmacytic meningitis (1/17), gliosis (1/17), and neuropil vacuolization (1/17) were observed. One pigeon had eosinophilic intracytoplasmic inclusions measuring 3-5 µm in neurons in the gray matter of the spinal cord. In the kidneys, multifocal lymphoplasmacytic and rare macrophages were

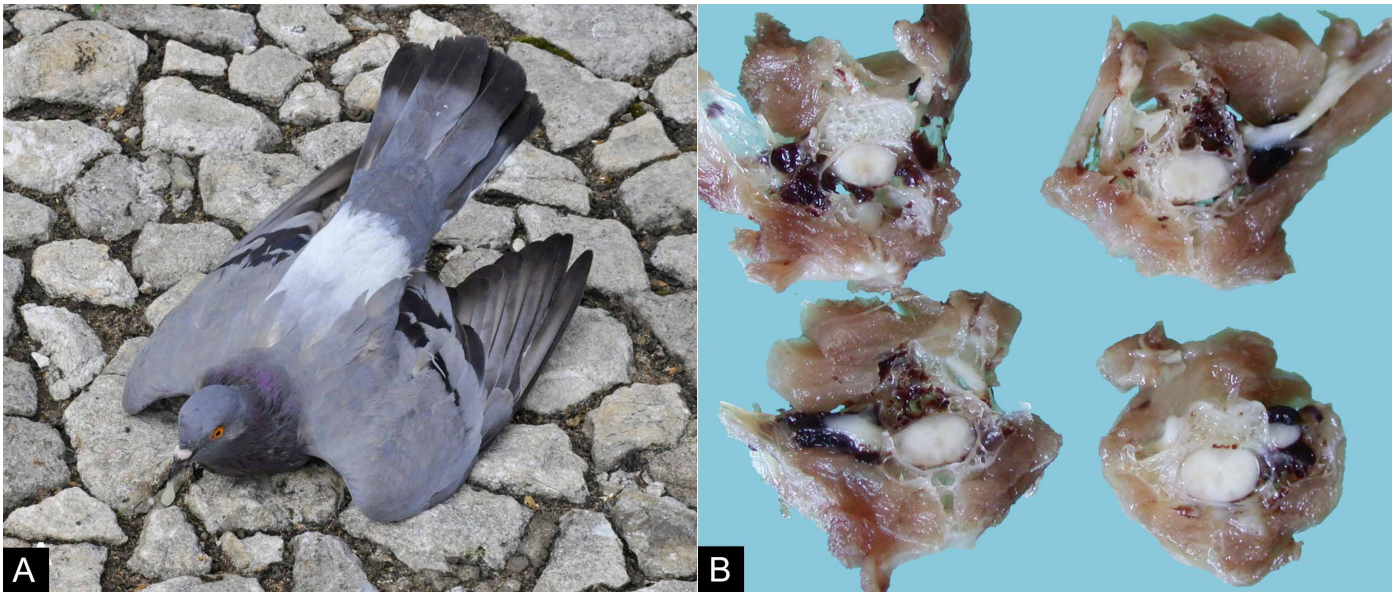

Fig.1. Clinical-pathological characterization of Paramyxovirus infection in Columbiformes. (**A**) Pigeon. Note the impaired locomotion causing it to stand on its wings. (**B**) Pigeon, spinal cord. Cross section, areas of subdural hemorrhage.

Table 2. List of animal outbreaks and the period in which they were assessed, affected organs and the results of RT-PCR and immunohistochemistry analyses

Bird no.	Week of receipt	Affected organs	RT-PCR M protein	Immunohistochemistry
1	1ª	B, Li, P, K, S	ND	+B
2	2ª	B, P, K, S	+	+P +B +K
3	2ª	P, K	-	-
4	2ª	P, K, S	+	-
5	2ª	B	-	+B
6	2ª	B, Li, S, P, K	-	-
7	2ª	B	+	+B
8	3ª	B, SC, S, Li, K, T	-	-
9	4ª	B, SC, Li, S, K, P	-	+B
10	4ª	SC, S, K, Li, P, T	+	+Li
11	4ª	B, P, K, Li, B	-	-
12	4ª	B, P, K, Li, B	-	-
13	4ª	B, P, K, Li, B	+	+Li
14	4ª	B, SC, P, K, Li, S	+	+Li +B
15	5ª	B, SC, P, K, Li, S	-	-
16	5ª	B, Li, SC, P, K, O	-	+Li
17	5ª	S, P, Li, K	-	-
18	5ª	B, P, K, Li, S, T	-	-
19	5ª	B, Li, K	-	-
20	5ª	K, Li, P, T	-	-
21	5ª	B, SC, P, K, Li, S	-	-
22	5ª	B, P, K. Li, S	-	-
23	5ª	B, SC, Li, K, T	-	-
24	5ª	P, K, Li	-	-

- Negative, + positive; B = brain, P = pancreas, Li = liver, K = kidney, S = spleen, SC = spinal cord, O = oviduct, T = testicles, ND = not done.

observed in the interstitial nephritis (22/24) (Fig.2C), as well as necrosis of tubular epithelial cells (3/24) and hemorrhage (2/24). In the spleen, hemosiderosis (14/24), lymphoid necrosis associated with fibrin deposition (10/24), lymphoid depletion (6/24), macrophage infiltration, and hemorrhage (2/24) were observed. In the liver, periportal lymphocytes, plasma cells, macrophage, and heterophil infiltration (20/24) were observed (Fig.2D). In the pancreas, lymphocytic infiltrate (17/24) was found and was occasionally associated with extensive necrosis (6/24) (Fig.2E). Lymphocytic infiltration was observed in two female oviducts, and five males had testicles with tubular degeneration that was occasionally associated with lymphocytic infiltration.

Immunohistochemistry

ND virus antigens were detected multifocally and were characterized by a granular immunostaining in the cytoplasm of neurons (6/24) (Fig.2F), macrophages in the liver (4/24), epithelial cells in the exocrine pancreas (1/24), and in epithelial cells in the kidney (1/24)(Table 2).

RT-PCR and phylogenetic analysis

Six out of the 23 pigeons analyzed were positive for NDV by RT-PCR for the matrix (M) protein gene (Table 2). The complete gene and the coding sequence of the fusion (F) gene, according to the unified NDV classification system, indicated that the strain had a cleavage site, ^{112}RRQKRF^{117}, which was characteristic of velogenic strains. Phylogenetic analysis of protein F showed that this strain could be classified into class II and genotype VI (Fig.3).

Additional exams

A total of six pigeons tested negative for Influenza virus A, West Nile virus, *Mycoplasma gallisepticum*, and *Mycoplasma synoviae* by PCR assay.

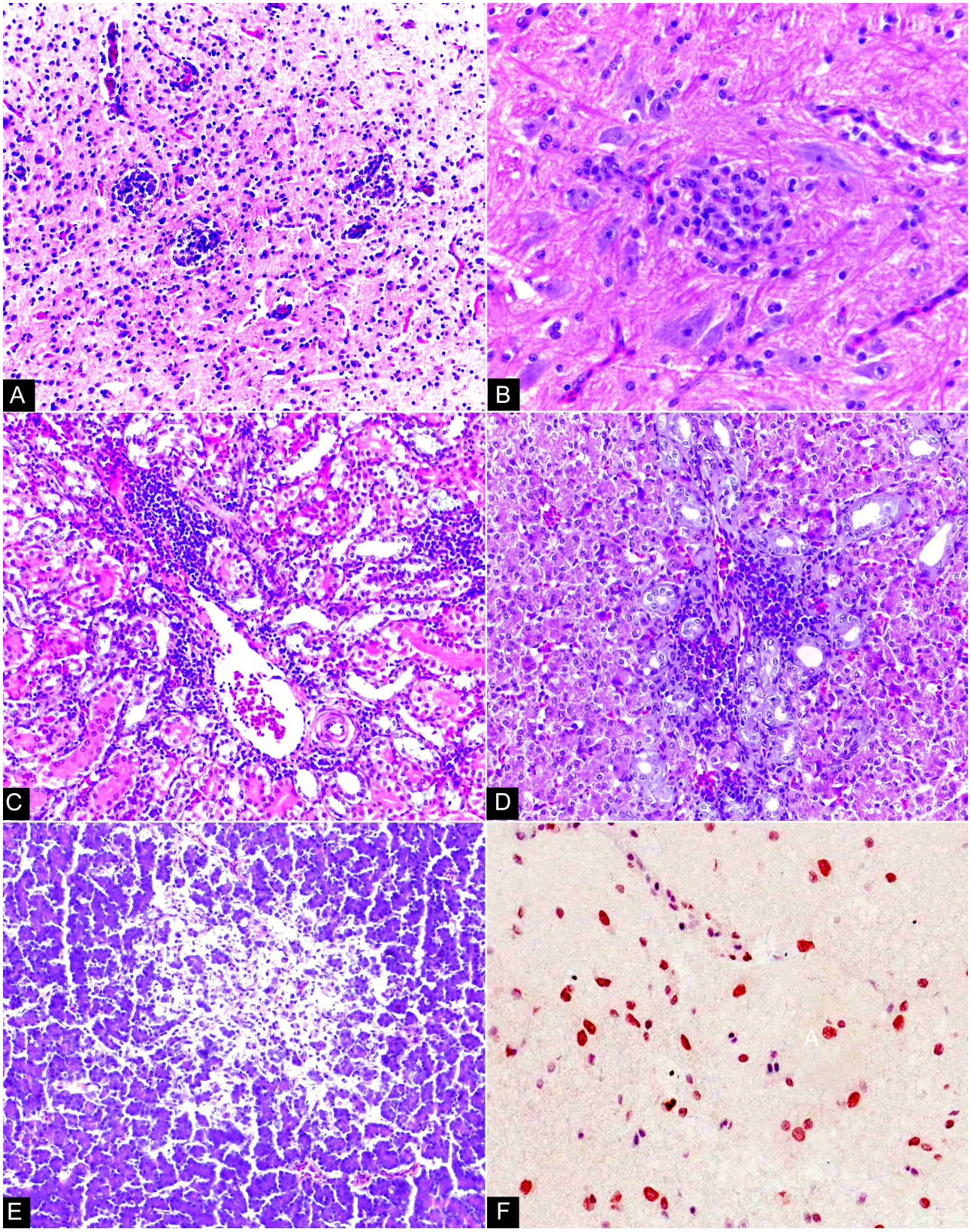

Fig.2. Histopathologic and immunohistochemical characterization of Paramyxovirus infection in Columbiformes. (**A**) Brain. Perivascular lymphoplasmacytic cuffing and mild microgliosis. HE, obj.20x. (**B**) Spinal cord. Mild nodular microgliosis. HE, obj.40x. (**C**) Kidney. Lymphoplasmacytic multifocal interstitial infiltrate associated with rares macrophages and necrosis of tubular epithelial cells. HE, obj.40x. (**D**) Liver. Focal area of mild periportal inflammatory infiltrate constituted by lymphocytes, plasma cells, macrophages, and heterophils, in addition to mild bile duct proliferation. HE, obj.40x. (**E**) Pancreas. Focal area of necrosis of pancreatic acini. HE, obj.40x. (**F**) Brain. Positive immunostaining in the cytoplasm of neurons for Newcastle Disease virus. AEC, obj.40x.

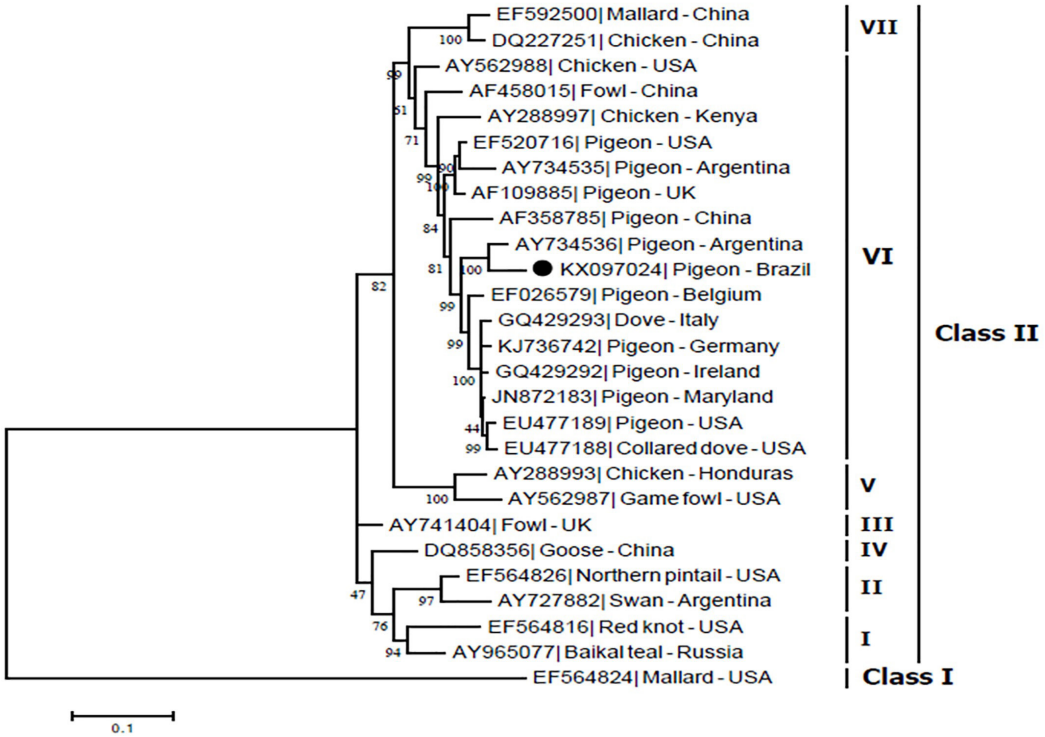

Fig.3. Molecular phylogenetic analysis of paramyxovirus type I F protein by Maximum Likelihood method. Phylogenetic analysis showed that this strain could be classified into class II and genotype VI clade, which includes the strain described in the present study and the reference strain AY734536.1/Pigeon-Argentina.

DISCUSSION

The diagnosis of paramyxovirus infection was confirmed through histological, immunohistochemical, and molecular analyses. The pigeons in this study presented neurological signs that included ataxia, imbalance, head tremors, incoordination, torticollis, and inability to fly, which are the same as those described as the primary signs of this disease observed in the order Columbiformes (Barton et al. 1992). The gross findings were nonspecific. However, most of the pigeons presented a focally extensive area of hemorrhage in the dorsal cervical region that extended into the subcutis. Columbiformes are known to exhibit a prominent vascular plexus in the cervical region (Werther 2007), which may have been injured in these birds due to the tremors and head rotation they displayed.

The histological lesions observed in the central nervous system and organs of the coelomic cavity of these pigeons were similar to those described in the literature (Bhaiyat et al. 1994, Brown et al. 1999, Kuiken et al. 1999, Ecco et al. 2011). The large amount of AAvV-1 found in the brains of experimentally infected pigeons suggests a high concentration of this viral strain in the central nervous system (Kommers et al. 2002), justifying the presence of lesions in the CNS found in the majority of pigeons analyzed in our study.

Immunohistochemical positive staining was found in nine pigeons of 24 tested. The absence of staining in the tissue samples from the other pigeons could be related to the period of infection; in some cases, there was no association between injury and the presence of the virus. The absence of positive staining may also indicate that undetectable amounts of virus can generate inflammatory lesions (Ecco et al. 2011) due to the greater sensitivity of nervous tissue (Brown et al. 1999). Viral replication may not be the only mechanism of neuronal damage; some damage likely occurs due to vascular impairment, and direct neuronal necrosis may be caused by the virus (Ecco et al. 2011). One bird exhibited the deposition of eosinophilic granular material in neuron bodies in an experimental infection study (Kommers et al. 2002), and another showed the same structures in respiratory epithelial cells and neuroglia cells (Hamid et al. 1991). In our study, immunohistochemical analysis did not reveal positive staining in these spinal cord structures. Therefore, it is not possible to state that these are paramyxovirus particles.

The pigeons examined in the last week of the outbreak were not RT-PCR positive, possibly because they were close to the end of the clinical course of the disease. It was not possible to identify viral material in the cloaca and trachea swabs or in the organs. Experimental infection studies with AAvV-1 strains have shown that pigeons primarily eliminate the virus in the first few days after infection (Dortmans et al. 2011). In tissues, positive PCR results were observed more frequently until the sixth day after infection (Hamid et al. 1991). RT-PCR analysis and sequencing were effective for the virus detection and in the deduction of virulence (Alexander et al. 2012). These techniques have been widely used because they are faster and less laborious than *in vivo* tests (Alexander 2000b), allowing for phylogenetic studies to determine the source and possible spread and mutation of a given strain. These techniques are also a good alternative in outbreaks (Aldous & Alexander 2001). In this study, they were effective and

essential diagnostic tools. In the present study, it was possible to observe that infections with a longer period of evolution can exhibited histological lesions without the presence of the virus, considering that the cases observed in the last week of the outbreak were negative in the immunohistochemical assay and the PCR.

Currently, ND is defined as a poultry infection caused by AAvV-1 that either has an ICPI greater than 0.7 or has multiple basic amino acids in the C-terminus of the F2 protein and the N-terminus of the F1 protein (OIE 2012). Sequencing enables the detection of a strain that may be highly pathogenic for chickens and may cause a Newcastle disease outbreak upon crossing biosafety barriers and infecting poultry.

Differential diagnoses include Influenza virus A, West Nile virus, *Mycoplasma gallisepticum*, and *Mycoplasma synoviae*, agents that can cause damage to the central nervous system (Swayne et al. 2001, Swayne 2008). None of the pigeons tested was positive for these agents. Therefore, it is extremely important to use histology in conjunction with other diagnostic tools. Whereas immunohistochemical analysis enables the differentiation of ND from other agents, RT-PCR analysis and sequencing directly determines virulence.

The potential of the carrier pigeon to introduce ND in certain areas has been reported (Alexander 2000b), including in South American countries (Zanetti et al. 2001, Castro et al. 2012). The strain identified in the present study demonstrated homology with the strain that was identified in an Argentinian outbreak (Zanetti et al. 2001), relatively close to the state of Rio Grande do Sul. The mortality outbreak in this study occurred near the Guaiba River (in the eastern portion of the Rio Grande do Sul State, at the intersection of 50º and 52º west longitude and 30º and 31º south latitude). The hypothesis that the infection was acquired from aquatic and wild birds inhabiting this region (Accordi & Barcellos 2006) cannot be ruled out.

NDV is a pathogen with zoonotic potential, and the most common sign of infection in humans is conjunctivitis that develops within 24 hours of NDV exposure to the eye (Swayne & King 2003). Isolation of a pigeon-like APMV-1 from the lung tissue, urine, and feces of an immunocompromised patient who died of pneumonia has been reported (Goebel et al. 2007). Urban pigeons (*Columba livia*) may serve as reservoirs, carriers, and transmitters of various pathogens, and pose a risk to public health (Werther 2007). The free-living pigeons (*Columba livia*) in the present study were transient and living in the town square in the center of the city where they were fed, thus, they may be in frequent contact with humans.

After natural transmission from pigeons to chickens, PPMV-1 strains may become more virulent and lead to major outbreaks (Meulemans et al. 2002). The specific strain of PPMV-1 carried by pigeons can cause severe lesions in infected chickens (Kommers et al. 2002). Commercial poultry may be susceptible to the identified strain responsible for the outbreak presented in this study. The identified strain is pathogenic for pigeons because the clinical signs that are presented in this study are similar to those reported in the literature (Brown et al. 1999). The lesions that were found were mostly present in the central nervous system and were associated with the identification of the agent through immunohistochemical examination, which strongly suggests that the strain involved in this study is neurotropic. These results indicate the presence of an AAvV-1 with the potential to cause Newcastle disease if it is transmitted to poultry. Biosafety measures and virus surveillance must be increased to prevent this disease from causing further infections in this region.

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Conflict of interest statement.- The authors do not have any potential conflicts of interest to declare.

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Original Article
Animais Selvagens/*Wildlife Medicine*

Blackleg in a free-range brown brocket deer (*Mazama gouazoubira*)[1]

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ABSTRACT.- Echenique J.V.Z., Soares M.P., Uzal F.A., Ladeira S.R.L., Albano A.P.N., Bandarra P.M. & Schild A.L. 2018. **Blackleg in a brown brocket deer (*Mazama gouazoubira*).** *Pesquisa Veterinária Brasileira 38(12):2262-2265.* Laboratório Regional de Diagnóstico, Faculdade de Veterinária, Universidade Federal de Pelotas, Campus Capão do Leão, Pelotas, RS 96010-900, Brazil. E-mail: gmpsoares@gmail.com

A case of blackleg in a brown brocket deer (*Mazama gouazoubira*) associated with trauma from being hit by a car in southern Rio Grande do Sul is reported. The clinical signs included fever, dehydration and lethargy that worsened progressively until 36 hours after the accident, when the animal died. In the fore right limb, there was a comminuted closed fracture of the radius and ulna but no skin wounds were observed. Grossly, the musculature of the pelvic limbs presented hemorrhage, edema and emphysema. Microscopically, the muscles of both rear legs had necrosis, edema, hemorrhage and mild inflammatory infiltration of neutrophils. *Clostridium chauvoei* was cultured from affected skeletal muscles, and it was also detected by immunohistochemistry, confirming a diagnosis of blackleg. The overlapping habitat of cattle and brown brocket deer is proposed as a predisposing factor in this case and alerts to spillover cases maybe happening in this region. In addition, blackleg should be included as differential diagnoses of deer with post-traumatic myositis.

INDEX TERMS: Blackleg, brown brocket deer, *Mazama gouazoubira, Clostridium chauvoei*, cervid, spillover, myositis, wildlife animals.

RESUMO.- [**Carbúnculo sintomático em veado virá de vida livre (*Mazama gouazoubira*)**.]Descreve-se um caso de carbúnculo sintomático em um veado-virá (*Mazama gouazoubira*), macho, jovem, resgatado após atropelamento em uma rodovia na região sul do Rio Grande do Sul. O cervídeo apresentou febre, desidratação e letargia, progredindo para a morte em 36 horas. No membro torácico direito foi observado fratura cominutiva fechada de rádio e ulna sem a presença de feridas perfurantes. Na necropsia foi observada hemorragia, edema e enfisema na musculatura dos membros pélvicos. Microscopicamente, os músculos dos membros pélvicos apresentaram necrose, edema, hemorragia e discreto infiltrado inflamatório neutrofílico. Houve o isolamento de *Clostridium chauvoei* e marcação positiva na técnica de IHQ com anticorpo monoclonal anti-*C. chauvoei,* confirmando o diagnóstico de carbúnculo sintomático. A sobreposição de habitat entre bovinos domésticos e cervídeos pode ser um fator de risco para esta doença e chama a atenção para casos de "spillover" que podem estar ocorrendo na região. Adicionalmente, sugere-se que o carbúnculo sintomático seja incluído nos diagnósticos diferenciais de cervídeos que apresentam miosite pós-traumática.

TERMOS DE INDEXAÇÃO: Carbúnculo sintomático, veado virá, vida livre, *Mazama gouazoubira*, *Clostridium chauvoei*, cervídeo, spillover, miosite, animais silvestres.

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INTRODUCTION

Blackleg is a clostridial disease characterized by necrotizing myositis and caused by *Clostridium chauvoei*, which leads to significant economic losses in cattle around the world (Maboni et al. 2010, Abreu & Uzal 2016, Abreu et al. 2017), including Brazil (Riet-Correa 2007). Although the pathogenesis of blackleg has not been definitely established, current dogma states that the animals become infected by ingesting spores from the pasture. After one or more replication cycles in the intestine, the spores are absorbed into the systemic circulation and get eventually phagocytized by macrophages within the striated musculature. When a traumatic injury occurs with a consequent decrease in redox potential, the bacteria proliferate and produce several potent toxins that are responsible for the clinical signs and lesions of the disease (Abreu & Uzal 2016, Cooper & Valentine 2016). Because of this proposed pathogenesis, black leg is frequently referred to as an endogenous infection.

Blackleg needs to be differentiated from gas gangrene, considered an exogenous infection that is usually associated with skin and/or mucosal wounds, which allow entry of spores or vegetative forms of the organisms involved into the tissues of the host (Abreu & Uzal 2016, Silva et al. 2016, Abreu et al. 2017). Gas gangrene can be produced by one or more of the following clostridial species: *Clostridium septicum, C. chauvoei, C. perfringens, C. novyi* and *C. sordelli*. Gas gangrene has been described in several ruminant species including deer, associated with a variety of wounds such as those produced by surgery, accidental trauma, darting and parturition (Herron et al. 1979, Buxton 1994, MacKintosh et al. 2002, Silva et al. 2016).

Although black leg is mainly a disease of cattle, cases have been reported, albeit infrequently, in wild ruminants, including white-tailed deer (*Odocoileus virginianus*), reindeer (*Rangifer tarandus*), and wapiti (*Cervus elaphus* spp.) (MacKintosh et al. 2002). To our knowledge, however, black leg has not been described in free-range brown brocket deer (*Mazama gouazoubira*) before. This is a South American cervid that can be found in southern Brazil and the Bolivian Chaco. The population of this deer is declining due to illegal hunting, habitat loss and road killing (Duarte & Reis 2012, Black-Decima & Vogliotti 2016). This paper describes a case of blackleg in a wild brown brocket deer associated with a road accident injury.

MATERIALS AND METHODS

A juvenile male brown brocket deer was found on the side of a road in Southern Brazil after being hit by a car, and transported immediately to the Núcleo de Reabilitação da Fauna Silvestre of Universidade Federal de Pelotas (NURFS-UFPel) for examination and treatment. On arrival, approximately six hours after the accident, the animal had hyperthermia (39.2°C), lethargy, dehydration and swelling of the left pelvic limb musculature. Support treatment with meloxicam and fluids was initiated, but the deer died 36 hours after arrival. The carcass was sent for necropsy to the Laboratório Regional de Diagnóstico. A full necropsy was performed immediately after death and samples of skeletal muscle from both thigs, skin, spleen, small and large intestine, rumen, reticulum, omasum, abomasum, liver, kidney, lung, heart and nervous system were collected, fixed by immersion in 10% buffered formalin and routinely processed for histology. Selected sections of muscle were stained with hematoxylin and eosin (HE) and Gram and processed by immunohistochemistry (IHC) for *Clostridium perfringens, C. chauvoei, C. septicum, C. novyi* and *C. sordellii* as previously described (Assis et al. 2005, Nyaoke et al. 2018). The primary antibodies were produced in rabbit against each of the clostridia species mentioned above (VDRM, Pullman/WA, USA). Positive controls included bovine tissues from which each of those clostridia had been isolated. No cross-reactivity was observed between these clostridial IHC reactions. Samples of rear leg muscles were aseptically collected and cultured on 5% sheep blood agar in anaerobiosis, MacConkey agar and cooked meat media and incubated at 37° C for 48-72 hours.

RESULTS

The carcass was in good nutritional condition; it had adequate amounts of fat reserves and was well fleshed. There was multifocal hemorrhage, edema and emphysema of the subcutaneous tissue and muscles of both pelvic limbs (Fig.1A). These lesions were more severe on the left side (Fig.1B). The anterior right limb had close, comminuted, complete fractures of the radius and ulna and severe hemorrhage of the soft tissues surrounding the fractured bones. A careful examination of the skin in this area did not reveal punctures.

Histologically, the muscle fibers of the pelvic limb showed coagulation necrosis, characterized by hypereosinophilia, hyalinization, loss of striations, and flocculation of cytoplasm (Fig.2A), vacuolation and hypercontraction bands. In addition, there was interstitial edema, hemorrhage (Fig.2B) and mild neutrophilic inflammatory infiltrate (Fig.2C). The blood vessels showed discrete, multifocal vasculitis with fibrinoid necrosis. Large gram-positive rods, many of them with sub-terminal spores were seen single and in clusters in the muscular interstitium and occasionally within myocytes and blood vessels. These organisms stain positively for *Clostridium chauvoei* IHC (Fig.2D), but negatively for the other clostridia tested. In the microbiological culture of the affected muscle fragment, there was no growth when incubated aerobically on MacConkey agar and blood agar. On cooked meet medium, gram-positive and sporulated rod-shaped bacteria grew. This organism was re-plated on blood agar in an anaerobic system (Probac do Brasil, Anaerobac®). The colonies were surrounded by large zone of hemolysis. Gram-stained impression smear of those colonies revealed large gram-positive rods with oval, sub terminal or central spores. No other microorganisms were isolated from muscle samples of this animal.

DISCUSSION

A presumptive diagnosis of blackleg was based on gross, microscopic findings, and by isolation of an anaerobic sporulated gram-positive rod. This diagnosis was confirmed by detection of *Clostridium chauvoei* in muscle by IHC. Moreover, examination of IHC sections revealed that *C. chauvoei* was intimately associated with muscle lesions, which confirms that this microorganism was indeed responsible for the lesions observed.

In this case, trauma occurring when a car hit this deer was considered the likely triggering factor for blackleg. If this was so, the deer must have been harboring spores of *C. chauvoei* in the musculature before the accident and tissue anaerobiosis associated with muscle trauma triggered spore germination followed by a chain of events similar to those described for

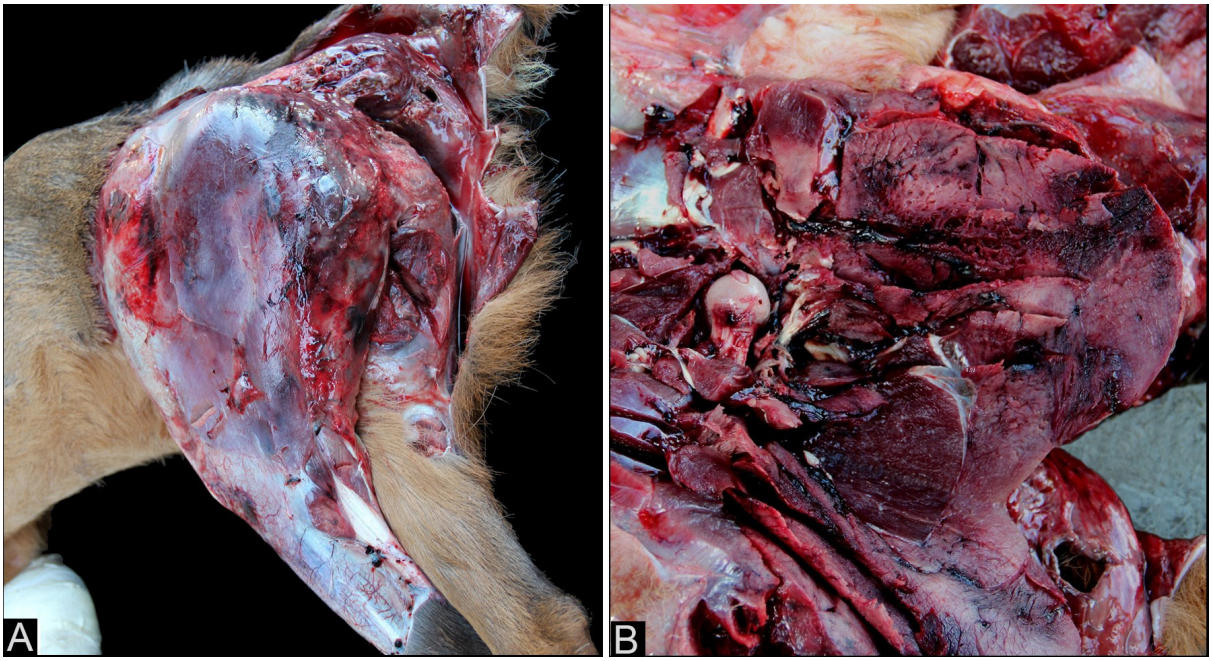

Fig.1. Brown brocket deer, male, left pelvic limb. (**A**) Musculature with increased volume and hemorrhage. (**B**) musculature whit hemorrhage, discrete discoloration, necrosis and gas bubbles.

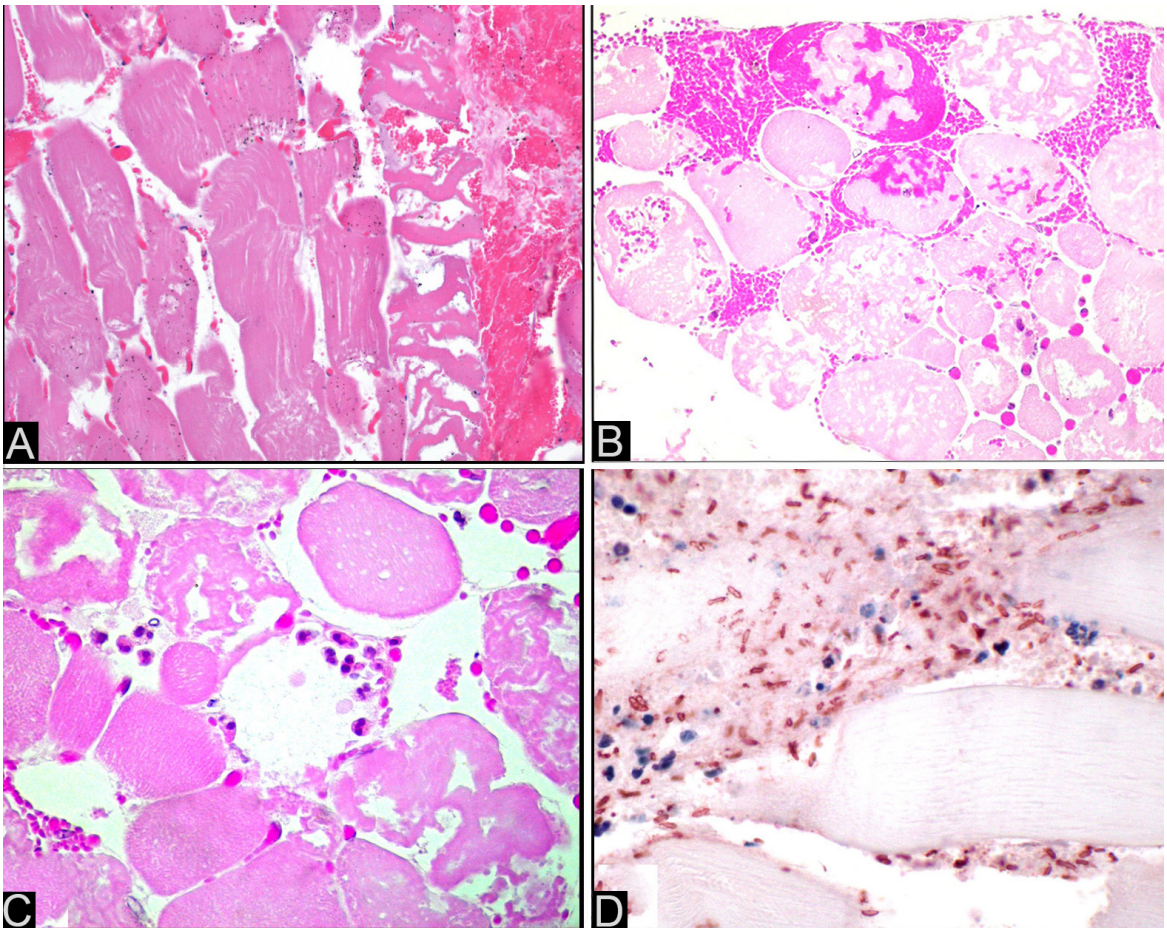

Fig.2. Brown-brocket-deer, skeletal muscle. (**A**) Longitudinal section of the muscular fibers showing segmental necrosis and hemorrhage. HE, obj.40x. (**B**) Degenerative changes in the muscle fibers with flocular necrosis, edema, hemorrhage. HE, obj.40x. (**C**) Discrete inflammatory infiltrate of neutrophils between the muscle fibers. HE, obj.40x. (**D**) Bacterial rods positive staining with anti-*Clostridium chauvoei*. IHC, 40x.

blackleg pathogenesis in cattle (Abreu & Uzal 2016, Silva et al. 2016, Abreu et al. 2017). The most severe injuries produced by the accident occurred in the front legs and yet, blackleg lesions were only observed in the rear legs. It is possible that spores were present only in the rear leg musculature and blunt trauma affected also these areas triggering spore germination. Alternatively, it is possible that spores were present initially in the front legs where bone fractures occurred and a small blackleg lesion developed in this area initially but it was not discovered during gross and microscopic examination. If this was the case, septicemia may have ensued with metastatic larger lesions occurring in the rear legs. The large muscular masses of the hind legs are amongst the most frequently affected in cattle with blackleg; the reason for this preference is unknown (Abreu et al. 2017).

Although *C. chauvoei* is best known for its role in blackleg, this microorganism can also be, albeit very rarely, part of the gas gangrene complex (Abreu & Uzal 2016, Abreu et al. 2017). Although blackleg affects mostly muscle and gas gangrene tends to affect primarily sub-cutis, some overlap between both diseases may occur, and lesion location is not always conclusive in the differentiation of both diseases. In this case, however, no skin or mucosal lesions were seen, which rules out a diagnosis of gas gangrene.

The gross and microscopic lesions found in the deer of this study were very similar to those found in cattle diagnosed with blackleg, which also supports a similar pathogenesis between cattle and deer blackleg. Differential diagnoses considered in this case were gas gangrene and capture myopathy. They were ruled out based on clinical, gross and microscopic findings, while other bacterial myositis were ruled out based, microscopic, microbiological and IHC findings (Abreu et al. 2017).

Cattle production is the main economic activity of Rio Grande do Sul state (Pinto & Coronel 2015), and the habitat of the brown brocket deer overlaps with areas of large cattle density (Rodrigues et al. 2014). Furthermore, cases of bovine blackleg were previously diagnosed in the area where the brown brocket deer of this report was found (Riet-Correa 2007). It is therefore possible that spores of *C. chauvoei* of bovine origin contaminated the pastures in the area and that this deer became contaminated by grazing in areas where cattle has been grazing before. Proximity of wild animals to livestock is a determining factor for the spread of several infectious diseases (Brahmbhatt et al. 2012, Gortazar et al. 2014) and this seems to have been the case with this deer.

CONCLUSIONS

Blackleg should be considered amongst the differential diagnoses in cases of myositis associated with muscular blunt trauma in brown brocket deer.

The close interaction between cattle and deer may have provided an opportunity for infection spillover from domestic to wild animals.

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Tópico de Interesse Geral
Animais Selvagens/*Wildlife Medicine*

É possível integrar pecuária à conservação da biodiversidade? Estudo de casos de depredação de ovinos por onça-parda (*Puma concolor*)[1]

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ABSTRACT.- Ubiali D.G., Weiss B.A., Ubiali B.G., Colodel E.M., Valderrama-Vasquez C., Garrido E.P., Tortato F.R. & Hoogesteijn R. 2018 [**Is it possible to integrate livestock into biodiversity conservation? Case study of sheep predation by puma (*Puma concolor*)**] É possível integrar pecuária à conservação da biodiversidade? Estudo de casos de depredação de ovinos por onça-parda (*Puma concolor*). *Pesquisa Veterinária Brasileira 38(12):2266-2277*. Setor de Anatomia Patológica, Departamento de Epidemiologia e Saúde Pública, Instituto de Veterinária, Universidade Federal Rural do Rio de Janeiro, Seropédica, RJ 23890-000, Brazil. danielubiali@hotmail.com

In several parts of Brazil and Colombia, the expansion of agriculture over the years has caused loss and reduction of wild fauna natural habitat. Recently, deaths of sheep and cattle have increased due to predation by large carnivores and the resulting retaliation to predators. Consequently, populations of these top predators have been reduced or even locally extinct, leading to imbalances on ecosystems, as alteration in the prey dynamics played by carnivore. The objective of this study is to point out preventative and mitigating measures of sheep predation by puma (*Puma concolor*) through the analysis of case studies in Central Brazil and the Colombian Andes. We discuss potential sustainable solutions that can be implemented by farmers. From 2005 to 2014, we visited a ranch in Alto Paraguay, Mato Grosso state for diagnostic purposes and we compared the death of sheep from diseases and predation attacks. In 2014, we visited a rural area in the central region of *Departamento del Valle del Cauca*, at 2814m of altitude in the Colombian Andes, to diagnose sheep predation, implement preventive measures, and evaluate their effectiveness. The results reveal that economic losses due to predation are critical on both studied regions and similar to losses by diseases in Mato Grosso state, Brazil. Thus, we recommend the integration of health management, preventive measures as well as mitigation of predatory attacks at the local scale. We recommend that public policies should incorporate scientific results on human-wildlife conflicts to be effective regarding livestock management and biodiversity conservation.

INDEX TERMS: Livestock, carnivore conservation, Mato Grosso, Brazil, predation, *Puma concolor*, puma, wildlife medicine, sheep diseases, diagnosis, livestock, conservation medicine, wild animals.

RESUMO.- Em diversas partes do território brasileiro, a perda e diminuição do habitat natural de animais silvestres ocorre em função do aumento das atividades agropecuárias. Nos últimos anos o número de mortes de animais de criação por depredação tem aumentado, bem como a consequente retaliação aos predadores. Como resultado destas ações, ocorre à extinção ou redução das populações destes predadores de topo, provocando perdas ecológicas. Esse estudo teve como objetivo apontar medidas preventivas e mitigatórias da depredação de ovinos por onça-parda (*Puma concolor*). Através da análise de dois estudos de casos de depredação no Centro-Oeste brasileiro e na região dos Andes Colombianos, levantamos soluções alternativas sustentáveis para que

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profissionais e criadores possam se prevenir desta ameaça ao rebanho ovino. Um estudo de caso foi realizado em fazenda no município de Alto Paraguai, Mato Grosso. Entre os anos 2005 e 2014 houve visitas na propriedade para diagnóstico de doenças e realizou-se estudo comparativo da quantidade de mortes por doenças com as mortes por depredação. No ano de 2010 ocorreu um ataque depredatório que resultou em morte de seis ovinos. Em 2014, realizou-se um estudo na região central do *Departamento del Valle del Cauca*, há 2814m de altitude dos Andes colombianos, para diagnosticar a depredação de gado na região e implementar medidas para prevenir sua ocorrência e avaliar sua eficácia. No total, foram implementadas medidas anti-depredação sobre oito propriedades, e entre elas, um curral com cerca elétrica para ovelhas foi implementada em uma fazenda no município de Tuluá. Os resultados mostram que as perdas econômicas por depredação são graves nas duas regiões estudadas e se equiparam a perdas por doenças no estudo de caso em Mato Grosso, Brasil. Portanto recomenda-se a combinação entre o manejo sanitário, métodos de prevenção e, se necessário, mitigação de ataques depredatório. Além disso, legisladores devem se associar a pesquisadores para traçar estratégias efetivas para esse sério problema ad América Latina.

TERMOS DE INDEXAÇÃO: Pecuária, conservação de carnívoros, predação, depredação, ovinos, onça-parda, Mato Grosso, *Puma concolor*, onça-parda, animais silvestres, doenças de ovinos, diagnóstico, conservação da biodiversidade, medicina da conservação.

INTRODUÇÃO

Na América Latina, a expansão da agropecuária resulta na fragmentação e perda de *habitats* de animais como a onça-parda (*Puma concolor*) e a onça-pintada (*Panthera onca*) (Calaça et al. 2010). Esses felinos, desempenham o papel de predadores de topo da cadeia alimentar e necessitam de extensas áreas para sobreviver (Caruso et al. 2015). O hábito alimentar está ligado à disponibilidade de presas, disponibilidade de habitat adequado e variações sazonais. É importante ressaltar que características do *habitat* e ações humanas intensas são capazes de interferir na dieta desses felinos (Schulz et al. 2014). A conversão do *habitat* natural para agricultura e formação de pastagens tem gerado perda de biodiversidade animal e, consequentemente, depredação de herbívoros domésticos por felinos silvestres (Novack et al. 2005, Palmeira et al. 2008), fato que ocorre em diversas partes do mundo e está associado a fatores ecológicos, sócio econômicos e políticos (Polisar et al. 2003, Graham et al. 2005). Como uma tentativa de solução à depredação, tem sido comum, pecuaristas eliminarem predadores (Zimmermann et al. 2005, Hoogesteijn & Hoogesteijn 2010), atividade que ameaça severamente populações selvagem desses carnívoros (Schulz et al. 2014).

A biodiversidade do Brasil e Colômbia é reconhecida mundialmente pela riqueza de fauna e é objeto de grande interesse em vários setores. Portanto, a importância do uso de estratégias alternativas para a preservação da diversidade é ressaltada (Machado et al. 2016). A onça-parda (*Puma concolor*) ocorre desde o sul do Chile até o Canadá e no Brasil a espécie pode ser encontrada em uma variedade de *habitats* incluindo florestas de baixa altitude, florestas montanas, campos e pântanos (Nielsen et al. 2015) Algumas de suas presas naturais são animais silvestres como capivaras, veados, tatus e emas (Crawshaw & Quigley 2002) ou animais domésticos como os ruminantes e equídeos (Hoogesteijn & Hoogesteijn 2011, Tortato et al. 2015). A onça-pintada (*Panthera onca*) ocorre desde a Argentina até os Estados Unidos, com a maior parte das populações concentradas na Bacia Amazônica (Quigley et al. 2017) devido à forte associação da espécie com a presença de cursos d´água (Jackson & Nowell 1996). Alimenta-se de presas grandes como a capivara, cervos-do-pantanal, jacarés, veados-mateiros e de presas de porte menor como queixadas, porcos selvagens e tamanduás, além de ser um importante predador de animais de interesse pecuário (Campos Neto et al. 2011, Tortato et al. 2015). A espécie necessita de vasto território de vegetação natural para sua sobrevivência (Terborgh et al. 2001, Angelo et al. 2011), isso faz com que a perda populacional seja muito mais acelerada quando comparada com a perda de remanescentes naturais (Quigley et al. 2017).

O acesso humano ao *habitat* (Terborgh et al. 2001) das onças aumenta de acordo com o avanço do desmatamento. Com isso ambas espécies de felinos são perseguidas, seja por retaliação ao abate de criações domésticas ou por motivo cultural, geralmente associado ao medo. Os prejuízos econômicos causados por depredação podem ser equiparados às perdas causadas por doenças de herbívoros domésticos (Hoogesteijn et al. 1993, Palmeira et al. 2008). Portanto, o manejo preventivo da depredação deve ser aplicado conjuntamente a medidas de sanidade animal em cada fazenda, para minimizar danos ambientais e econômicos (Hoogesteijn & Hoogesteijn 2010, 2011). Enfrentamentos entre humanos e predadores selvagens têm sido estudados no âmbito da conservação da biodiversidade (Jackson & Nowell 1996, Conforti & Azevedo 2003, Zimmermann et al. 2005, Santos et al. 2008, Schulz et al. 2014), fornecendo informações importantes para o gerenciamento local de tais conflitos (Pitman et al. 2002, Hoogesteijn & Hoogesteijn 2011, Marchini et al. 2011).

Neste trabalho o conceito **predação** é atribuído ao ataque e consumo de presas naturais ou silvestres (por exemplo cervos, queixadas, antas, capivaras e jacarés) por carnívoros (por exemplo felinos, caninos, ursos, águias). O conceito de **depredação** é o ataque e consumo de animais domésticos (por exemplo ovinos, caprinos, bovinos e equinos) por qualquer espécie de carnívoro. A literatura científica veterinária sobre depredação de onças a criações animais é escassa. Portanto, são objetivos deste trabalho: 1) Listar as causas de mortalidade em um rebanho ovino entre 2005 e 2014 em uma fazenda no Estado de Mato Grosso, Brasil. 2) Relatar e descrever dois estudos de caso de ataque depredatório de *P. concolor* a ovinos em fazendas no Brasil e na Colômbia e 3) Discutir soluções e alternativas sobre ataques de carnívoros silvestres a animais de produção.

MATERIAL E MÉTODOS

Área de estudo 1 em Mato Grosso, Brasil. Propriedade localizada no município de Alto Paraguai, Mato Grosso, Brasil, na bacia do Rio Paraguai, próximo de seus afluentes, Rio Lavrinha e Rio Curupira e da Serra das Araras. O bioma local é o Cerrado, e o uso do solo consiste em pastagens para ovinos e bovinos, com *Brachiaria brizantha* (Poaceae) como forrageira predominante. O rebanho de ovinos da raça Santa Inês era composto por cerca de 60 a 100 animais e o rebanho de bovinos Nelore era de cerca de 30 animais durante o período de estudo (2005-2014). A propriedade possui 25 hectares

de vegetação natural na forma de matas de galeria. No entorno há um assentamento com propriedades de pecuária e agricultura familiar de médio porte. Existem também, nas proximidades, fazendas de grande porte de pecuária extensiva com gado para corte predominante da raça Nelore.

Em sete de fevereiro de 2010 ocorreu um ataque depredatório que resultou em morte de ovinos. Foram encontrados mortos, durante o período da manhã, seis ovinos que estavam alocados em um recinto móvel de 15x15m, com 1,5m de altura, feito com tubos metálicos e tela de arame (Fig.1). Realizou-se necropsia em cinco ovelhas e coleta de amostras de diversos órgãos, que foram fixados em formalina a 10% e processadas rotineiramente para histopatologia no Laboratório de Patologia Veterinária da Universidade Federal de Mato Grosso (UFMT). Em uma das ovelhas submetidas à necropsia, realizou-se maceração das vértebras cervicais. A carcaça de uma ovelha consumida pelo predador foi avaliada macroscopicamente sem manipulação e mantida na posição na qual foi encontrada em solicitação dos proprietários (Fig.3A-C).

Entre os anos 2005 e 2014 realizou-se visitas na mesma propriedade para coleta de dados sanitários do rebanho de ovinos e realização de necropsias para diagnóstico de doenças. Foram também realizadas entrevistas com os proprietários para coleta de dados referentes ao manejo sanitário dos ovinos e a ocorrência de ataques de onça-parda a ovinos. Adicionalmente, realizou-se consulta com patologista e clínicos de animais de produção do Brasil para avaliar qual a situação em outras regiões do país.

Área de estudo 2. Andes Colombianos, Colômbia. Em 2014 a fundação *Panthera* em convênio com a *Corporación Autónoma Regional del Valle del Cauca* (CVC) realizaram um estudo de diagnósticos sobre a depredação nesta região para implementar medidas anti-depredação e avaliar a efetividade dessas medidas. A região possui topografia acidentada e a principal atividade pecuária é a bovinocultura leiteira. Há também uma considerável produção de ovinos para produção de carne e lã. Implementaram-se medidas em oito propriedades; em uma delas se construiu um curral anti-depredação para ovelhas na fazenda *El Silencio* (município de Tuluá, localizado na região central do departamento do Vale do Cauca (N3°55´11.3¨, W75°55´24.9¨), a 2814m de altitude nos Andes Colombianos. Nesta propriedade, o proprietário afirmou que cinco ovelhas foram mortas por depredação por onça-parda nos últimos dois anos e afirmou que não teve perdas de bovinos.

Nas oito fazendas, foram coletadas informações e instalaram-se armadilhas fotográficas. Em casos de depredação eram realizadas a avaliação macroscópica das presas através de exame de necropsia.

RESULTADOS E DISCUSSÃO

Relato de ataque de onça-parda (*Puma concolor*) em Mato Grosso, Brasil

Na manhã do dia sete de fevereiro de 2010, seis ovinos, alocados em um recinto móvel de 225m^2 para rotação de pastagens (Fig.1A), foram encontrados mortos (Fig.1B).

À necropsia dos outros cinco ovinos, mortos pelo predador, observaram-se bom estado nutricional e peso estimado de 30-35kg. Nos cinco havia perfurações cervicais dorsal (n=2), ventral (n=2) e dorso lateral (n=1), em média estimada de 4cm entre as perfurações (Fig.2A-C) associada aos dentes caninos à do felino, variaram entre as regiões. A maceração das vértebras cervicais revelou perfurações vertebrais (Fig.2D). Notou-se também incisões na pele causadas pelas garras (Fig.2E). Ao exame interno havia equimoses e sufusões no tecido subcutâneo e músculos esqueléticos (Fig.1F) e moderada quantidade de espuma na traqueia. Nas cinco ovelhas, nenhum tecido foi consumido pela onça-parda.

Uma das ovelhas mortas no recinto estava em decúbito lateral esquerdo e carcaça parcialmente consumida pelo predador (Fig.3A). A cabeça e o pescoço estavam íntegros. O esterno, o gradil costal direito, o coração e pulmões e parte do gradil costal esquerdo estavam ausentes (Fig.3B). A cavidade abdominal estava aberta com ausência dos músculos da parede abdominal. No interior da carcaça observou-se apenas rúmen, retículo, omaso e abomaso, os quais estavam dilatados com timpanismo pós-mortal e o retículo se projetava para a cavidade torácica. Não foram encontradas na carcaça as demais vísceras das cavidades abdominal e pélvica, bem como, também estavam ausentes os músculos do membro posterior direito (Fig.3C). No dia do ataque, somente a carcaça deste ovino foi parcialmente consumida pela onça-parda.

No dia seguinte a onça-parda retornou ao local da carcaça e foi abatida por caçadores contratados para tal fim. Os autores são expressamente contrários ao abate de carnívoros silvestres e somente reportam o fato, afim de manter a descrição dos eventos de maneira imparcial. A Lei nº 5.197, de 3 de janeiro de 1967 dispõe sobre a proteção à fauna e dá outras providências. O Artigo 1º protege a fauna silvestre, sendo proibida a sua utilização, perseguição, destruição, caça ou apanha (Brasil 1967).

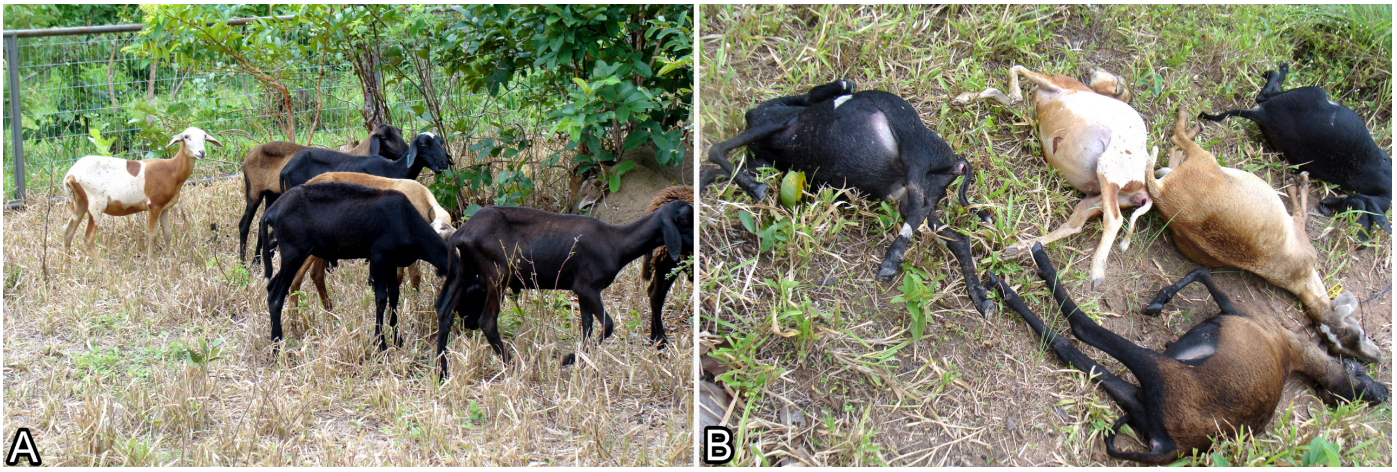


Fig.1. (**A**) Ovinos mantidos em recinto antes do ataque de *Puma concolor*, janeiro de 2010 (**B**) Carcaças de cinco ovelhas após ataque depredatório de *Puma concolor* a ovinos. Alto Paraguai, MT, fevereiro de 2010.

Fig.2. (**A**-**C**) Feridas perfurantes na região cervical atlanto-axial dorsal. (**D**) Perfuração de vértebras cervicais em ovino atacado por *Puma concolor*. fevereiro de 2010. (**E**) Feridas perfurantes na região cervical atlanto-axial dorsolateral e hemorragia. (**F**) Músculos cervicais com sufusão da região submandibular à região cervical profunda.

Outros casos de depredação, dos quais não foi possível de ser realizadas necropsias, o diagnóstico foi baseado nos sinais característicos deixados nas presas (Hoogesteijn et al. 1993). Os diagnósticos realizados nesta mesma propriedade entre 2005 e 2014 estão apresentados no Quadro 1 e os casos de depredação representaram 46% das mortes no período estudado. As principais doenças diagnosticadas nesta propriedade foram a calcinose enzoótica (Guedes et al. 2011) e a pitiose rinofacial (Ubiali et al. 2013).

Relato de ataque de onça-parda (*Puma concolor*) na Colômbia, nos Andes Colombianos

Através das armadilhas fotográficas, a depredatório de onça-parda foi registrada na região estudada e um evento predatório de uma ovelha foi confirmado na fazenda *El Silencio* (Fig.4A-C). Da mesma forma a necropsia foi executada no indivíduo predado e se confirmou a depredação com a evidenciação de extensas hemorragias na musculatura e tecido subcutâneo da região cervical da ovelha, assim como feridas

Fig.3. (**A**) Carcaça de ovelha parcialmente consumida por *Puma concolor*. Alto Paraguai, MT. Fevereiro de 2010. (**B**) Membro posterior direito com exposição do fêmur e da tíbia e ausência dos músculos esqueléticos. (**C**) No tórax observa-se ausência dos pulmões e coração, porções distais das costelas, músculos intercostais, cartilagens costais, esterno. Note o retículo mantido intocado na cavidade torácica.

Fig.4. Depredação de ovinos por onça-parda, tomadas com armadilhas fotográficas pela equipe da *Panthera* Colômbia nos Andes Colombianos, em 3.000m de altitude. (**A**) onça-parda se deslocando em direção ao cercado dos carneiros (**B**) onça-parda com uma ovelha mordida na garganta (**C**) onça-parda regressou ao local do ataque depredatório para consumir a carcaça da ovelha.

Quadro 1. Diagnósticos clínico-patológicos em ovinos em comparação com depredação

Diagnóstico	Mortes (nº)	Período
Depredação	41[a]	2005-2014
Calcinose enzoótica	30[b]	2005-2010
Pitiose rinofacial	9[c]	2006-2012
Aborto por causa indeterminada	2	2009
Broncopneumonia purulenta	1	2009
Empiema basilar	1	2009
Intoxicação por Brachiaria brizantha	1	2009
Osteomielite abscedativa e compressão de medula espinhal	1	2009
Doença renal policística	1	2010
Urolitíase obstrutiva	1	2010
Linfadenite caseosa	1	2011

[a] Seis casos com diagnóstico pela necropsia e em 35 o diagnóstico foi baseado no histórico de sinais característicos deixados nas presas conforme Hoogesteijn et al. (1993). [b] Guedes et al. (2011). [c] Ubiali et al. (2013).

circulares de pele compatíveis com mordedura do felino. Nesta ovelha predada foi constatado que o felino abriu a cavidade abdominal e consumiu as vísceras, assim como consumiu o músculo esquelético do coxal e membros posteriores.

Devido aos resultados e à análise da fazenda, foi recomendado implementar um curral para abrigar as ovelhas à noite com cercas elétricas de estrutura anti-depredação para protegê-las durante os horários de maior atividade desses grandes felinos. O curral foi construído perto da casa e, durante dois anos após a sua implementação, não tiveram problemas de depredação, com a excecão de um evento noturno de depredação, por falha humana, pois as ovelhas não foram fechadas no curral durante a noite. Infelizmente depois de dois anos, houve mudanças na gestão da fazenda, e decidiram abandonar o manejo do curral anti-depredação com cerca elétrica das ovelhas para protegê-las durante a noite no curral. Após essas mudanças na fazenda ocorreram três eventos de depredação durante um período de três meses; a onça-parda predou a totalidade das ovelhas desta fazenda.

Embora o abandono do projeto não tenha sido positivo, a gestão do curral anti-depredação realizada durante dois anos, a confirmação por armadilhas fotográficas, a necropsia realizada e os eventos depredatório registrados após descontinuar a medida anti-depredação, confirmaram

a eficiência dessa estratégia, para minimizar as perdas causadas pela depredação de ovinos. Portanto essa medida pode ser implantada em algumas fazendas de pecuária com intuito de evitar o conflito e a consequente caça de grandes felinos por retaliação.

Levantamento da situação junto a patologistas e clínicos no Brasil

Realizamos consultas junto aos patologistas ou clínicos de animais de produção, Professor de Clínica de Ruminantes, Carlos E.P. Santos, da Universidade Federal do Mato Grosso (UFMT), Professor de Patologia Veterinária Ricardo A.A. Lemos, da Universidade Federal do Mato Grosso do Sul (UFMS), Professor de Patologia Veterinária, Luciano da Anunciação Pimentel, da Universidade Federal do Recôncavo da Bahia (UFRB), o Professor de Patologia Veterinária David Driemeier da Universidade Federal do Rio Grande do Sul (UFRGS) e a Professora Josi Seixas da Universidade Federal de Lavras (UFLA) com o objetivo de obter informações adicionais a respeito de casos de depredação a animais domésticos.

O Professor Carlos Santos afirmou ter registros visuais de felinos selvagens que tendem a atacar e matar sem consumo da presa. Foi relatado ataques por onça-parda no Pantanal mato-grossense na região do Pirizal, município de Nossa Senhora do Livramento/MT. Na ocasião um bezerro foi atacado, outros ataques também ocorreram, mas na zona rural do município de Poconé/MT, onde um potro foi atacado por uma onça-parda. O potro não morreu porque a mãe o defendeu com vigorosos coices, embora tenha sido registrado marcas de garra no dorso da égua e dias depois houve formação de tecido de granulação nas lesões de pele. Na mesma região houve um outro ataque por onça pintada à 15 bezerros. Em Apiacás/MT dois potros foram atacados por onça-parda e pintada. A carcaça de um dos potros foi parcialmente recoberta por folhas e o esterno foi consumido, mais tarde o informaram que na primeira ocasião a onça-pintada parida foi a responsável pelo ataque e na segunda a onça-parda. Em outras regiões do estado de MT, também há relatos de ataques por cães domésticos a ovinos nas cidades de Santo Antônio do Leverger (duas ovelhas), Cuiabá/MT, distrito de Bandeira (quatro ovelhas) e Nossa Senhora da Guia (cinco ovelhas). Além dos casos citados, o professor Carlos confirmou ter tido acesso a outros históricos de depredação de animais domésticos, com maior incidência na região norte do estado, mais especificamente Sinop, Alta Floresta e Apiacás (Santos 2017).

O Professor Ricardo Lemos também verificou históricos de depredação por cães domésticos. Dois casos a ovinos, ambos no rebanho da faculdade, no Hospital Veterinário da UFMS; apesar dos prejuízos importantes, ele entende que são casos esporádicos na rotina de diagnóstico. Outro caso foi relatado em bovinos de uma propriedade rural do município de Campo Grande/MS. O professor informou não haver históricos de depredação por grandes felinos (onças), embora veterinários de campo e produtores já tenham relatado esse tipo de ocorrência (Lemos 2017).

O Professor Luciano Pimentel confirmou um histórico de ataques de cães domésticos a ovelhas em Cruz das Almas no Recôncavo da Bahia, através da necropsia, o professor considera haver uma média anual de dois casos de depredação à animais domésticos (Pimentel 2017).

O Professor David Driemeier também verificou no Setor de Patologia Veterinária da UFRGS através de históricos clínicos e necropsias que houve casos depredação por cães a cabras e ovelhas (Driemeier 2017).

A professora Josi Seixas afirmou que no campus da universidade, em Lavras/MG, especialmente nos setores de ovinocultura e caprinocultura há relatos de casos esporádicos de ataques de cães às criações, que por vezes são animais em experimentação. Em um destes episódios foram encaminhados ao Setor de Patologia Veterinária nove ovinos para necropsia. As principais lesões foram perfurações puntiformes e lacerações de pele, associadas a hemorragia e edema subcutâneo e muscular. As lesões mais graves na região cervical, lombar, inguinal ou perianal. Em alguns casos, além das lacerações musculares houve também perfurações de órgão como traqueia. E embora não seja um problema frequente há de considerar o impacto que estes casos podem representar para os proprietários envolvidos. Além de insegurança e perdas econômicas, neste caso houve ainda grande transtorno para os pesquisadores (Seixas 2017).

Condições associadas aos ataques

Uma crendice que dificulta muito a conservação de grandes felinos selvagens na América Latina, especialmente enraizada em populações rurais, é que eles são perigosos e atacam seres humanos. Embora inúmeros casos, na África e na Ásia, de grandes felinos do gênero *Panthera* (leão, tigre e leopardo) predarem humanos, casos de ataques de onças a humanos são raros (Campos Neto et al. 2011, Hoogesteijn et al. 2016d), pois os grandes felinos americanos evitam confrontos e os humanos não estão incluídos na dieta de onças pintadas ou pardas (Hoogesteijn et al. 2016d). Campos Neto et al. (2011) relataram três casos de ataque de onça pintada a humanos no estado de Mato Grosso, nos quais dois pacientes sobreviveram aos ataques e um morreu. A imensa maioria dos ataques de felinos americanos se deve ao fato de estarem se defendendo, feridos ou encurralados em situações de caça. Os grandes felinos estão no continente americano há cerca de 150-200.000 anos, mas aparentemente a escolha de fuga à predação de humanos se deve a provável chegada e dispersão de humanos no continente (há 20-30.000 anos) já caçadores hábeis, armados e capazes de se defender. Mais detalhes são descritos na revisão de Hoogesteijn et al. (2016d).

O desequilíbrio no ecossistema pode ser considerado um dos principais fatores relacionados aos casos de depredação por carnívoros selvagens sobre criações de animais domésticos. Áreas de pastagens localizadas próximas a matas representam locais onde a depredação é esperada (Pitman et al. 2002). Essa condição foi constatada nas propriedades estudadas no Brasil e na Colômbia.

Os ataques depredatório podem causar mortes isoladas de grandes animais ou mortes de grupos de pequenos ruminantes como no presente relato. O fato de a onça ter consumido apenas uma carcaça dos seis ovinos abatidos no caso em Mato Grosso, Brasil, mostra o comportamento oportunista do predador devido ao porte e à vulnerabilidade das presas confinadas em um recinto de 225m^2. Tal comportamento tem como base o instinto de sobrevivência do predador, também pode ser atribuído à intenção de consumo da presa posteriormente ao ataque, às supostas exigências nutricionais da família do predador ou pela influência da perseguição de

outros carnívoros oportunistas (Elbroch et al. 2014). O retorno à presa é um comportamento característico da onça-parda. Além disso, o felino predador pode alimentar-se do animal abatido por até quatro dias (Schaller & Crawshaw 1980).

Pumas matam com mordidas na região cervical dorsal causando fraturas vertebrais ou por sufocamento através de mordida e compressão traqueal (Verdade & Campos 2004). No ataque predatórios deste relato, a *causa mortis* de cinco ovelhas as duas situações foram observadas; observaram-se lesões em vértebras cervicais e sufusão em região ventro-cervical. Marchini et al. (2011) pontuaram que nas presas mortas por onças-pardas há lesões de pele perfurantes causadas pelos dentes na região cervical e lesões incisas causadas por marcas aleatórias de garras (Marchini et al. 2011), essas lesões foram observadas nos cinco ovinos submetidos à necropsia no presente estudo; assim através de exame de necropsia, foi possível observar que essas lesões de pele estavam associadas a hemorragias subcutâneas e musculares. Ressalta-se a importância de realizar a necropsia para confirmar os casos de depredação ou diagnosticar outras doenças (Wobeser 1996, Peixoto & Barros 1998).

Diagnósticos diferenciais

Durante exame *post-mortem* de animais com suspeita de depredação é importante avaliar possíveis fragilidades da presa, como doenças do aparelho locomotor ou desnutrição, assim como a possibilidade de morte por outras causas com posterior consumo da carcaça pelo predador (Hoogesteijn et al. 1993). Ações predatórias por urubus (*Coragyps atratus*) também podem ocasionar perdas significativas em bezerros recém-nascidos ao bicar o umbigo e os olhos, caso as vacas não os protejam efetivamente (Hoogesteijn & Hoogesteijn 2011). Aves de rapina em geral podem lacerar parcialmente carcaças de animais mortos por causas diversas, no entanto as aves consomem apenas tecidos moles, enquanto na depredação por mamíferos há evidências de consumo de ossos.

O caso de depredação em Mato Grosso, ocorreu provavelmente devido à fragilidade dos ovinos, especialmente por se tratar de um ambiente cercado próximo à mata (Fig.1). Os bovinos de cria geralmente não são predados por onça-parda (Verdade & Campos 2004, Zimmermann et al. 2005), esse fato foi também constatado nesta propriedade. Estudos de causas de mortes em fazendas de cria de bovinos na Venezuela revelaram perdas de bezerros por diversas causas incluindo flebite e poliartrite, raiva, carbúnculo sintomático, desnutrição, afogamentos, acidentes de manejo ou transporte, acidentes ofídicos e roubo. Nessas fazendas, as mortes anuais de bezerros atribuídas à depredação variaram entre 6-30% e o diagnóstico foi baseado nos sinais característicos apresentados pelas presas (Hoogesteijn et al. 1993). Tortato et al. (2015) avaliando as perdas anuais no rebanho em uma fazenda no Pantanal relatam uma perda máxima de 2.8% do rebanho bovino provocadas por ataques de onças-pintadas, principalmente bezerros ou garrotes com peso entre 26kg e 360kg.

Os cães domésticos (*Canis familiaris*) também podem atacar ovinos, caprinos, bezerros ou potros; grande parte desses ataques geralmente é atribuída a predadores selvagens (Pitman et al. 2002). As matilhas que realizam esses ataques não necessariamente precisam ser asselvajadas (Hoogesteijn & Hoogesteijn 2011). Na Universidade Federal Rural do Rio de Janeiro (UFRRJ) diversos ataques de cães a ovinos e caprinos entre os anos de 2004 e 2010 foram observados (Tokarnia 2015). No município de Quatis, RJ, eventos depredatório de diversos cães domésticos a bezerros em fazendas de cria de vacas Nelore causaram sérios prejuízos econômicos (Galvão 2015). Os cães normalmente causam feridas na presa na região cervical, escapular e nas orelhas (Verdade & Campos 2004). Ao contrário das onças-pardas, os cães atacam também a região posterior das ovelhas, com evidências de mordidas antes da morte das presas. Por ser uma espécie doméstica, os cães não são eficientes durante o ataque. Muitas vezes a presa não morre pelo ataque dos cães e nos casos de morte, geralmente pouca carne é consumida ou a carcaça pode não ser consumida pelos cães (Pitman et al. 2002, Verdade & Campos 2004). Os ataques depredatório causam sinais macroscópicos característicos nas presas e remetem a achados epidemiológicos e situações peculiares, nas quais as demais causas de morte são facilmente descartadas e podem ser apenas remotamente consideradas como diagnóstico diferencial.

ESTRATÉGIAS PARA DIMINUIR A DEPREDAÇÃO DE ANIMAIS DOMÉSTICOS

Descrevem-se os principais métodos de manejo *in loco* que podem ser utilizados por criadores de animais com objetivo de minimizar ataques depredatório, considerando-se também aspectos produtivos (Jackson & Nowell 1996, Pitman et al. 2002, Hoogesteijn & Hoogesteijn 2007, 2008, 2010, 2011, Hoogesteijn et al. 2016b, Cavalcanti et al. 2011, Marchini et al. 2011). Separaram-se as técnicas em preventivas e mitigatórias, sendo as preventivas importantes para propriedades rurais com criação de animais de uma forma geral e as mitigatórias mais importantes para propriedades que já tenham histórico de predação por animais silvestres.

Não existe uma técnica totalmente eficaz, assim se recomenda utilizar uma combinação de métodos, que variam de acordo com a região e as condições socioeconômicas e ambientais de cada propriedade rural. Nota-se que é importante priorizar sempre as técnicas preventivas e as mitigatórias adicionalmente, caso tão somente as preventivas não sejam suficientes para reduzir de forma significativa a depredação. Vale ressaltar que os felinos predadores têm grande capacidade de adaptação, podendo se habituar a algumas das técnicas abaixo mencionadas. Portanto, as recomendações têm como objetivo minimizar os ataques depredatório, o que não significa, necessariamente, que os mesmos deixarão de ocorrer.

Medidas preventivas

Conservação da vegetação e da fauna nativa e corredores de vegetação. O desmatamento total de uma fazenda, ao contrário do que alguns produtores rurais acreditam, não soluciona o problema de depredação atribuído aos felinos silvestres. Ao contrário, o ato de não conservar áreas de vegetação nativa como reservas legais e áreas de preservação permanente (APPs) pode aumentar o problema de depredação em fazendas devido à diminuição das populações de presas silvestres (Marchini et al. 2011), que são preferidas por onças como capivara (*Hydrochoerus hydrochaeris*), cateto (*Pecari tajacu*), queixada (*Tayassu pecari*), anta (*Tapirus terrestris*) e jacarés (Família Alligatoridae), além de outros animais menores como tatus, iguanas, tejus e pequenos roedores(Quigley et al. 2017, Nielsen et al. 2015) o que indica a importância da

conservação de habitats naturais e da prevenção da caça desses animais também para a minimização de ataques a animais domésticos (Hoogesteijn & Hoogesteijn 2011).

Evitar áreas de risco. Os sistemas de pastejo rotacionado que incluem uma "praça" ou área central equipada com bebedouros e cochos de sal mineral ou suplementos podem gerar ótimos resultados produtivos, comparando-se com sistemas de pastejo extensivo. A área central deve ser alocada em áreas distantes de florestas para evitar a concentração dos animais domésticos em locais onde os predadores possam atacá-los com facilidade; ou deve ser rodeada de cercas elétricas com desenho anti-depredação).

Devido à presença de área central, o contato do rebanho com os responsáveis pelo manejo dos animais é frequente. Dessa maneira, torna-se possível quantificar a mortalidade com maior precisão e investigar as causas de morte no rebanho. Esse tipo de sistema pode ser circundado por cercas elétricas para repelir os felinos (Castaño-Uribe et al. 2016). Em áreas inundáveis, o deslocamento dos animais para áreas altas durante a época de cheias evita que os rebanhos fiquem isolados e enfraquecidos pelas inundações, o que os torna mais frágeis e suscetíveis à depredação (Hoogesteijn & Hoogesteijn 2011).

Programa sanitário. Grande parte das perdas em propriedades pecuárias acontece em decorrência da falta de planejamento. As boas práticas de manejo incluem a identificação individual dos animais, o registro de entrada e saída, assim como as anotações de ocorrência de eventos ou morte de animais. As causas das mortes de animais de produção devem ser investigadas a fim de se programar medidas de controle e profilaxia (Cruz et al. 2011, Hoogesteijn & Hoogesteijn 2014). Sabe-se que na bovinocultura a mortalidade por doenças causadas pelo consumo de plantas tóxicas, botulismo e raiva são os principais problemas sanitários (Tokarnia et al. 2012).

Nos principais levantamentos de causas de morte em ovinos (Rissi et al. 2010, Almeida et al. 2013), caprinos (Rosa et al. 2013, Bassuino et al. 2018), bovinos (Lucena et al. 2010, Rondelli et al. 2017) e equinos (Pierezan et al. 2009, Marcolongo-Pereira et al. 2014) no Brasil; nenhum caso de depredação foi registrado.

No que se refere aos rebanhos de cria de bovinos, há grande queixa de morte de bezerros por ataque de onças. No entanto se avalia que as maiores perdas em fazendas de bovinos de cria são decorrentes de doenças, como as que causam aborto. O diagnóstico das causas de aborto deve ser baseado na coleta e envio do feto e placenta para equipe de patologistas para realizar o exame de necropsia e complementares (Antoniassi et al. 2013). Esses diagnósticos, juntamente com os dados sistematizados do programa sanitário, permitem que o pecuarista controle seu rebanho de acordo com as reais causas de perdas e faça esforços profiláticos significativos priorizando as principais causas de perdas econômicas.

Tipo de produção pecuária. Bovinos acima de um ano de idade são presas menos frequentes, portanto, a recria e a engorda são opções produtivas com menor risco de perdas por depredação que a cria, por evitarem a presença de bezerros (Hoogesteijn & Hoogesteijn 2011).

Uso estratégico de cercas. Sempre que possível deve-se cercar áreas de mata ou floresta para impedir a entrada do gado, que pode se tornar uma presa vulnerável nessas áreas (Hoogesteijn & Hoogesteijn 2011, 2014). Cercas elétricas (especialmente desenhadas para evitar a entrada dos felinos e a saída das criações domésticas) podem diminuir a depredação de animais domésticos, podendo ter alta eficácia em algumas situações (Hoogesteijn & Hoogesteijn 2014, Hoogesteijn et al. 2016b). Para que evitem a passagem de felinos, as cercas elétricas devem ser utilizadas em áreas pequenas como currais de fechamento noturno, piquetes pequenos, piquetes maternidade ou áreas com histórico de depredação. Deve-se considerar que, assim como os animais de criação, os animais silvestres aprendem a evitar cercas elétricas. Contudo, os felinos têm maior capacidade de adaptação para invadir as áreas cercadas, o que ressalta a necessidade de considerar um profissional capacitado para realizar o planejamento e a instalação das cercas elétricas (Cavalcanti et al. 2011), e que sejam especialmente desenhadas com essa finalidade (Valderrama-Vásquez et al. 2016, Hoogesteijn et al. 2016b). Observe na Figura 5 o diagrama de cerca elétrica.

Estação de monta e pastos de maternidade. O estabelecimento de estação de monta de curta duração

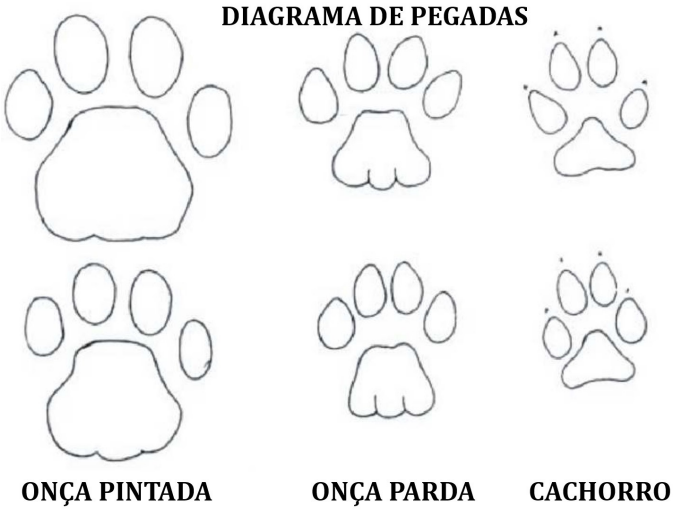
Fig.5. (**A**) Curral anti-depredação com cerca elétrica, como uma amostra do que pode ser feito de forma efetiva para controlar a depredação. *Vereda Santa Lucia, Municipio Tuluá, Cordillera Central, Valle del Cauca*, *Colombia*. (**B**) Detalhe da cerca em funcionamento, com oito fios eletrificados e as ovelhas no interior do curral anti-depredação.

em bovinos (2 a 4 meses), além de melhorar a eficiência dos resultados pecuários em animais de cria, permite que os piquetes maternidade sejam alocados em áreas com pouco ou nenhum problema de depredação e em curto período de controle mais intensivo (Hoogesteijn & Hoogesteijn 2011). Adicionalmente, é importante manter vacas paridas e seus bezerros até os 3 meses de idade em pastos limpos e longe de áreas de florestas, em locais que possam ser supervisionados frequentemente (Pitman et al. 2002, Hoogesteijn & Hoogesteijn 2011). Experiências da Costa Rica demostram que em áreas com depredação só por Onças-Pardas, a depredação pode ser controlada, colocando sinos nos pescoços de um mínimo de 25% dos bezerros (Corrales-Gutiérrez et al. 2016a).

Currais de confinamento noturno. O fechamento de animais durante a noite e perto de habitações humanas, pode ser aplicado, principalmente em pequenas propriedades. Essa medida se torna mais eficiente aliada ao uso de iluminação, cães de guarda e cercas elétricas. O manejo se intensifica e os animais se habituam a essa prática, que pode ser associada com o fornecimento de sal mineral ou suplementação proteico-energética. Contudo essa prática deve ser bem planejada, com utilização de instalações protegidas contra os predadores, para estes não consigam invadir os currais e matar diversos animais em um só ataque (Pitman et al. 2002, Hoogesteijn & Hoogesteijn 2011, 2014).

Medidas mitigatórias

Reconhecer o predador. Devem-se identificar os sinais de animais predados por felinos e diferenciar dos ataques por cães ou outros predadores. Tanto os felinos quanto os cães deixam certos rastros característicos como pegadas, fezes e pelos, que podem fornecer informações sobre a espécie (Fig.6) (Hoogesteijn & Hoogesteijn 2011). A necropsia pode ser um método auxiliar na detecção do predador, através de lesões em regiões anatômicas que mais frequentemente os predadores atacam (Wobeser 1996, Peixoto & Barros 1998, Pitman et al. 2002).



Hoogesteijn & Hoogesteijn (2011) modificado de Shaw 1993, Aranda 1994

Fig.6. Diagrama de pegadas de onça pintada, onça-parda e cão doméstico.

Eliminação de carcaças. Carcaças de animais mortos por diversas causas devem ser eliminadas para evitar que felinos se aproximem para se alimentar e possivelmente possam predar outros animais da mesma fazenda (Hoogesteijn et al. 1993). Tal medida é recomendada adicionalmente, como profilaxia para o botulismo (Curci et al. 2007).

Cães pastores. O uso de cães pastores foi muito eficaz na prevenção de problemas de depredação de ovinos e caprinos por onça-parda. No entanto os resultados não foram satisfatórios na prevenção de ataques por onça-pintada em rebanhos de bovinos mantidos em sistema extensivo (Hoogesteijn & Hoogesteijn 2011). A manutenção de cães para dar alarme é aconselhada quando utilizadas raças de grande porte e em grande número. É aconselhado o uso de pelo menos cinco cães que devem ficar contidos numa área próxima à possível área de acesso dos predadores (Pitman et al. 2002).

Animais defensivos. Os bovinos têm comportamento gregário, ou seja, vivem em grupos e há uma hierarquia social, na qual bovinos dominantes geralmente são machos com idade avançada. A presença de alguns bovinos experientes, preferencialmente com chifres, ensina ao grupo de animais jovens como se defender de ataques depredatório. Pode-se, também, utilizar sino em alguns bovinos com a intenção de usar o som para afastar os felinos (Pitman et al. 2002, Hoogesteijn & Hoogesteijn 2011).

Búfalos e raças bovinas nativas. Os bubalinos (*Bubalus bubalis*) de uma maneira geral, diferentemente de algumas raças bovinas, apresentam comportamento defensivo contra predadores. Os bovinos taurinos e zebuínos foram domesticados há pelo menos 7.000 anos, quase que na ausência de predadores, enquanto búfalos selvagens e tigres asiáticos (*Panthera tigris*) coexistiram por um longo período de tempo, o que acarretou no comportamento defensivo das manadas de búfalos (Hoogesteijn & Hoogesteijn 2007, 2008). As vacas búfalo, quando ameaçadas por um predador, se comportam em marcha ao redor de seu bezerro e o touro búfalo marcha em direção ao predador, impedindo muitas vezes o ataque do predador. A mortalidade por depredação em rebanhos pecuários pode ser reduzida mantendo-se búfalos mansos e bem manejados junto com bovinos nos mesmos pastos ou utilizando-se apenas búfalos. Normalmente ambas as espécies têm comportamento agregado. No entanto, em piquetes menores que 100ha, búfalos e bovinos dividem espaços próximos e, nessas situações, búfalos adultos podem proteger bezerros bovinos (Hoogesteijn & Hoogesteijn 2007, 2008, Hoogesteijn et al. 2016c). Em fazendas com introdução recente de Búfalos mansos, e convenientes fecharem eles na noite junto com o gado da fazenda no mesmo curral, durantes uma a duas semanas para eles se acostumarem a ficar juntos (Corrales-Gutiérrez et al. 2016a, 2016b).

Na maioria dos casos de ataque, vacas da raça Nelore (*Bos indicus*) se afugentam com a presença do predador, gerando tumulto e tornando o bezerro uma presa fácil. Outras raças crioulas (*Bos taurus*), introduzidas nas Américas há 300-400 anos, se adaptaram ao clima e apresenta comportamento mais defensivo contra predadores, semelhante ao dos búfalos. A raça Pantaneira é um exemplo de gado crioulo, rústico, com boa fertilidade, resistente às enchentes e com capacidade de consumir gramíneas debaixo da água, no entanto com desempenho produtivo baixo, em comparação com outras raças taurinas. Igualmente a raça Sanmartinero

na Colômbia que tem dado um bom resultado em provas anti-depredação (Hoogesteijn et al. 2016a). Assim, alguns pecuaristas optam por manter bovinos de raças crioulas com bovinos da raça Nelore para promover proteção contra a depredação. Muitos pecuaristas não desejam touros crioulos como estratégia anti-depredação já que não engajam com os programas genéticos atuais da pecuária, pois os bezerros mestiços são menos valorizados, em comparação com raças como Nelore ou Angus. Uma das soluções anti-depredação que pode resolver o problema da genética é operar esses touros crioulos (Pantaneiros ou Caracus) como detectores de cio nas vacas (rufião), e preferentemente mochos ou com as pontas dos chifres reduzidas para evitar ferimentos aos touros reprodutores. Com isso tem-se a vantagem da defesa do touro crioulo no rebanho, sem problemas de desvalorização de bezerros (Pitman et al. 2002, Hoogesteijn & Hoogesteijn 2011, Hoogesteijn et al. 2016a).

Repelentes físicos e químicos. Técnicas não letais que envolvem tecnologia são objetos de estudo, como exemplos existem os pastores eletrônicos, os detectores de movimento, disparos e colar sônico que servem como alternativas para repelir os predadores. O método químico consiste no uso de colares com substâncias irritantes aos predadores. O cloreto de lítio, por exemplo, é usado nos colares para provocar uma aversão condicionada no predador. Assim, ao morder o pescoço da presa, o predador terá liberado em sua boca o produto químico (Pitman et al. 2002, Hoogesteijn & Hoogesteijn 2011).

CONSIDERAÇÕES FINAIS

No contexto pecuário da América Latina, ainda não existe um sistema de compensação econômica por perdas causadas pela depredação. A opção adotada por muitos criadores (retaliação através do extermínio de carnívoros silvestres) não soluciona o problema e tal ação constitui um crime ambiental. Mostraram-se, nesse artigo, medidas preventivas e mitigatórias da depredação para que profissionais e criadores possam lidar *in loco* da melhor forma possível com esse problema comum do contexto pecuário latino americano.

Os ataques de felinos silvestres a animais domésticos ocorrem em situações peculiares e causam sérios prejuízos, no entanto existe uma tendência da supervalorização deste problema no meio rural. Entendemos que há necessidade de conhecer quais são as reais causas de perdas econômicas em rebanhos de pecuária na América Latina, para que as ações de manejo sejam focadas para os principais problemas de cada região.

Nota-se que há necessidade de se testar cientificamente a efetividade de métodos para prevenir e mitigar a depredação de carnívoros predadores a animais de criação. As técnicas e métodos aqui apresentados devem, também, ser adaptados para a realidade de cada fazenda. Por fim, para que se atinjam níveis de sustentabilidade em uma escala maior, ressalta-se a importância que os órgãos governamentais e legisladores considerarem essas questões e se associam a pesquisadores e associações de pecuaristas, a fim de traçar estratégias efetivas em nível de paisagem para minimizar danos ambientais, econômicos e sociais relacionados à depredação de animais de produção por carnívoros silvestres.

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Original Article
Morfofisiologia/*Morphophysiology*

Evaluation of the applicability of musculoskeletal ultrasonography of the thoracolumbar and lumbar spine segment of healthy dogs[1]

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ABSTRACT.- Lopes E.R., Bellegard G.M.C., Cury F.S., Abreu F.A.S., Ambrósio C.E., Carregaro A.B. & Hage M.C.F.N.S. 2018. **Evaluation of the applicability of musculoskeletal ultrasonography of the thoracolumbar and lumbar spine segment of healthy dogs.** *Pesquisa Veterinária Brasileira 38(12):*2278-2283. Setor de Diagnóstico por Imagem, Faculdade de Zootecnia e Engenharia de Alimentos, Universidade de São Paulo, Av. Duque de Caxias Norte 225, Zona Rural, Pirassununga, SP 13635-900, Brazil. E-mail: crishage@usp.br

Changes in the spine of dogs are usually detected in clinical and in surgical practice. Few studies exist on musculoskeletal ultrasound anatomy of the thoracolumbar and lumbar segments of the normal spine of dogs. This study aimed to compare the normal musculoskeletal ultrasound anatomy of the T10-S1 vertebral segments with images obtained with magnetic resonance imaging (MRI), computed tomography (CT), and anatomical structures, and to establish the ability to identify structures using these modalities. Ultrasound scans allowed visualization of the muscles of the region, articular processes, spinous process, interspinous ligament, and yellow ligament in the lumbosacral window. Computed tomography images provided better bone details, compared to ultrasound images. Low-field MRI allowed the identification of the same structures identified with ultrasound imaging, and allowed the identification of cerebrospinal fluid, transverse processes, and provided improved detail of the intervertebral discs and spinal cord. Knowledge of ultrasound anatomy of the region may allow the the identification of muscle and ligament injuries. Thus, in cities where CT and MRI are inaccessible, ultrasonography of the region could be a good alternative to identify possible changes not observable with radiographic examination or to complement radiographic examination.

INDEX TERMS: Musculoskeletal ultrasonography, thoracolumbar, lumbar spine, healthy dogs, skeleton, muscle, vertebra.

RESUMO.- [**Avaliação da aplicabilidade da ultrassonografia musculoesquelética do segmento toracolombar e lombar da coluna vertebral de cães hígidos.**] Alterações na coluna vertebral de cães são comumente encontradas na rotina clínica e cirúrgica veterinária. Existem poucos estudos sobre a anatomia ultrassonográfica musculoesquelética do segmento toracolombar e lombar da coluna vertebral normal de cães. O objetivo deste trabalho foi comparar a anatomia ultrassonográfica musculoesquelética normal dos segmentos vertebrais T10-S1 com imagens obtidas pela ressonância magnética, tomografia computadorizada e peças anatômicas visando demonstrar a sua capacidade de identificação de estruturas. A varredura ultrassonográfica permitiu a visibilização da musculatura da região, processos articulares, processos espinhosos, ligamentos interespinhosos e ligamento amarelo na janela lombossacra. A tomografia computadorizada forneceu imagens com melhor detalhamento ósseo quando comparada ao exame ultrassonográfico. A ressonância magnética de baixo campo permitiu a identificação das mesmas estruturas

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que o exame ultrassonográfico acrescido da identificação do líquido cerebroespinal, processos transversos e melhor detalhamento dos discos intervertebrais e medula espinhal. Com o conhecimento da anatomia ultrassonográfica da região, acredita-se que lesões musculares e ligamentares possam ser identificadas. Vale salientar que em cidades onde a tomografia computadorizada e a ressonância magnética não estejam acessíveis a ultrassonografia da região pode ser uma boa alternativa para identificar possíveis alterações não visibilizadas ao exame radiográfico, ou complementá-lo.

TERMOS DE INDEXAÇÃO: Ultrassonografia musculoesquelética, toracolombar, coluna lombar, cães sadios, esqueleto, musculatura, ultrassom, vértebra.

INTRODUCTION

Several imaging techniques are used to assess the canine spine. The most common techniques include simple radiography, myelography, computed tomography (CT), and magnetic resonance imaging (MRI) (Emery et al. 2018, Noyes et al. 2017).

Ultrasonography is a widely available and low-cost imaging diagnostic technique, compared to CT and MRI. The combination of high-definition ultrasound equipment and trained professionals can provide similar information or additional information as that obtained through MRI of the vertebral musculature (Samii & Long 2005).

The literature contains extensive descriptions of the use of ultrasonography on equine limbs (Dowling et al. 2000) and on the spinal column (Fonseca et al. 2006, Vandeweerd et al. 2007, Fuglbjerg et al. 2010) to aid the collection of cerebrospinal fluid from the cisterna magna and to evaluate the lumbosacral space (Aleman et al. 2007). In small animals, the use of ultrasonography to assess the musculoskeletal system has been growing (Samii & Long 2005). There are reports on its use to evaluate the shoulder (Long & Nyland 1999), long bones (Risselada et al. 2003), elbow (Lamb & Wong 2005), cisterna magna in dogs with Chiari malformations (Schmidt et al. 2008), and ultrasound anatomy of the cervical spine (Sarto et al. 2014). However, few studies exist on the ultrasound anatomy of the lumbar spine and guided lumbar puncture (Garcia et al. 2018, Monticelli et al. 2017, Etienne et al. 2010), and no study exists on the ultrasound anatomy of the T10-S1 region. This study thus aimed to test the ultrasonographic technique to assess the thoracolumbar and lumbar segments of the spine of dogs, and to evaluate its potential applicability in this region.

MATERIALS AND METHODS

After obtaining approval by the ethics committee of the Faculty of Animal Science and Food Engineering at the University of São Paulo, Faculdade de Zootecnia e Engenharia de Alimentos, Universidade São Paulo (FZEA-USP) (Pirassununga/SP, Brazil, approval no. 14.1.1469.74.1), studies were conducted on anatomical structures obtained from the FZEA-USP Veterinary Anatomy Laboratory and from animals that died in private clinics in the city of Pirassununga, Brazil. After anatomically identifying the structures, 10 live dogs were screened radiographically. All animals with radiographic evidence of alterations in the structures were excluded from the experiment. The selected animals underwent a complete blood count and measurement of the levels of urea and creatinine, alanine aminotransferase, aspartate aminotransferase, and alkaline phosphatase. Dogs with no abnormal blood test results were anesthetized for radiographic examination of the laterolateral and ventrodorsal projections along the entire spine. After screening, the T10-S1 segments of the spine of 10 healthy animals were assessed ultrasonographically by using the dorsal approach. After the descriptive studies of the anatomical structures and x-ray and ultrasonographic evaluations, an ultrasonically examined animal was selected to undergo CT and MRI scans.

Local. The anatomical study and the preparation of anatomical structures were conducted at the Veterinary Anatomy Laboratory of the Faculty of Animal Science and Food Engineering of the University of São Paulo (FZEA-USP, Pirassununga/SP, Brazil). The thoracolumbar and lumbar segments of the spine of the dogs were also evaluated using radiography and ultrasound in the Diagnostic Imaging Division of the FZEA-USP. The CT scans were performed in the Diagnostic Imaging Division of the School of Veterinary Medicine and Animal Science, Universidade Estadual Paulista (Unesp), Botucatu/SP, Brazil), and the MRI scans were conducted at the Provet Veterinary Diagnostic Medicine in Moema/SP, Brazil.

Anatomical structures assessment. The anatomy laboratory provided anatomical structures and other three animals that died in private clinics in the city of Pirassununga. The three corpses were frozen in an upright position. Thereafter, sections were cut in the transverse plane with the aid of a band saw (Siemens, Munich, Germany).

The anatomical structures were photographed. The collected data and images of the anatomical structures were compared with the images obtained by radiography, ultrasonography, MRI, and CT scans of the region (Dyce et al. 2010, Evans & De Lahunta 2012, Wisner & Zwingenberger 2015).

Radiographic examination. The selected animals underwent chemical restraint with preanesthetic medication (PAM), which consisted of acepromazine (0.05mg/kg) and morphine (0.5mg/kg) administered intramuscularly. Radiographic examinations were administered to some animals in which PAM medication provided sufficient relaxation. Animals that did not relax with PAM were induced with intravenous propofol (5mg/kg), and anesthesia was then maintained by isoflurane inhalation. Heart rate, respiratory rate, and rectal temperature were monitored while the dogs completely recovered. On recovery, they were returned to their owners.

After anesthesia, the animals were placed in the laterolateral and the ventrodorsal decubitus positions, and serial radiographs of the entire spine were obtained in each position. The radiographic apparatus used was the Altus ST generator (Sawae Tecnologia Ltd., Nova Lima/MG, Brazil) 630mA and 125 kV with rotating anode x-ray ampoule, and equipped with a radiological table with a fixed grid. The radiographic films were placed in a metal chassis equipped with intensifying screens that were suitable for the size of the animal. The development and fixation of the films took place in an automated x-ray film processor (LX-2; Lotus, Curitiba, Paraná, Brazil).

Radiographic examinations were conducted using radiological protection standards. The veterinarians involved in the examination used lead aprons that were 0.5mm thick in the front and 0.25mm thick in the back, a 0.50-mm thick thyroid lead protector, and 0.50mm lead gloves. The technique was based on the correlation between kilovoltage and milliamperage, according to the thickness of the evaluated segment. The animals were radiographed in the ventrodorsal and laterolateral projection to assess the entire spine.

Ultrasound examination. Ultrasonography was conducted with the MyLab Class C Vet ultrasound device (Esaote, Genoa, Italy), which was equipped with an electronic linear transducer (LA 533) of 8-13 MHz. The procedure started with trichotomy of the region of

study with a shearing machine and subsequent application of acoustic gel. The animals were maintained in left lateral decubitus with the thoracic limbs parallel and cranially extended and the pelvic limbs parallel and cranially extended. First, the images were acquired in B-mode in the median longitudinal and paramedian plane and later in the transversal plane to analyze the segments of the sacrum at the 10th thoracic vertebra. Ultrasound scanning started with L7-S1 because this region allowed easier visualization, and was followed cranially segment by segment until reaching T10-T11.

Computed tomography scan. An animal undergoing a CT scan was anesthetized. The PAM included acepromazine (0.02mg/kg) and morphine (0.5mg/kg), and was administered intramuscularly. The animal was then induced with intravenous propofol (3.5mg/kg), and anesthesia was maintained with isoflurane until the end of the procedure. Heart rate, respiratory rate, and rectal temperature were monitored until stabilization. The animal was then returned to the owner.

The images used in the study were obtained with a CT scanner[1]. The technique settings were 120 kV and 160mA with 2 seconds of acquisition time, a cut thickness of 2mm, and a cut-off interval of 2mm; scanning was conducted without contrast from the 10th thoracic vertebra to the lumbosacral region. The CT images were obtained in transverse plane and then the sagittal reconstructions were performed. The soft tissue and bone windows were used in the tomographic images, based on the technique of Tidwell (2010).

Magnetic resonance imaging. Dogs that underwent MRI were anesthetized. Acepromazine (0.03mg/kg) and pethidine (3mg/kg) were administered intramuscularly. The animals were then induced with propofol (3mg/kg) and midazolam (0.2mg/kg). Isoflurane was used to maintain anesthesia until the end of the procedure. Heart rate, respiratory rate, and rectal temperature were monitored until complete stabilization. The animal was then returned to the owner.

The images were acquired through MRI[2]. The imaging planes were the transverse and sagittal planes. The images were acquired in T1-weighted spin echo (SE) and T2-weighted fast spin echo (FSE) sequences.

RESULTS

In this study, it was possible to observe the hyperechoic laminar appearance of the skin with ultrasonographic examination. The subcutaneous tissues had hypoechogenic areas with hyperechogenic sites.

The lumbar multifidus and longissimus muscles in the longitudinal plane were hypoechogenic and the muscular fascias exhibited echogenic streaks. In the transverse plane, the muscles were hypoechogenic with echogenic foci (Fig.1).

The bone surface of each vertebra had a hyperechogenic line that produced an acoustic shadow (Fig.1).

The 2-mm thick vertebral segments evaluated through the tomographic examination provided good bone detail of the vertebrae.

Magnetic resonance imaging allowed the visualization of more structures with better image definition, compared to other imaging techniques (Table 1). Yellow and interspinous ligaments were only observed through ultrasonography examination (Fig.2).

The T1-weighted SE MRI images allowed visualization of the musculature with improved definition, compared with T2-weighted FSE images. The T2-weighted images allowed

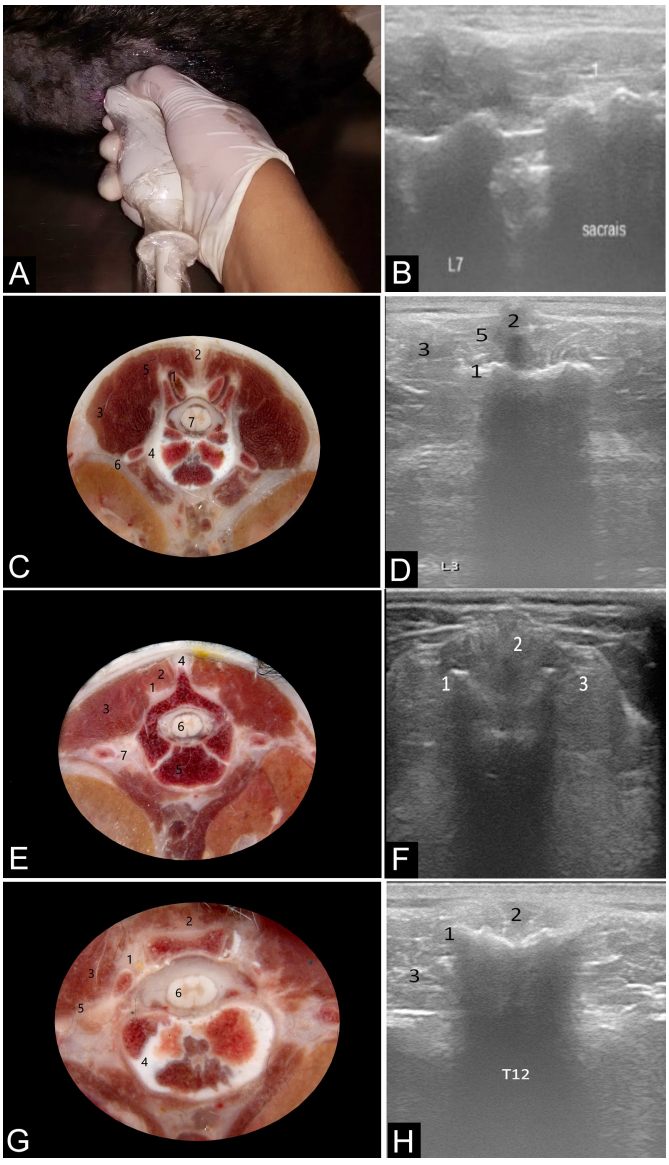

Fig.1. Ultrasound planes and images. (**A**,**B**) Paramedian longitudinal plane of a dog's lumbosacral region. The number "1" indicates the lumbar multifidus muscle. Transverse planes in the anatomical section and the corresponding ultrasound image. (**C**,**D**) The third lumbar vertebra (L3) region. The numbers indicate the following anatomical structures: joint processes (1), spinal process (2), lumbar longissimus muscle (3), third lumbar vertebra (4), lumbar multifidus muscle (5), transverse process (6), spinal cord (7). (**E**,**F**) The 13th thoracic vertebra (T13) region. The numbers indicate the following anatomical structures: joint process (1), thoracic and lumbar multifidus muscle (2), longissimus thoracic and lumbar muscle (3), spinous process (4), 13th thoracic vertebra (5), spinal cord (6), transverse process (7). (**G**,**H**) The 12th thoracic vertebra (T12) region. The numbers indicate the following anatomical structures: joint process (1), thoracic multifidus and lumbar muscles (2), longissimus thoracic and lumbar muscle (3), twelfth thoracic vertebra (4), transversal process (5), spinal cord (6).

[1] Shimadzu, model SCT-7800. Kyoto, Japan.

[2] Esaote, model Vet MR large 0.3 Tesla and column coil number 13. Genoa, Italy.

Table 1. Visible structures in the imaging scans and the respective quality of the visualization

Structures	Imaging techniques			
	XR	US	MR	CT
Skin	—	+++	++	—
Subcutaneous	—	+++	++	—
Muscle fascia	—	+++	—	—
Epaxial muscles	—	+++	+++	+
Hypaxial muscles	—	—	+++	+
Cortical bone	++	+*	+++	+++
Spinal cancellous bone	—	—	+++	+++
Joint processes	+	++	+	+++
Spinous process	++	++	+++	+++
Transverse process	++	+	+++	+++
Vertebral body	++	—	+++	+++
Spinal canal	++	—	+++	+++
Spinal cord	—	—	+++	+
Interspinous ligament	—	+++	—	—
Yellow ligament	—	++	—	—
Intervertebral space	++	++	+++	+++
Intervertebral disc	—	—	+++	—
Cerebrospinal fluid	—	—	+++	—

XR = X-ray, US = ultrasound, CT = computed tomography, MR = magnetic resonance; ___ unidentified structure, + poorly visible structure, +* bone surface only, ++ moderate visibility of the structure, +++ optimal visibility of the structure.

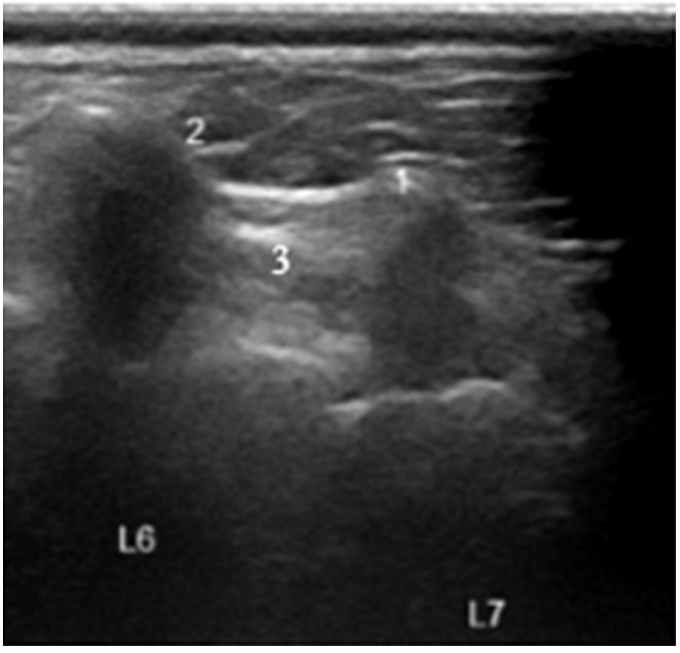

Fig.2. Ultrasound image of the spine in the longitudinal paramedian oblique plane demonstrates the interspinous ligament between L6 and L7. The numbers indicate the following anatomical structures: L7 spinous process (1), L6 spinous process (2), interspinous ligament (3).

the intervertebral disc nucleus and cerebrospinal fluid to be observed.

The correlation between diagnostic imaging methods and anatomical structures are shown in Table 1.

DISCUSSION

This study confirmed that it is necessary to have anatomical knowledge of the evaluated structures for a correct ultrasonographic evaluation of the spine. Thus, the study of corpses and the execution of cross-sections in frozen anatomical structures were essential for correctly applying the ultrasonographic technique, which was also confirmed by other authors (Berg et al. 2003).

The lumbar and thoracic vertebrae were easily identified on ultrasound examination. However, it is worth mentioning some difficulties associated with the examination such as maintaining an animal in the correct position, the difficulty of maintaining the transducer in the same place to facilitate counting the vertebrae, the veterinarian's experience in performing the procedure, and the lack of studies on the topic.

A linear 8-13 MHz transducer provided adequate definition of the evaluated vertebral structures. This finding was similar to the results obtained by other researchers (Samii & Long 2005, Sarto et al. 2014) who used 5-10 MHz linear transducers in ultrasound studies of the cervical spine of dogs. The better definition of the image using linear transducers is because of their high frequency, which provides a higher resolution of the image.

Visualization of the musculature details may vary with different types of equipment and with the frequency of the transducer; however, the findings are usually a hypoechogenic structure interspersed by hyperechogenic lines with variable organization, depending on the plane approach. The quality of the devices and transducers and the varying frequencies will impact image quality. An acoustic pad may be required when using a transducer with a frequency lower than the frequency suggested in the literature. The trichotomy of the region may also provide enhanced acoustic coupling in these instances, and thereby improve the image. Despite these variations, the image of the musculature is characteristic and easy to recognize

Similar to the results obtained by Kramer et al. (1997), the bone surface of each vertebra revealed a hyperechogenic line that produced a posterior acoustic shadow. This phenomenon is associated with the difference in acoustic impedance between the soft structures and the bone tissue. This difference causes a sharp reflection of the sound beams back to the transducer, which provides a hyperechogenic line effect on the bone surface and consequently an acoustic shadow. In the current study, the interspinous yellow ligaments were observed, which was in contrast to the findings of previous reports (Chhem et al. 1994) that claim that these ligaments cannot be observed because of their small size and proximity to the bone surfaces (with the exception of the ligaments of the knee). The differences between the equipment used may have contributed to the discrepancy in the observation of the aforementioned ligaments.

In this study, the use of CT, as previously described by Assheuer & Sager (1997), provided better bone detail. However, accurately differentiating soft tissues was not possible. Therefore, the use of ultrasonography was important as a complementary technique that provided better detail of the muscles of the T10-S1 segment of the spine; allowed visualization of the musculature; and, because it is a dynamic examination, allowed for muscle contractions, as reported by Kramer et al. (1997). Thus, ultrasonography provides a

dynamic evaluation of the musculature and can be explored in dogs while placing the spine in different positions (i.e., neutral, extended, and flexed), which allows the ability to investigate variations in muscle thickness and possibly view adhesions between skin and musculature, cicatricial retractions, muscular atrophy, et al. Of course, it loses with respect to the potential of evaluation in humans who are able to perform muscular movements and contractions in a voluntary and directed way, further improving the possibilities of interpretation of the images.

Ultrasound imaging showed a wealth of details in muscle tissue and adjacent regions (e.g., skin, the subcutaneum, muscular fascia) and the possibility of real-time evaluation, compared with CT imaging in which muscle tissues exhibited the same attenuation, which hindered the identification of individual muscles (it was also a static image). By contrast, the ultrasound examination allowed the differentiation of the longissimus and multifidus muscles. The thoracic (T10-T13) and lumbar vertebrae were identified with better detail in the bone window where it was possible to view the articular processes, dorsal arch, vertebral canal, vertebral body, spinal process, and transverse processes. No contrast was administered in the CT scan; therefore, individualization of the spinal cord was not possible.

The 2-mm thick sections in the CT scan allowed adequate detail of the structures, which was consistent with the findings of some authors who studied the cervical spine and recommended a 2-mm maximum. A greater cutting thickness can result in loss of detail because the larger voxel will show the average of the Hounsfield units in the pixel used to form an image, and dilute the minimum and maximum values that would create contrast.

Magnetic resonance imaging allowed the visualization of more structures and allowed improved definition, compared to other imaging techniques (Table 1). This finding was consistent with that of Bagley et al. (2009) who stated that MRI is the gold standard for evaluating the spine and spinal cord. However, visualization of the yellow and interspinous ligaments was only possible through ultrasonography examination. For instance, if 1.5-7 T closed-field MRI equipment was used, the signal conspicuity may be more intense, even in anatomical regions with low signal. This factor allowed a contrast between these structures and improved their visualization. The latter should be investigated in future studies. In this study, two pulse sequences and two imaging planes (i.e., transversal and sagittal) were used to identify most anatomical structures. The T1-weighted SE images allowed visualization of the musculature with enhanced definition, compared with the T2-weighted FSE images. The T1-weighted images provide an optimal representation of the anatomy, although these images have lower water contrast, compared to T2-weighted images (Dennis 2011). The contrast between differences in the concentrations of water molecules in the tissues was higher in T2-weighted images, which allowed visualization of the intervertebral disc nucleus and the cerebrospinal fluid; however, the identification of muscular structures presented a greater degree of difficulty in these images, compared with the T1-weighted images. A better quality signal of the musculature may be possible by using equipment with a greater magnetic field intensity. However, the detail of the musculature was superior with the ultrasonographic method, compared to the 0.3 T signal intensity open field equipment used.

Computed tomography and MRI scans of the spine were performed on a single animal to obtain illustrative images of our own for comparison purposes. Computed tomography and MRI atlases describing spinal findings have been published. Therefore, this focus was not the purpose of this study.

The three imaging methods (i.e., ultrasound, CT, and MRI) have limitations and advantages that complement each other, as presented in Table 1. However, the high availability and low cost of ultrasound equipment, compared to other equipment; its portability; and the ability to identify various structures of the spine region using this modality indicate it as the reference screening method of the spine that can be used by trained technicians.

CONCLUSION

In this study, B-mode ultrasound examination allowed optimal visualization of the perivertebral musculature and the interspinous and yellow ligaments, compared with other imaging techniques.

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Original Article
Morfofisiologia/*Morphophysiology*

Interval in the replacement of *in vitro* culture medium affects the integrity and development of equine preantral follicles[1]

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ABSTRACT.- Bizarro-Silva C., González S.M., Búfalo I., Lindquist A.G., Sarapião F.D. & Seneda M.M. 2018. **Interval in the replacement of *in vitro* culture medium affects the integrity and development of equine preantral follicles***. Pesquisa Veterinária Brasileira 38(12):2284-2288.* Laboratório de Reprodução Animal, Universidade Estadual de Londrina, Rodovia Celso Garcia Cid PR-445 Km 380, Cx. Postal 10.011, Campus Universitário, Londrina, PR 86057-970, Brazil. E-mail: camilabizarros@gmail.com

The efficiency of a culture system is related to the elaboration and replacement of a medium with conditions suitable for follicular development. Recent investigations suggested that *in vitro* culture medium should be replaced after specific time periods in various species. However, the suitable interval for the exchange of *in vitro* culture medium has not yet been established in equine species. The objective of this investigation was to evaluate the effect of medium exchange intervals of 24 hours (T24) or 48 hours (T48) for *in vitro* culture of preantral follicles at 2 or 6 days. At the end of the culture period, the fragments were processed using classical histology. Equine preantral follicles were classified according to morphological integrity and developmental stage. Data analysis was performed using Fisher's test with a significance level of $p<0.05$. Out of a total of 399 follicles evaluated, 174 (43.6%) were primordial follicles, 225 (56.4%) were in development, and 63.76% were morphologically intact. In the *in vitro* culture performed over two days, there was no significant difference in relation to follicular integrity after medium replacement ($p>0.05$). Compared to the medium replacement at six days of culture, there was a statistically significant difference for T24 (68.9%, $p<0.05$). Therefore, we suggest changing the medium for equine species at 48 hours after the start of culture followed by subsequent daily replacements.

INDEX TERMS: *In vitro* culture, equine preantral follicles, ovary, horses, culture *in vitro* period, follicular morphology.

RESUMO.- [**Intervalo na substituição do meio de cultivo *in vitro* afeta a integridade e o desenvolvimento de folículos pré-antrais equinos.**] A eficiência de um sistema de cultivo está relacionada à elaboração e substituição do meio de cultivo com condições adequadas ao desenvolvimento folicular. Pesquisas recentes sugerem que o meio de cultivo *in vitro* deve ser substituído após períodos de tempo específicos para várias espécies. No entanto, o intervalo adequado para a troca de meio de cultivo *in vitro* ainda não foi estabelecido na espécie equina. O objetivo desta investigação foi avaliar o efeito de intervalos de troca média de 24 horas (T24) ou 48 horas (T48) para cultivo de folículos pré-antrais aos 2 ou 6 dias. No final do período de cultivo, os fragmentos foram processados usando histologia clássica. Os folículos pré-antrais equinos foram classificados de acordo com a integridade morfológica e o estágio de desenvolvimento. A análise dos dados foi realizada utilizando o teste de Fisher com um nível de significância de $p<0,05$. De um total de 399 folículos avaliados, 174 (43,6%) foram folículos primordiais, 225 (56,4%) estavam em desenvolvimento e 63,76% estavam morfologicamente intactos. No cultivo *in vitro* realizado ao longo de dois dias, não houve diferença significativa em relação à integridade folicular após a substituição do meio ($p>0,05$). Comparado com a substituição média aos seis dias de cultivo, houve

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diferença estatisticamente significativa para T24 (68,9%, p<0,05). Portanto, sugerimos alterar o meio para as espécies equinas às 48 horas após o início da cultura, seguindo as subsequentes substituições diárias.

TERMOS DE INDEXAÇÃO: Cultivo *in vitro*, folículos pré-antrais, equinos, ovário, período de cultivo *in vitro*, morfologia folicular.

INTRODUCTION

Reproductive biotechnologies allow people to overcome several obstacles of reproductive physiology, optimize the genetic material of animals and accelerate and facilitate genetic improvement. This technology has been especially useful for mitigating the high level of follicular loss that occurs naturally throughout the lives of mammals (Carmo et al. 2002). Since the ovarian reserve of female mammals consists of 90% primordial follicles in the ovarian tissue, approximately 99% of these follicles do not reach the ovulatory phase (Markström et al. 2002).

In this context, the *in vitro* culture (IVC) of preantral follicles has been used widely to minimize the apoptotic fate or follicular loss by degeneration that occurs through the physiological process of atresia (Haag et al. 2013). Therefore, several researchers have sought the efficient development of the in vitro culture systems in the various species, such as sheep (Magalhães et al. 2011, Bandeira et al. 2015), goats (Magalhães et al. 2011, Duarte et al. 2013, Pessoa et al. 2014), bovines (Andrade et al. 2012, Sun & Li 2013), buffalos (Gupta et al. 2008), humans (Telfer et al. 2008), canines (Serafim et al. 2015) and in mice (Demeestere et al. 2002), with the purpose of finding clarification related to the follicular loss, making the follicles that would never reach ovulation can be used through this tool.

The efficiency of the culture system is related to the elaboration and substitution of a base medium that allows for the conditions necessary for follicular development. This system is indispensable for producing substances that are favorable and/or harmful for cultured cells. However, it has not yet been possible to standardize protocols for replacing the medium during culture. In equines, the IVC and the medium replacement interval has not been fully established, and great efforts are required to advance the technique, which is extremely important due to some reproductive peculiarities that the species present (Gomes et al. 2015).

In the last decade, the expansion of reproductive biotechnologies applied to Equidae has promoted an increase in the equestrian industry, directly reflecting the interest in this species (Gomes & Seneda 2013). Thus, it is notable the execution of applied research to continue the advancement in this segment. Therefore, the objective of the present investigation was to verify the effect of the medium replacement protocol after 24 and 48 hours at 2 or 6 days of *in vitro* culture using equine preantral follicles.

MATERIALS AND METHODS

Collection and transportation of ovaries. Ovaries (n=5) from five mares in seasonal anestrus of unknown age, body condition and reproductive status were collected from a slaughterhouse located approximately 40 km from the laboratory (latitude 23°17'34" S and longitude 51°10'14" W). At the slaughterhouse, the ovaries were washed in 70% alcohol followed by a wash in PBS (PBS; Embriolife®, Vitrocell, Brazil). The ovaries were transported to the laboratory in a temperature-controlled container, following the method used by Gomes & Seneda (2013).

Experimental protocol. Each ovary was carefully dissected to remove adipose and connective tissue. The ovary was sectioned along the sagittal plane, and ovaries containing abundant CL or antral follicles were discarded. The portion of the parenchyma (internal) of the five selected ovaries was cut into fragments of approximately 3x3x1mm. A fragment from each ovary was randomly selected and immediately fixed in Bouin. The remaining fragments (n=8) were individually cultured in 1ml aliquots of the culture medium in 24-well dishes with a 5% CO2 atmosphere in air and saturated humidity at 38.5°C.

The base culture medium used was the minimal essential medium (MEM, Gibco BRL, Rockville/MD, USA; osmolarity 300mOsm/L, pH 7.2) supplemented with ITS (insulin 6.25mg/mL, transferrin 6.25ng/mL, and selenium 6.25ng/mL), 0.23mM pyruvate, 2mM glutamine, 2mM hypoxanthine, 1.25mg/mL bovine serum albumin (BSA Gibco BRL, Rockville/MD, USA), 20 IU/ml of penicillin and 200mg/ml of streptomycin. Two fragments were intended for *in vitro* culture for two or six days, and the medium was completely replaced according to the treatment at either 24 (T24) or 48 (T48) hours. After the culture period, the ovarian fragments were evaluated using the classical histology technique. The staining was performed with Schiff's periodic acid (PAS) and hematoxylin.

Follicular classification. All sections were examined using light microscopy (Nikon®, Tokyo, Japan). Equine preantral follicles were classified according to the integrity of their structure as morphologically intact or degenerate, according to Andrade et al. (2012). Briefly, follicles were classified according to the integrity of their structure in morphologically normal (an intact oocyte, surrounded by granulosa cells well organized in one or more layers and absence of pycnotic nuclei) or degenerate (atretic follicles: pynotic nucleus and/or withdrawn, disorganization of the granulosa cells and cytoplasmic vacuoles. Preantral follicles were also evaluated for their development according to Gomes et al. (2015); these were classified as primordial or developing (primary or secondary) follicles. Briefly, primordial follicles (a layer of granulosa cells flattened around the oocyte) or primary (a single layer of granulosa cuboid cells), or secondary (two or more layers of granulosa cuboid cells).

Morphometric analysis. The slides were examined, and images were captured using a MOTICAM 2500 digital camera (5.0 M Pixel) then analyzed using the Motic Images Plus 2.0 ML software. The measurements of follicles and oocytes were recorded, according to Silva-Buttkus et al. (2008), and the oocyte and follicular diameters were calculated from the arithmetic mean of two perpendicular measurements.

Statistical analysis. Follicular integrity data from the five replicates were submitted for analysis of normality and homoscedasticity. The multiple comparisons between the experimental groups were performed using Fisher's test. The follicular development data in the five replicates were compared using Student's t-test. All analyses were performed using the software Action 3.1 version of R 3.0.2 (Campinas/SP, Brazil). The level of statistical significance was set at $p<0.05$.

RESULTS

The follicles were present in only 5.8% (217/3,750) of the fragments at different stages of development (Fig.1). Of the 399 follicles evaluated, 174 (43.6%) were primordial

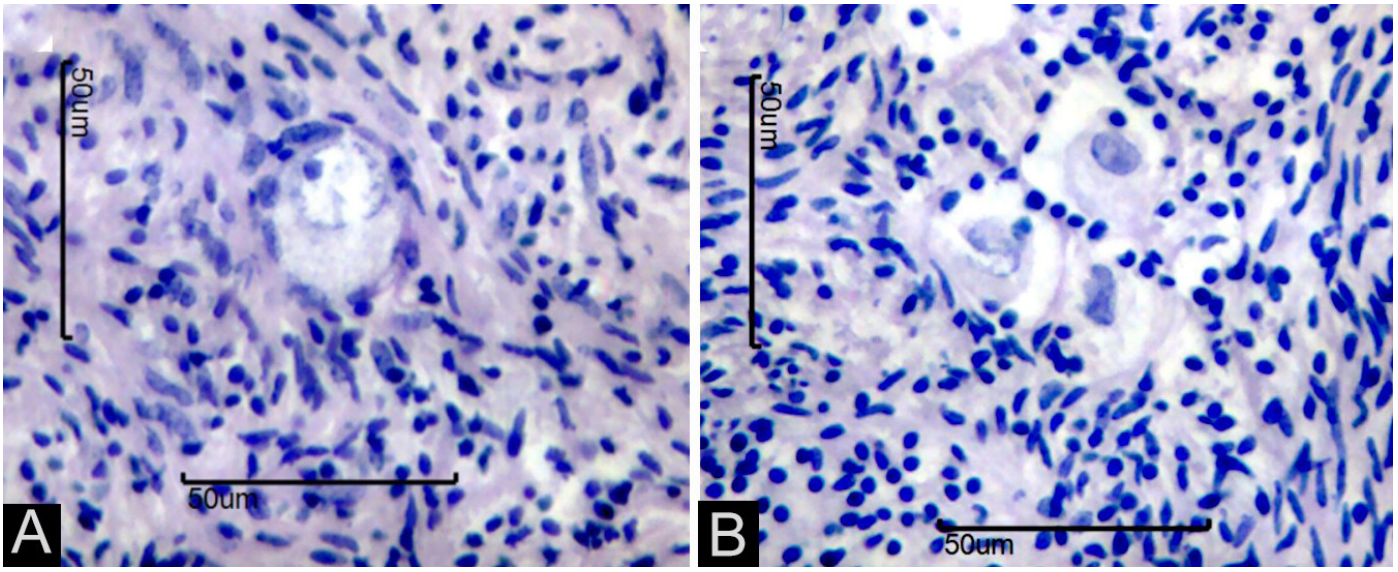

Fig.1. Morphological aspects of preantral follicles. (**A**) Intact primordial follicles and (**B**) degenerate primary follicles. PAS and hematoxylin, bar = 50µm.

follicles, 225 (56.4%) were in development and 63.76% were morphologically intact. On average, 91.8 follicles were found per treatment.

When the different protocols for medium replacement were analyzed, there was a significant difference in relation to the follicular integrity in the replacement of the medium at T48 compared to the daily exchange (T24) after two days of culture ($p<0.05$; Fig.2). When comparing the types of medium replacement over a six-day culture period of the intact ovarian follicles, the daily intervention demonstrated statistical significance ($p>0.05$; Fig.2). In this way, the culture over a two-day period presented the best result as a percentage of preantral follicles intact compared to the other treatments (T48 D2, T24 D6).

The morphometric analysis was performed on the follicle and oocyte (Fig.3). The mean follicle and oocyte diameter was measured at days two and six of the culture. The mean diameter of the follicles was found predominantly at the primary stage of development in most treatments. After the culture period, the mean diameter of the preantral follicles was similar for T24 and T48 replacement treatments after 2 days of culture (27.6±2.6µm, 29.2±2.3µm, respectively; $p>0.05$). While the mean follicle diameters did not show significant differences, replacements T24 h and T48 h showed mean diameters of 24.1±3.2 and 24.5±4.5µm, respectively ($p<0.05$). Compared to the diameter of the oocytes present in the follicles, there was no statistically significant difference according to the mean replacement and the culture period ($p>0.05$).

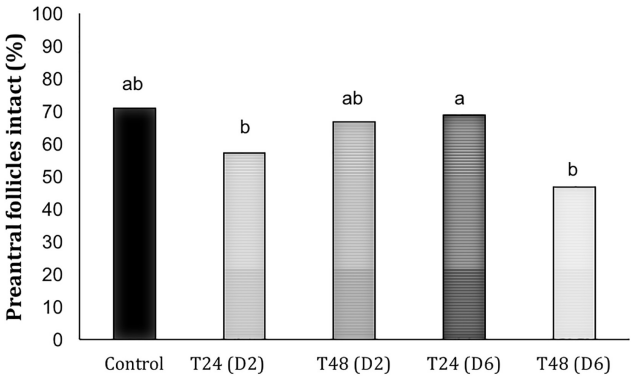

Fig.2. Percentage of preantral follicles cultured *in vitro* according to morphological integrity in relation to medium replacement in the following groups: daily exchange of the medium for two days (T24 D2), exchange of the medium every two days until the second day (T48 D2), daily exchange of the medium for six days (T24 D6) and exchange of the medium every two days until the sixth day (T48 D6). Values followed by lower case letters (a, b) differ statistically ($p<0.05$).

DISCUSSION

We evaluated the efficiency of replacing *in vitro* culture medium for preantral follicles in the ovarian tissue. We propose two methods of total substitution of the culture medium that consist of medium exchange every 24 and 48 hours. Our results showed a difference in the percentage of total follicles at six days after total daily culture medium exchange. On the other hand, during the first days of *in vitro* culture, the medium replacement could be done at 24h or 48h intervals for without causing interference in the morphological integrity of the follicle.

After 6 days of culture, the daily exchange treatment was the only treatment that could maintain the integrity of the cultured follicles (68.9%). Haag et al. (2013) performed *in vitro* culture for 7 days using ovarian fragments obtained by biopsy. They completely changed the medium every two days and obtained 65.5% of total intact follicles. Our research found similar results (59.2%); however, in the culture period (D6), which was similar to the study by Haag et al. (2013), we found that daily exchange was beneficial and had repercussions on the maintenance of follicular integrity. In contrast, similar studies in cattle (Andrade et al. 2012), sheep (Bandeira et al. 2015) and buffaloes (Gupta et al. 2008) obtained positive results

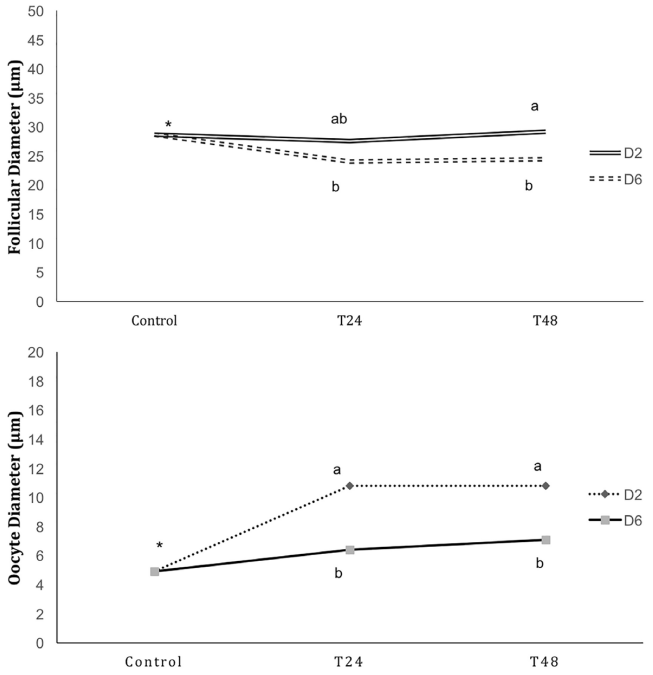

Fig.3. Percentage of mean diameter follicular and oocyte the preantral follicles (primordial and primary) and equine oocytes in fragments of uncultured ovarian tissue (control) and after two or six days in medium supplemented with different methodologies of total medium replacement: 24 and 48 hours exchange. *Not compared; values followed by lower case letters (a, b) differ statistically ($p<0.05$).

with the replacement of the medium every two days. In the present study, the replacement of the medium every 48 hours was beneficial for maintaining follicular integrity only at two days of culture.

The medium exchange interval affected the development of isolated cultured follicles after replacement every 2 days in caprine species (Magalhães et al. 2011). Our study showed that the replacement of the medium every two days did not have harmful repercussions for the ovarian follicles of equine species, and it could contribute to the development of these follicles. This finding opposes what was proposed in the abovementioned study.

For the in vitro culture of ovarian follicles, it is considered essential to replace the medium. The medium refreshment allows the withdrawal of the substances metabolized by the growing follicles, as well as the inclusion of new components to the follicles keep the development (Figueiredo et al. 2002).

We can infer that the follicular diameter observed on days 2 and 6 of *in vitro* culture did not change. This can be justified by the absence of substances in the culture medium that provide for the increase in follicular diameter. Replacement of culture medium is considered essential for *in vitro* culture technology using ovarian follicles since it washes away substances metabolized by the growing follicles and brings in new culture medium to support development (Figueiredo et al. 2002).

In most *in vitro* culture systems, the medium is totally or partially replaced with fresh medium every two days regardless of the culture period (O'Brien et al. 2003, Muruvi et al. 2005, Matos et al. 2007). In our work, we found that the total exchange of equine ovarian follicle in *in vitro* culture medium can initially be performed daily or every two days and subsequently done daily.

We identified few preantral follicles in this study, even after the removal of internal fragments of the equine ovary. This difficulty finding preantral follicles was also reported by Driancourt et al. (1982) and in recent investigations proposed by Gomes et al. (2015), Alves et al. (2015) and Gonzalez et al. (2017), in which they found little quantitative homogeneity of follicles in the evaluated fragments.

CONCLUSIONS

We found that the total exchange of *in vitro* culture medium for equine ovarian follicles can be performed daily in cultures up to 6 days. However, it is possible to carry out an exchange on the two days of cultivation and to continue the replacement with daily intervals.

This information contributes to the standardization of *in vitro* culture protocols through the adequate replacement of the medium during the culture of preantral follicles.

Conflict of interest statement.- The authors have no competing interests.

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Original Article
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Biochemical profile in dairy cows with artificial induction of lactation[1]

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ABSTRACT.- Paiano R.B., Lahr F.C., Poit D.A.S., Costa A.G.B.V.B., Birgel D.B. & Birgel Junior E.H. 2018. **Biochemical profile in dairy cows with artificial induction of lactation.** *Pesquisa Veterinária Brasileira 38(12):2289-2292.* Departamento de Reprodução Animal, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Avenida Prof. Dr. Orlando Marques de Paiva 87, São Paulo, SP 05508-270, Brazil. E-mail: renanpaiano@hotmail.com

The objective of this study was to determine the biochemical profile of dairy cows with induced lactation. For comparison, another group of normally calved cows was used as control. Lactation was induced in multiparous Holstein cows (*n=10*) with two norgestomet implants (3mg each implant) on day 1. The testing continued with intramuscular norgestomet (3mg/animal) on days 1, 3, 5, 7, 9, 11, 13 and 15. On days 1, 9, 16 to 18 and then every 14 days, bSTr (500mg/animal) was added. On day 16, the intravaginal implant was removed and intramuscular prostaglandin F2α (0.530mg/animal) and intramuscular estradiol benzoate (5mg/animal) were added. On days 16 to 18 dexamethasone (10mg/animal) was added, and from days 18 to 20 intramuscular metoclopramide (100mg/animal) was added. Milking began on day 19 of the induction. Blood was collected for a biochemical profile after 21 days in milk. It was found that urea and triglyceride concentrations were significantly higher in the induced cows ($P<0.05$). Therefore, it was concluded that the animals that had lactation induced did not present disorders related to the biochemical profile indicating that the hepatic function, renal function and lipidogram of the animals were not affected by the use of the drugs to induce lactation.

INDEX TERMS: Biochemical profile, dairy cows, hormonal induction, lactation, Holstein cows, cattle.

RESUMO.- [**Perfil bioquímico de vacas leiteiras submetidas a indução artificial de lactação.**] O objetivo deste estudo foi determinar o perfil bioquímico de vacas leiteiras com submetidas a indução de lactação. Para comparação, outro grupo de vacas que apresentaram parto normal foi usado como controle. A lactação foi induzida em vacas Holandesas (n=10) utilizando dois implantes de norgestomet (3mg cada implante) no dia 1. O protocolo continuou com a aplicação de norgestomet intramuscular (3mg / animal) nos dias 1, 3, 5, 7, 9, 11, 13 e 15. Nos dias 1, 9, 16 a 18 e depois a cada 14 dias, foi adicionado bSTr (500mg / animal). No dia 16, o implante intravaginal foi removido e adicionou-se prostaglandina F2a intramuscular (0,530 mg / animal) e benzoato de estradiol intramuscular (5mg / animal). Nos dias 16 a 18 foi adicionada dexametasona (10mg / animal) e dos dias 18 a 20 foi adicionada metoclopramida intramuscular (100 mg / animal). A ordenha começou no dia 19 da indução. O sangue foi coletado para mensuração do perfil bioquímico após 21 em leite. Verificou-se que as concentrações de ureia e triglicérides foram significativamente maiores nas vacas induzidas (P<0,05). Portanto, concluiu-se que os animais que tiveram a lactação induzida não apresentaram distúrbios relacionados ao perfil bioquímico, indicando que a função hepática, a função renal e o lipidograma dos animais não foram afetados pelo uso das drogas para induzir a lactação.

TERMOS DE INDEXAÇÃO: Perfil bioquímico, vacas leiteiras, indução hormonal, lactação, vaca Holandesa, bovinos.

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INTRODUCTION

The disposal of animals due to reproductive failures represents a great economic loss for dairy cattle, and one of the main factors for this is a non-pregnant cow at the end of lactation. Artificial induction of lactation is a tool that is based on the combination of synthetic hormones that mimic gestation in the final trimester and stimulates milk synthesis. This approach represents an excellent alternative for owners to maintain a genetically superior herd with good potential for milk production, avoid unproductive animals in the herd, generate new reproductive opportunities and increase profits (Magliaro et al. 2004, Freitas et al. 2010, Radavelli et al. 2016).

For more than 60 years, reports of use of lactation induction protocols have been described (Jewell 2002), including hormonal induction in heifers in the 1940s (Walker & Stanley 1941). However, the period of hormonal induction was too long, lasting about 180 days (Freitas et al. 2010), generating pain and discomfort in the animals (Pestano et al. 2015). Over time, the protocol was shortened to seven days due to a study by Smith & Schanbacher (1973), the cows produced 70% of the milk of a normal lactation. The protocols currently have duration of about 21 days, allowing the production of milk with normal solids content, although there is great variability in the milk induction response and milk production (Pestano et al. 2015).

The response to the reported lactation induction protocol was 100% in 98 cows used in the study by Mellado et al. (2006) and 78% in the study by Ramgattie et al. (2014). Based on milk production, a production volume of 77.2% was described from the previous lactation (Freitas et al. 2010), and 78% of the dairy production of cows that calved normally. (Mellado et al. 2006). The is a discrepancy in the data regarding the reproductive efficiency of the animals, whereby 41.4% of pregnant Holstein cows after lactation induction protocol performed in Brazil (Freitas et al. 2010), while Mellado et al. (2006) did not observe a difference between the proportion of pregnancies in induced dairy cows (71% of pregnancies) when compared to non-induced cows (75% of pregnancies) in a study carried out in Mexico.

Despite studies showing the efficiency of lactation induction protocols, no publications were found in scientific journals to evaluate the effects of its use on the metabolism of Holstein dairy cows. Therefore, the present study had the objective of evaluating the influence of lactation induction on the biochemical profile of Holstein cows.

MATERIALS AND METHODS

Cows and herd management. This study was conducted at the commercial dairy farm, located in Águas da Prata, State of São Paulo, Brazil. The cows were housed in free-stall barns and fed a total mixed ration twice daily. During the administration of hormonal protocol for induction lactation, cows received a diet formulated for dry period composed of 30kg corn silage, 0.6kg pre-dried oat, 1.0kg sorghum wet distillers grain, 1.9kg soybean meal, 0.02kg urea and 0.29kg anionic salt (Bovigold Beta prepartum®) per head, and was designed to contain 2.79% of ethereal extract, 14.53% of crude protein, 38.73% of neutral detergent fibre, 0.55% of calcium and 0.32 of phosphor.

With the beginning of milk production, all cows received the ration composed of 19.35kg corn silage, 2.0 kg tifton 85 haylage, 4.2kg corn grain, 3.4kg soybean meal, 3.0kg wet brewer grains, minerals and vitamins (Bovigold Beta postpartum®) and 3.3kg commercial concentrate per head, and was designed to contain 3.34% of ethereal extract, 17.76% of crude protein, 33% of neutral detergent fibre, 0.73% of calcium and 0.37% of phosphor. The cows were milked twice a day. A total of 20 multiparous (2 to 3 lactations and 3-4 years old) Holstein dairy cows that had the average milk yield 9,200kg in the previous lactation were included in the study. Two groups of cows were formed: 10 dairy cows with at least 45 days dry prior to the start of the hormonal protocol were artificially induced into lactation and 10 dairy cows naturally calved.

All animal procedures were approved by the Bioethics Committee of the School of Veterinary Medicine and Animal Sciences, University of São Paulo, São Paulo, Brazil (approval number: 8022150216/2018).

Hormonal protocol. The following hormonal protocol was used: Holstein cows (*n=10*) were implanted with two norgestomet 3mg each implant (Crestar, MSD Animal Health) on day 1. The testing continued with intramuscular norgestomet 3mg/animal (Crestar, MSD Animal Health) on days 1, 3, 5, 7, 9, 11, 13 and 15. On days 1, 9, 16 to 18 and then every 14 days, bSTr 500 mg/animal (Boostin, MSD Animal Health) was added. On day 16, the intravaginal implant was removed and intramuscular prostaglandin F2α 0.530mg/animal (Ciosin, MSD Animal Health) and intramuscular estradiol benzoate 5mg/animal (Fertilcare, MSD Animal Health) were added. On days 16 to 18 dexamethasone 10mg/animal (Azium, MSD Animal Health) was added, and from days 18 to 20 intramuscular metoclopramide 100mg/animal (Noprosil, Isofarma) was added as described by Silva et al. (2017). Milking began on day 19 of the induction.

Blood sampling and measurement of body condition score. Blood samples were collected at 21 days in milk (DIM) in the morning before feeding, from the middle coccygeal vein or artery puncture and stored in tubes without anticoagulant. Within 3 h of collection, blood samples were centrifuged at 2500rpm for 20 min. Serum samples were stored in Eppendorf tubes at -20°C until analysis measurements. Simultaneous with the blood collection, the body condition score (BCS) was measured by the same observer, using the five-point scale (Ferguson et al. 1994).

Measurement of biochemical samples. The concentration of total protein, albumin, globulins, urea, creatinine, β-hydroxybutyrate (BHBA), triglycerides, total cholesterol, gamma-glutamyltransferase (GGT) and aspartate aminotransferase (AST) were determined using commercial kits from Randox in an automatic biochemistry system (RX Daytona - Randox Laboratories).

Statistical analysis. The ANOVA was performed with the SAS software package (version 9.3 SAS/STAT, SAS Institute Inc., Cary, NC) using PROC GLIMMIX. To compare the means of biochemical parameters and BCS in the different groups (induced lactation vs. control), Tukey's test was used for detecting differences among means. Statistical significance level was regarded as $P<0.05$ and $0.05<P<0.1$ was considered to indicate a tendency toward a significant difference.

RESULTS

All continuous data are presented as least squares means ± SE. Biochemical concentrations and BCS values are shown in Table 1. Serum creatinine and triglyceride concentrations were significantly higher in induced cows ($P<0.01$). In the other variables, no statistically significant differences were found.

Table 1. Biochemical profile and body condition score from control cows and induced cows at 21 days in milk

Variables	Control cows	Induced cows
Total Protein (g/dL)	7.73±0.18	7.68±0.18
Albumin (g/dL)	2.92±0.12	3.23±0.11
Globulins (g/dL)	4.92±0.30	4.41±0.24
Urea (mmol/L)	4.01±0.31	4.89±0.31
Creatinine (mg/dL)	0.97±0.06[b]	1.30±0.06[a]
BHBA (mmol/L)	0.50±0.03	0.43±0.04
Triglycerides (mg/dL)	10.23±1.51[b]	14.33±1.52[a]
Cholesterol (mg/dL)	97.17±8.57	78.18±7.56
GGT (U/L)	15.11±2.97	22.11±2.98
AST (U/L)	66.11±6.55	71.22±6.56
BCS (points)	3.04±0.24	3.50±0.20

[a, b] Different lower case letters indicate difference by Tukey's test ($P \leq 0.01$).

DISCUSSION

Analysis of the results showed that there were no alterations in liver function due to lactation induction. The evaluation of GGT and AST enzyme activity were within the recommended limit for the species (Smith 2014). Measurement of the activity of GGT and AST enzyme contributed to the diagnosis of acute hepatic lesions (Russell & Roussel 2007).

The liver is responsible for the synthesis of proteins, and this functionality is dependent on an adequate nutritional status (González & Silva 2006). Albumin is responsible for the transport of amino acids and bilirubin (González 2000). The reduction of albumin concentration can be observed in animals with hepatic lesions, or in animals with inflammatory processes, being considered as a negative acute phase protein (Russell & Roussel 2007, Bertoni et al. 2015, Trevisi et al. 2015). Increased albumin concentration may occur due to dehydration (González 2000, Russell & Roussel 2007). No difference was observed in serum concentration of total protein, albumin and globulins. To the contrary, high concentrations of albumin and globulins were reported in sheep submitted to lactation induction protocol (Radavelli et al. 2016).

The urea concentration was within of biological range (Radostits et al. 2007). Urea is a product of nitrogen metabolism excretion, produced in the liver, and elevated urea values are suggestive of energy deficit, high protein in the diet or dehydration (González 2000, Roy et al. 2011). High values of urea concentration were observed in ketotic cows (İssi et al. 2016). On the other hand, Oliveira (2017) noticed a decrease in the concentration of urea when evaluating the effect of lactation induction on blood tests of crossbreed cows.

Control group showed lower blood creatinine concentration than those recommended by the literature (Radostits et al. 2007). Similar values to ours were observed by İssi et al. (2016) in control cows when compared to cows with ketosis and by Piccione et al. (2012) in dairy cows during the second week of lactation when compared to other stages of lactation, late gestation and dry period. The measurement of creatinine associated with serum urea assists in the evaluation of renal function, and creatinine is a more reliable parameter for renal function because it is eliminated exclusively through the kidneys unlike other nitrogen compounds that can be eliminated by the kidneys as well as mammary gland through milk (Smith 2009). Increased creatinine and urea values indicate that functionality of the nephrons is below 75% (Kaneko et al. 2008). Because the creatinine values were not elevated in the control group, the hypothesis of renal injury was ruled out. Low values of creatinine concentration can be caused due to starvation, low amount of protein in the diet and liver damage (Kaneko et al. 2008). This hypothesis was also ruled out because total protein values and liver enzyme activity were within normal range, indicating that low creatinine concentration in control group during postpartum was a normal finding.

The triglyceride concentration was higher in induced cows than the control animals, and this value was higher than physiological range (Radostits et al. 2007). Triglyceride measurement can be used to assess body energy resources (Nozad et al. 2014). Triglyceride values higher than measured in this study were observed in induced crossbreed cows (Oliveira 2017), and they associated this increase with the reduction of lipase lipoprotein activity in adipose tissue due to the use of estradiol during the protocol. The BCS reflects the energy balance of the animal (Jeong et al. 2015). Values similar to those obtained here were reported in cyclic postpartum cows (Jeong et al. 2015) and for induced crossbreed cows (Oliveira 2017).

According to the cholesterol concentration values, no difference was observed between the groups studied. The assessment of cholesterol helps in the diagnosis of energy balance (Sevinc et al. 2003). Metabolic and physiological adaptations related to the development of the mammary gland for milk production can cause hypocholesterolemia (Krajnicakova et al. 2003). Low serum cholesterol levels were observed in cows with puerperal ketotic (Djoković et al. 2013) and for cows in different reproduction periods (Piccione et al. 2012).

CONCLUSION

The data collected for this study indicates that the animals that had lactation induced did not present disorders related to the biochemical profile, indicating that the hepatic function, renal function and the lipidogram of the animals were not affected by the use of the drugs to induce lactation.

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Conflict of interest statement.- None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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Tokarnia C.H., Brito M.F., Barbosa J.D., Peixoto P.V. & Döbereiner J. 2012. Plantas que afetam o funcionamento do coração, p.27-94. In: Ibid. (Eds), Plantas Tóxicas do Brasil para Animais de Produção. 2ª ed. Helianthus, Rio de Janeiro.

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Sant´Ana V.A.C. 2004. Proteinograma do leite de vacas: padrões e variabilidade. Tese de Doutorado, Clínica Veterinária, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, SP. 161p.

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Pierezan F., Lemos R.A.A., Rech R.R., Rissi D.R., Kommers G.D., Cortada V.C.L.M., Mori A.E. & Barros C.S.L. 2007. Raiva em equinos. Anais XIII Encontro Nacional de Patologia Veterinária, Campo Grande, MS, p.145-146. (Resumo)

(Note: Evitar na consulta o uso de Resumos ao invés de artigos na íntegra!)

GUIDE FOR AUTHORS

Papers to "Pesquisa Veterinária Brasileira" (PVB), a Brazilian Journal of Veterinary Research, are submitted in Word online through ScholarOne, link <https://mc04.manuscriptcentral.com/pvb-scielo>

The authors should submit their papers in English, with a Portuguese Summary. To prove the quality of the English, a certificate of the English language is required, with exception of authors native in English.

With the communication of acceptance of the paper, the author for correspondence will be asked for payment of a Paper Charge of US$ 480.00 (*R$ 1.500,00*) for each article submitted in English.

Papers should be prepared in all details according to the style of the journal (www.pvb.com.br), in order to be peer reviewed. Tables and Figures should be submitted separately from the text.

PVB publishes Original Articles, but also Critical Literature Reviews and Topics of General Interest; no Short Communications are accepted.

The Original Papers should contain research results not yet published and not submitted to other journals.

Literature Reviews should be critical and consist of subjects of the author's research line.

Topics of General Interest should be of great importance and based on large experience of the authors.

The opinions and concepts emitted are of the responsibility of the authors. The Editorial Board of the journal, assisted by the peer review, may suggest or ask for modification of the text.

The author rights of the accepted papers are preserved.

1. **The submitted article should be organized in TITLE, ABSTRACT, RESUMO (the last when authors are from a Portuguese speaking country), INTRODUCTION, MATERIALS AND METHODS, RESULTS, DISCUSSION, CONCLUSION(S) (the last three preferably as separate chapters), Acknowledgements, Conflict of interest statement and REFERENCES:**

a) The **TITLE** should be concise and indicate the content of the article; details of scientific identification should be put into MATERIALS AND METHODS.

b) **Authors with several first and family names should shorten their names for scientific publication,** as for example: Cláudio Severo Lombardo de Barros writes Cláudio S.L. Barros or Barros C.S.L., and Franklin Riet-Correa Amaral writes Franklin Riet-Correa or Riet-Correa F. **The papers should not have more than 8 (eight) authors.** Corresponding author should be one who guarantees the contact with the Editorial Board of PVB. Asterisks for call to the footnotes should be elevated once more, in order to appear larger.

c) The **heading of the ABSTRACT** should contain the shortened and inverted names of the authors, the year, the Title (in brakes when translated), and the postal address of the laboratory or institution where the main part of the research was done (Always compare the authors of the paper and their shortened and inverted in the heading of the Abstract to avoid discrepancies).

d) The **footnote of the first page** should contain the complete professional address of each author (in the language of the author's country where to correspondence could be posted, Portuguese, Spanish, English, etc.) as well as the underlined e-mail of the corresponding author.

e) The **ABSTRACT** should be a well explained version of the Portuguese RESUMO, followed by "INDEX TERMS" which should include terms of the title, as they are not only Additional Index Terms.

f) The **RESUMO** should contain (1) what have been investigated, indicating (2) materials and methods used, (3) the most important results, and (4) the conclusion, followed by "TERMOS DE INDEXAÇÃO" (which include also words of the title, as they are not only Additional Index Terms).

g) The **INTRODUCTION** should be short, with citation of the specific literature without assuming main importance, followed by the objective of the research.

h) In **MATERIALS AND METHODS** should be given all data necessary for other research workers to repeat the research.

i) In **RESULTS** are presented the data obtained in a concise form.

j) In **DISCUSSION** the results should be confronted with the literature. Research in development or future planning should not be mentioned, to avoid the obligation for the journal to publish the results.

k) The **CONCLUSIONS** should be based only on the results obtained.

l) **Acknowledgements** should not be mentioned in the text or in footnotes.

m) **Conflict of interest or none** should be mentioned.

n) The **REFERENCES** include all citations consulted and presented chronologically in the text. The List of References should be written in alphabetical and chronological order, beginning with the family name of the first author, followed by the names of all other authors of the respective reference, in capital and small letters, and each author divided only by a comma, followed by year, title and the data of the publication (extensively in case of doubt about abbreviation) according to www.pvb.com.br.

2. During the elaboration of the paper, **the style of the journal has to be attended**, as follows:

a) Font **Cambria at 10 pitch, simple space between lines;** page **format A4, with 2cm margins** (superior, inferior, left and right), text in one column justified, with Figure captions below the list of References; without repeating the captions with the images of the Figures. Figures and Tables should be separately submitted.

b) **ABSTRACT** and **RESUMO** are written in only one paragraph and should not contain references.

c) The articles should be concise, always when possible in past tense and impersonal.

d) The scientific names should be presented in full (p.ex. *Palicourea marcgravii)* at the beginning of each chapter (Title, Abstract, Resumo, Introduction, etc.) when they appear for the first time, followed with abbreviation of the genus (p.ex. *P. marcgravii*).

e) In the Title of Tables and in Figure captions the scientific names are written in full.

f) In the text, calls to footnotes are given in Arabic numbers, in crescent order through the whole paper, without use of "Insert final note" of Word.

Note: To avoid separation in two lines, numbers should be presented without space to their units (p.ex.: 100ppm, 10mm, 50cm, 18x10cm, P<0.05, 15h; but 35 kg or 35kg, 5 h or 5h as convenient).

The abbreviation for number is "nº" and not "no"; for degree Celsius "ºC" and not "ºC".

g) Tables and Figures should be cited in the text with their respective numbers in crescent order.

h) Abbreviations of institutions when presented in the first place should be put within parentheses, after the full name of the institution.

i) Citations of the literature in the text are given by "author and year" (p.ex. Caldas 2005); papers with two authors are cited with the two names (p.ex. Pedroso & Pimentel 2013); citations with more than two authors are cited in the text by the name of the first author followed by "et al." and the year (p.ex. Brito et al. 2015). If two articles are not to distinguish, the differentiation is obtained through the addition of small letters after the year (p.ex. Barros 2017a, 2017b). The order of citation in the text should be chronological (p.ex. Barbosa et al. 2003, Armién et al. 2004).

j) **All cited articles should be consulted in full text**; if not possible, the original reference is put into the text as p.ex. Bancroft (1921); but in the List of References this should appear as: Bancroft 1921. ..... title. ... journal .... (Apud Suvarna & Layton 2013). The consulted reference should be also included in full in the List.

k) The use of "personal communication" and "non-published data" should be exceptional and cited in the text as Author and Year, and in the List of References as p.ex. Barbosa 2016. Personal Communication (Universidade Federal do Pará, campus Castanhal, Brazil).

l) **Figure captions** (p.ex. "Fig.3. ……") should be sufficiently informative for understanding (because Figures are independent from the text).

m) The **Title of Tables** should be written in **bold** and the **Heading** (titles of the columns) should be in clear (not bold), written in capital and small letter and separated by two long horizontal lines. There are no vertical lines and no grey bottom; exceptionally can exist horizontal lines. The calls for footnotes should be in small letters or other signs, but not in Arabic numbers. Tables should be submitted in Word (not as images) to allow corrections according to the style of the journal.

n) Complex data should be presented as **graphics (but named Figures)** in 2D without grey bottom and horizontal lines.

3. **Figure apresentation:**

a) Save images at 300 dpi, TIF files.

b) Number each Figure separately (1, 2, …).

c) Similar subjects as images should be grouped into a composition divided with spaces of about 1mm width. Label each subimage with a letter (A, B, ...) at the lower left corner, preferably with white, 14-point Arial font inside a black board with no border.

d) Use preferably scale bars for micrographs. For optical micrographs indicate at the legend finally the staining method and the objective used, for example: HE, obj.40x.

e) Figure legends should contain initially what is seen on the image, followed by additional information (Legend example = Fig.1. Sentence description. Diagnosis, organ or tissue, animal species, case number. Staining method and objective used.).

4. **All references cited in the text should be included in the List of References**; before the submission of the paper, discrepancies have to be corrected by the author (as the system ScholarOne blocks automatically if such discrepancies exist).

Exemples for References:

- Articles published in scientific journals:

Ubiali D.G., Cruz R.A., De Paula D.A., Silva M.C., Mendonça F.S., Dutra V., Nakazato L., Colodel E.M. & Pescador C.A. 2013. Pathology of nasal infection caused by *Conidiobolus lamprauges* and *Pythium insidiosum* in sheep. J. Comp. Pathol. 149(2/3):137-145.

Hooiveld M., Smit L.A., Wouters I.M., Van Dijk C.E., Spreeuwenberg P., Heederik D.J. & Yzermans C.J. 2016. Doctor-diagnosed health problems in a region with a high density of concentrated animal feeding operations: a cross-sectional study. Environ. Health 17:15-24.

(Note: The first letters of the words in the title of papers published in journals are small. It is preferable to indicate the number of the respective issue.)

- Books:

Marsh P. & Martin M. 1992. Oral Microbiology. 3rd ed. Chapman and Hall, London, p.167-196.

Tokarnia C.H., Brito M.F., Barbosa J.D., Peixoto P.V. & Döbereiner J. 2012. Plantas Tóxicas do Brasil para Animais de Produção. 2ª ed. Helianthus, Rio de Janeiro, p.305-348.

(Note: The first letter in the words of the title of books should be capital.)

- Chapters of books:

Uzal F.A., Plattner B.L. & Hostetter J.M. 2016. Alimentary system, p.1-257. In: Maxie M.G. (Ed.), Jubb, Kennedy and Palmer´s Pathology of Domestic Animals. Vol.2. 6th ed. Elsevier, St Louis, Missouri.

Barros C.S.L. 2007. Doenças víricas: leucose bovina, p.159-169. In: Riet-Correa F., Schild A.L., Lemos R.A.A. & Borges J.R.J. (Eds), Doenças de Ruminantes e Equídeos. Vol.1. 3ª ed. Pallotti, Santa Maria, RS.

Tokarnia C.H., Brito M.F., Barbosa J.D., Peixoto P.V. & Döbereiner J. 2012. Plantas que afetam o funcionamento do coração, p.27-94. In: Ibid. (Eds), Plantas Tóxicas do Brasil para Animais de Produção. 2ª ed. Helianthus, Rio de Janeiro.

- Dissertations and Theses:

Silva R.M.M. 2016. Prevalência, identificação e distribuição das lesões abscedativas em caprinos e ovinos abatidos em um matadouro frigorífico no Estado da Bahia. Dissertação de Mestrado, Universidade Federal do Recôncavo da Bahia, Cruz das Almas. 56p.

(Note: Use articles which originated from dissertations or theses instead of these).

- Abstracts published in Events:

Massa A.T., Potter K.A. & Bradway D. 2016. Epizootic bovine abortion outbreak in Eastern Nevada cattle. Annual Meeting American College of Veterinary Pathologist (ACVP), New Orleans, Lousiana. (Abstract D-50)

Mendonça F.S., Almeida V.M., Albuquerque R.F., Chaves H.A.S., Silva Filho G.B., Braga T.C., Lemos B.O. & Riet Correa F. 2016. Paralisia laríngea associada à deficiência de cobre em caprinos no semiárido de Pernambuco (IX Endivet, Salvador, BA). Pesq. Vet. Bras. 36(Supl.2):50-51. (Resumo)

Pierezan F., Lemos R.A.A., Rech R.R., Rissi D.R., Kommers G.D., Cortada V.C.L.M., Mori A.E. & Barros C.S.L. 2007. Raiva em equinos. Anais XIII Encontro Nacional de Patologia Veterinária, Campo Grande, MS, p.145-146. (Resumo)

(Note: Consult entire papers instead of only Abstracts)

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