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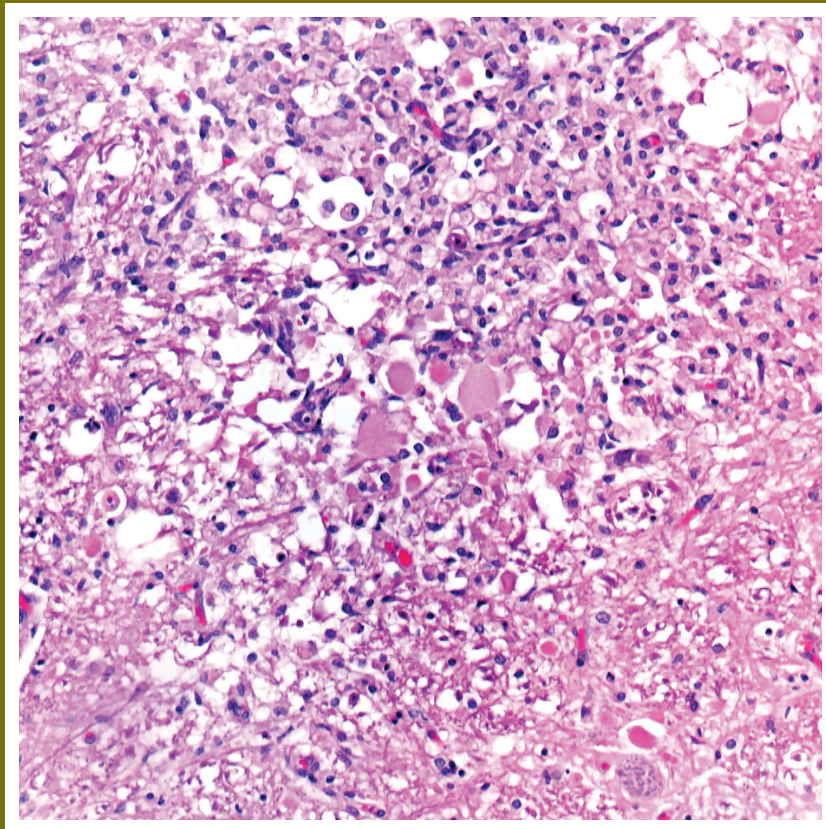
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Cover illustration: Microabscess with few neutrophils, axonal spheroids and neuropil vacuolation in brainstem of a buffalo with listeriosis. HE, obj.20x. (Prado et al., p. 301)

Nervous form of listeriosis in buffaloes¹

Robert Gustavo S. Prado², Tarcísio Anderson O. Domiciano²,
Laura Jamille A. Paredes³, Pedro S. Bezerra Junior², Washington Luiz A. Pereira³,
Valéria D. Cerqueira², David Driemeier⁴ and Gabriela Riet-Correa^{2*} 

ABSTRACT- Prado R.G.S., Domiciano T.A.O., Paredes L.J.A, Bezerra Jr P.S., Pereira W.L.A., Cerqueira V.D., Driemeier D. & Riet-Correa G. 2019. **Nervous form of listeriosis in buffaloes.** *Pesquisa Veterinária Brasileira* 39(5):299-303. Programa de Pós-Graduação em Saúde Animal na Amazônia, Universidade Federal do Pará, BR-316 s/n, Castanhal, PA 68740-000, Brazil. E-mail: griet@ufpa.br

Listeriosis is a disease that affects several animal species, including humans, and has three different forms of presentation: encephalic, reproductive, or septicemic. The nervous form is caused mainly by the bacterium *Listeria monocytogenes*. In Brazil, this disease has already been described in sheep, goats, and cattle. There are no reports of the disease in buffaloes in Brazil and worldwide. The objective of this study was to describe an outbreak of listeric meningoencephalitis in buffaloes in the state of Pará, Brazil. The outbreak occurred in a property located in the municipality of Bujaru, in the eastern Amazon, from May to July 2016. In a herd of 47 buffaloes, three animals (Cases 1, 2 and 3), aged <40 days, presented a neurological condition with locomotion difficulty characterized by paralysis of the four limbs, hypoesthesia, lateral recumbency, and death. Morbidity was 6.38% and lethality was 100%. At necropsy, no significant macroscopic lesions were found. Samples of the central nervous system were collected, fixed in 10% buffered formalin, and routinely processed for histopathological analysis. The main microscopic changes observed were unilateral microabscesses in the brainstem composed predominantly of mononuclear cells, with fewer polymorphonuclear cells, and perivascular cuffs composed mostly of mononuclear cells and few neutrophils. Samples of Cases 1 and 2 revealed Gram-positive bacteria in the areas of necrosis by the Gram's stain technique. Samples of Case 1 were positive in immunohistochemistry for *L. monocytogenes*. Diagnosis of the nervous form of listeriosis was based on epidemiological data, clinical profile, and immunostaining for *Listeria monocytogenes*. Results showed that listeriosis should be considered in the differential diagnosis in buffaloes with nervous signs.

INDEX TERMS: Nervous form of listeriosis, listeriosis, buffaloes, *Listeria monocytogenes*, *Bubalus bubalis*, Pará state, Amazon region, bacterioses.

RESUMO.- [Listeriose nervosa em búfalos.] A listeriose é uma doença que afeta várias espécies animais, incluindo o homem, e possui três formas diferentes de apresentação:

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² Postgraduate Program in Animal Health in Amazon, Universidade Federal do Pará (UFPA), BR-316 s/n, Castanhal, PA 68740-000, Brazil. *Corresponding author: griet@ufpa.br

³ Universidade da Amazônia (Unama), Faculdade de Medicina Veterinária, Centro de Ciências Biológicas e Saúde, Av. Alcindo Cacela 278, Umarizal, Belém, PA 66065-205, Brazil.

⁴ Setor de Patologia Veterinária, Departamento de Patologia Clínica Veterinária, Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul (UFRGS). Av. Bento Gonçalves 9090, Prédio 42505, Porto Alegre, RS 91540-000, Brazil.

nervosa, abortiva ou septicêmica. A forma nervosa é causada principalmente pela bactéria *Listeria monocytogenes*. No Brasil a doença já foi descrita em bovinos, ovinos e caprinos, mas não foram encontrados relatos desta doença em búfalos no Brasil e no mundo. O objetivo deste trabalho foi descrever um surto de listeriose nervosa em búfalos no estado do Pará, Brasil. O surto ocorreu de maio a julho de 2016, em uma propriedade localizada no município de Bujaru, na Amazônia Oriental. Três bubalinos de um total de 47 animais (Casos 1, 2 e 3), menores de 40 dias, apresentaram um quadro clínico neurológico caracterizado por dificuldade de locomoção, paralisia dos quatro membros, diminuição da sensibilidade cutânea, decúbito lateral e morte. A morbidade foi de 6,38% e a letalidade de 100%. Na necropsia não foram encontradas lesões macroscópicas significativas. Amostras

do sistema nervoso central foram coletadas e fixadas em formalina tamponada a 10% e processadas rotineiramente para análise histopatológica. As principais alterações microscópicas observadas foram microabscessos unilaterais no tronco encefálico, compostos predominantemente por células mononucleares, com menor número de polimorfonucleares, e manguitos perivasculares compostos predominantemente por células mononucleares e poucos neutrófilos. Amostras dos Casos 1 e 2 revelaram bactérias Gram positivas nas áreas de necrose na técnica de Gram. Amostras do Caso 1 resultaram positivas na imuno-histoquímica para *L. monocytogenes*. O diagnóstico da forma nervosa da listeriose foi baseado nos dados epidemiológicos, no quadro clínico patológico e na imunomarcagem para *Listeria monocytogenes*. Os resultados demonstram que a listeriose deve ser considerada no diagnóstico diferencial em bubalinos com sinais nervosos.

TERMOS DE INDEXAÇÃO: Listeriose nervosa, búfalos, *Listeria monocytogenes*, *Bubalus bubalis*, Pará, região amazônica, bacterioses.

INTRODUCTION

Listeriosis is an infectious disease caused by the Gram-positive bacterium *Listeria monocytogenes* with worldwide distribution (George 2002). It affects several animal species, including humans. Three forms of this disease have been identified in domestic animals: septicemic, affecting ruminants, swine, rabbits, and young birds (Low & Renton 1985, Jones et al. 2000); reproductive, with abortion in sheep and bovines; encephalitic, which presents with meningoencephalitis in sheep, goats, and cattle (Low & Renton 1985, Schneider 1994, Summers et al. 1995, George 2002). Generally, only one of these three clinical forms is observed in listeriosis outbreaks. Only *L. monocytogenes* is associated with encephalitis outbreaks (Jones et al. 2000). *L. monocytogenes* presents worldwide distribution, occurring mainly in areas with temperate climate. *L. monocytogenes* inhabits the soil or decomposed vegetable matter, and can be isolated from the feces of several domestic and wild animals (George 2002). In Brazil, *L. monocytogenes* was isolated from the feces and liver of healthy and ill cattle (Hofer et al. 2005), demonstrating the epidemiological role of these potential sources of infection in cases of meningoencephalitis caused by *L. monocytogenes* in ruminants. In China, *Listeria* spp., including *L. monocytogenes* and *L. ivanovii*, were isolated from the feces of wild rodents, indicating a possible involvement of these animals in the epidemiology of human listeriosis in natural environments (Wang et al. 2017).

In ruminants, the encephalitic disease usually shows low rates of morbidity and high lethality (Vandegraaff et al. 1981, Rissi et al. 2006, 2010). In these cases, infection is often associated with silage feeding or contact with the feces or milk from carrier animals (Vandegraaff et al. 1981, Wilesmith & Gitter 1986, Rissi et al. 2006). In Brazil, most cases of meningoencephalitis caused by *L. monocytogenes* in ruminants occur in the hottest months of the year, and most often not in association with silage (Rissi et al. 2006, Sanches et al. 2000).

In Brazil, the encephalitic form of listeriosis in ruminants has already been described in cattle (Sanches et al. 2000, Konradt et al. 2017), goats (Rissi et al. 2006, Guedes et al. 2007, Konradt et al. 2017), and sheep (Mendes et al. 2005,

Ribeiro et al. 2006, Guedes et al. 2007, Rissi et al. 2010, Konradt et al. 2017), but this form of the disease has not been reported in buffaloes to date. Therefore, this study aimed to describe an outbreak of listeric meningoencephalitis in buffaloes.

MATERIALS AND METHODS

Clinical, epidemiological and necropsy data were provided by the veterinarian responsible for the buffalo herd in the property where the outbreak occurred.

Three Murrah buffaloes (Buffaloes 1, 2, and 3) aged <40 days were investigated in a farm located in the municipality of Bujaru in the microregion of Castanhal, Pará state, Brazil. The animals died spontaneously, were necropsied, and had samples collected from the central nervous system (CNS) and organs of the abdominal and thoracic cavities, which were fixed in 10% buffered formalin and sent to the animal pathology laboratories of the "Universidade Federal do Pará" (UFPA) (Buffaloes 1 and 2) and the "Universidade Federal Rural da Amazônia" (UFRA) (Buffalo 3).

The formalin fixed samples were sectioned and routinely processed for histopathological examination, sliced (5µ thick), and stained using the hematoxylin and eosin (HE) technique. In the CNS samples, sections of the spinal cord, brainstem (rostral and caudal colliculus, pons, and medulla oblongata), cerebral cortex, and cerebellum were analyzed. The Gram's stain technique was used in tissue sections of the pons in Cases 1 and 2.

Tissue sections of the pons of the three cases were embedded in paraffin and sent to the Department of Veterinary Pathology of the "Universidade Federal do Rio Grande do Sul" (UFRGS) for immunohistochemistry (IHC) for *L. monocytogenes*. IHC staining was performed according to the protocol described by Konradt et al. (2017), using a primary monoclonal antibody to *L. monocytogenes* (BD®, New Jersey) in a 1:200 dilution, in phosphate-buffered saline (PBS). Antigen retrieval was obtained with citrate buffer (pH 6.0) for 10 min in microwave at maximum power, and background reactions were blocked using a 5% skim milk solution. The samples were incubated with primary antibodies overnight (18h) at 4°C. The streptavidin-biotin method (LSAB-DAKO) and the substrate chromogen (DAB), followed by hematoxylin counterstaining, were used to develop the reaction. As positive control, positive samples were used in the IHC for *L. monocytogenes*. Tissue sections incubated in PBS were used as negative control instead of primary antibody.

RESULTS

The outbreak occurred during May-June, 2016 in buffaloes of a dairy farm located in the municipality of Bujaru (1° 37' 38.7" S; 48° 13' 21.3" W), microregion of Castanhal, Pará state, Brazil. This property held a herd of 750 Murrah buffaloes: 630 bulls and 120 calves. Calves were divided into batches according to age. Animals belonging to two batches sickened: Batches 1 and 2 composed of 12 and 35 animals, respectively, aged <40 days, kept in stalls with access to *Brachiaria brizantha* and *Panicum maximum* (cv. mombaça) pastures. Twice a day, the calves were placed with the suckling cows (after milking) and received commercial feed (approximately 400g per animal). Other animal species, such as chickens, horses and bulls, had access to the stalls and pastures where the calves were kept. In the farm where the outbreak occurred, the milk produced was used to make artisanal fresh cheese.

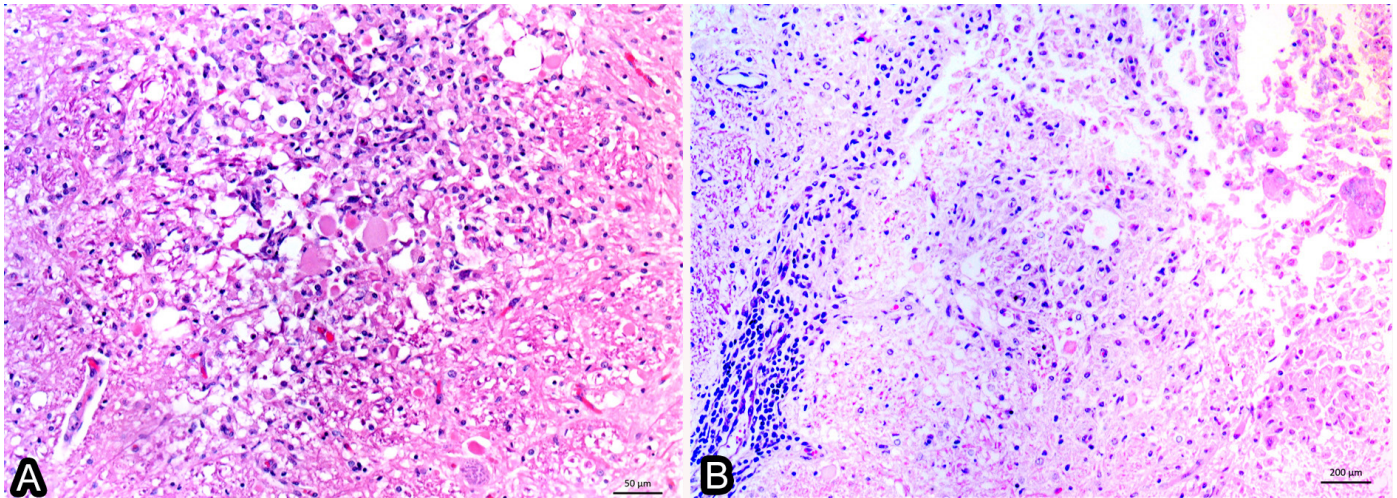


Fig.1. (A) Microabscess composed mainly of mononuclear cells, with few neutrophils. Axonal spheroids and neuropil vacuolation in brainstem of Buffalo 2. HE, obj.20x. (B) Malacia area with infiltration of Gitter cells, with some multinucleated cells, and adjacent mononuclear perivascular cuffs in brainstem of Buffalo 1. HE, obj.20x.

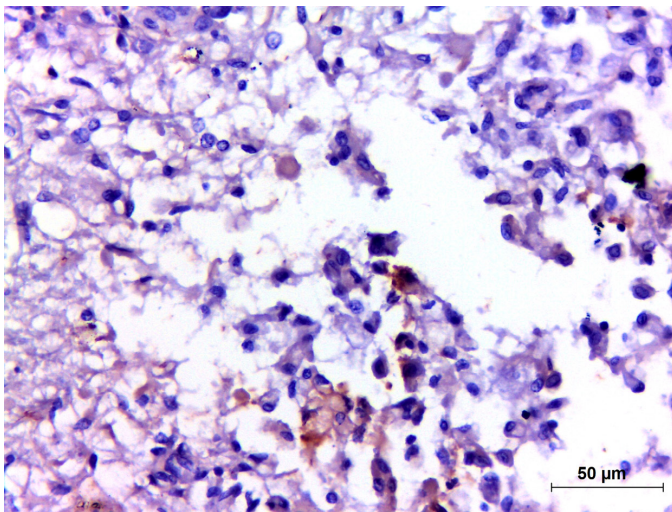


Fig.2. Anti-*Listeria monocytogenes* marking in the cytoplasm of macrophages in brainstem of Buffalo 1. IHC, obj.40x.

Two animals from Batch 1 (Calves 1 and 2) and one animal from Batch 2 (Calf 3) sickened and died. Morbidity was 6.38% (3/47) and lethality was 100%. According to the veterinarian, the three animals (Buffaloes 1, 2, and 3) presented clinical neurological condition consisting of mainly of ataxia, paralysis of the four limbs, decreased skin sensibility, lateral recumbency, and death, with 24-48h evolution. Sick buffaloes were treated with penicillin (20000UI/kg/bodyweight every 24h), but did not respond to the treatment. At necropsy, no significant macroscopic lesions were observed. Histopathology of the brainstem, tissue sections of the pons, showed multifocal, random microabscesses predominantly composed of mononuclear cells, with some polymorphonuclear cells (Fig.1A), and perivascular cuffs composed mostly of mononuclear cells and few neutrophils (Buffaloes 1, 2 and 3) (Fig.1B). In the areas adjacent to the microabscesses, neuronal necrosis, microgliosis, swollen astrocytes (Buffaloes 1 and 2), and fibrinoid necrosis

with thrombosis in blood vessels (Buffalo 2) were observed. Histopathology of tissue sections of the pons also showed Wallerian degeneration (Buffaloes 1 and 2) and malacia areas with neuropil vacuolation, with infiltration of Gitter cells, with some multinucleated cells (Fig.1B). In the cerebral cortex, areas of malacia were observed with gliosis (Buffalo 1) and mononuclear infiltrate in the meninges, which projected to the deep part of the white and gray matter (Buffaloes 1 and 2). The spinal cord showed Wallerian degeneration and unilateral demyelination in the lateral dorsal funiculi (Buffaloes 1 and 2) and unilateral (Buffalo 2) or bilateral (Buffalo 1) in the lateral ventral funiculi. There were also discrete perivascular cuffs (Buffaloes 1 and 2) and mixed inflammatory infiltrate with mononuclear and polymorphonuclear cells in the meninges (Buffalo 2). Discrete meningitis with mixed infiltrate was observed in the cerebellum of Buffalo 3. In this animal, changes in autolysis ranged from moderate to severe. No significant changes were found in the other organs.

The Gram's stain technique performed in tissue sections of the pons from Buffaloes 1 and 2 revealed Gram-positive cocci in the malacia area and adjacent neuropil. IHC was positive in the microabscesses of Buffalo 1, with moderate multifocal staining in the cytoplasm of macrophages and adjacent debris (Fig.2), whereas no staining was observed in Buffaloes 2 and 3.

DISCUSSION

In the present study, diagnosis of listeric meningoencephalitis was based on epidemiological data and on the pathological clinical condition of the animals, and was confirmed by immunostaining for *Listeria monocytogenes*. The histological changes observed in the buffaloes involved in the present outbreak are similar to those described in the literature in cattle and small ruminants (Oevermann et al. 2010, Headley et al. 2013).

The most significant lesions occurred in the brainstem, and were found unilaterally and characterized mainly by microabscesses, areas of malacia, and perivascular cuffs, similar to what has been observed in cattle (Sanches et al. 2000,

Oevermann et al. 2010, Margineda et al. 2012), goats (Rissi et al. 2006, Headley et al. 2013), and sheep (Rissi et al. 2010, Headley et al. 2013).

Microabscesses are considered as typical of this disease, and are formed by neutrophil infiltrates, with varying numbers of macrophages. The cell types that compose the microabscesses enable assessment of encephalopathy progression, classifying it as acute, when the microabscess presents a larger number of neutrophils, whereas in the most advanced cases, macrophages prevail over neutrophils and there is presence of multinucleated cells (Oevermann et al. 2010). In the present report, despite the short evolution time of the disease, which varied from 24 to 48h, the microabscesses were formed by large numbers of macrophages and giant multinucleated cells and few neutrophils. Oevermann et al. (2010) observed that the microabscesses show a larger number of macrophages in cattle than in small ruminants, and that occurrence of giant multinucleated cells, as observed in the present study, is significantly higher in cattle compared with sheep and goats. Perivascular cuffs were composed predominantly of mononuclear cells with rare polymorphonuclear cells, and extended from the brainstem to the cerebral cortex and spinal cord, findings also observed in cattle (Galiza et al. 2010, Margineda et al. 2012) and small ruminants (Campero et al. 2002, Headley et al. 2013).

When the neurological symptoms and the histopathological lesions are suggestive of listeriosis, diagnosis can be confirmed by culture, detection of the microorganism by special staining (Gram's stain), IHC, or polymerase chain reaction (PCR) (Campero et al. 2002, Loeb 2004). In this study, Gram-positive bacteria compatible with *Listeria* spp. were observed in the lesions of Buffaloes 1 and 2. However, only one of the animals tested positive by IHC. This finding differs from those reported in other studies conducted with cattle and small ruminants (Rissi et al. 2006, 2010, Oevermann et al. 2010, Margineda et al. 2012), which showed positive results by IHC in all of the animals analyzed. Nevertheless, differences in IHC staining have been observed in cases of listeriosis in goats, ranging from strong to absent, depending on the time of lesion evolution (Rissi et al. 2006). Pfister et al. (2002) demonstrated that, in listeriosis, the production capacity of nitric oxide is higher in cattle compared with that in small ruminants and that this substance may result in weak or absent immunostaining for the disease antigen, considering that it regulates intracellular bacterial growth. This fact could explain the absence of staining in Buffaloes 2 and 3. Another factor that may have interfered with the immunostaining in Buffalo 3 is the autolysis of the material available for analysis. In Buffaloes 2 and 3, the diagnoses were based on clinical signs, histopathological lesions suggestive of the disease, and similarity between the clinical and pathological condition of these animals and that observed in Buffalo 1, which was positive by IHC.

The encephalic form of listeriosis affects mainly ruminants regardless for gender and age, and the disease is most frequently observed in the first three years of life (Gray & Killinger 1996). In the present study, the disease affected calves aged <40 days. The main clinical signs observed by the veterinarian were ataxia, limb paralysis, lateral recumbency, and death, similar to those described for cattle (Margineda et al. 2012) and small ruminants (Rissi et al. 2006, 2010). Morbidity (6.38%) and

lethality (100%) were also similar to those described for these species. In Brazil, listeric meningoencephalitis has been reported mainly in ruminants under extensive farming with no history of feeding on silage, and occurring in the hottest months of the year (Rissi et al. 2010, Sanches et al. 2010) or in the transition period between the rainy and dry seasons (Guedes et al. 2007), epidemiological characteristics similar to those observed in the present outbreak, which occurred in the transition between the rainy and dry seasons in the region.

It was not possible to determine the source of infection in the present study, but chickens, horses, and bulls had contact with the calves, including access to the troughs where the feed was provided, and thus could serve as a source of infection. In outbreaks of listeriosis in goats and sheep in the state of Rio Grande do Sul, contaminated pasture or feed and direct contact with feces from other species such as chickens and pigs were considered as possible sources of infection (Rissi et al. 2006, 2010). According to George (2002), cases of meningoencephalitis not related to feed are commonly associated with environmental contamination. Clinically healthy bovines are carriers of *L. monocytogenes* and eliminate the bacterium along with their feces (Wesley 1999). Another source of infection that should be considered in this outbreak is the mother's milk, since for cattle elimination of *L. monocytogenes* through milk has been described after mastitis or abortions (Quinn et al. 2001).

Because the possibility of listeriosis was not considered during the clinical care of the animals, the oral cavities were not analyzed at necropsy. However, a possible gateway of the bacterium to the central nervous system could be the eruption of the teeth. In buffaloes, the first three pairs of primary incisor teeth erupt within the first three months of life, with the first pair erupting up to the 7th day and the others with intervals ranging from 14 to 30 days of life (Seixas et al. 2007), a period that coincides with the age, sickening, and death of the animals of the present study.

This is the first report of encephalic listeriosis in buffaloes, as well as the first report of disease occurrence in ruminants in the Brazilian Amazonia. The diagnosis of listeric meningoencephalitis in dairy buffaloes highlights the importance of these animals and their products as possible sources of infection by *L. monocytogenes* for humans. Consumption of bovine raw milk, pasteurized milk, and cheese, usually soft, fresh or mature, has been associated with outbreaks of human listeriosis (Silva et al. 2011).

CONCLUSIONS

The encephalic form of listeriosis occurs in buffaloes in the state of Pará, Brazil, and this disease should be considered in the differential diagnosis of buffaloes with neurological syndrome.

Occurrence of listeric meningoencephalitis in a herd of dairy buffaloes warns about the importance of this zoonosis, especially when the milk is used to produce artisanal fresh cheese.

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Osseous malformation in calves and its relationship with ingestion of apple pomace in pregnant cows¹

Nathalia S. Wicpolt² and Aldo Gava^{2*} 

ABSTRACT.- Wicpolt N.S. & Gava A. 2019. **Osseous malformation in calves and its relationship with ingestion of apple pomace in pregnant cows.** *Pesquisa Veterinária Brasileira* 39(5):304-307. Laboratório de Patologia Animal, Departamento de Medicina Veterinária, Centro de Ciências Agroveterinárias, Universidade do Estado de Santa Catarina, Avenida Luiz de Camões 2090, Lages, SC 88520-000, Brazil. E-mail: aldo.gava@udesc.br

Three outbreaks of osseous malformation in calves born of cows fed apple pomace throughout pregnancy are described. This study was carried out from historical surveys on properties where apple pomace was used in cattle feeding. The outbreaks occurred in the municipalities of Lages, Santa Catarina state, from 2007 to 2012, and Ipê, Rio Grande do Sul state, from 2011 to 2012. These calves presented bone deformity characterized mainly by arthrogryposis and chondrodysplasia of the anterior limbs. At necropsy, the observed changes were restricted to bending and thickening of the anterior limbs and skull. Microscopically, a disorganized, irregular, misaligned hypertrophic zone was observed in the humeral-radio-ulnar joints, in addition to absence of growth zone, thin bone trabeculae, and closed epiphyseal growth plate.

INDEX TERMS: Osseous malformation, calves, ingestion, apple pomace, pregnant cows, cattle, pathology.

RESUMO.- [Malformações ósseas em bezerros e sua relação com a ingestão de bagaço de maçã por vacas prenhes.] Descrevem-se 3 surtos de malformações ósseas em bezerros, nascidos de vacas prenhes alimentadas com bagaço de maçã. Estudos foram realizados a partir de levantamento de históricos em propriedades onde bagaço de maçã foi utilizado na alimentação de bovinos. Os surtos ocorreram nos municípios de Lages em Santa Catarina, de 2007 a 2012, e Ipê em Rio Grande do Sul, de 2011 a 2012. Dentre 20 e 69,2% dos bezerros apresentavam deformidade de membros, caracterizada principalmente por membros anteriores curvos e curtos. Na necropsia as alterações observadas restringiam-se ao encurvamento e engrossamento dos membros e do crânio. Microscopicamente nas articulações úmero-radio-ulnar, foi observado zona hipertrófica desorganizada, irregular e sem alinhamento, além de ausência da zona de crescimento e trabéculas ósseas finas e placa epifisária fechada.

TERMOS DE INDEXAÇÃO: Malformação óssea, bezerros, ingestão, bagaço de maçã, vacas prenhes, bovinos, patologia.

INTRODUCTION

In the south region of Brazil, apples (*Malus* spp.) are grown on a large scale, and pomace is the main by-product generated in the agroindustry, consisting of skin and flesh (94.5%), seeds (4.4%), and stem (1.1%) (Kennedy et al. 1999), used as an alternative energy source in the feeding of ruminants, mainly bovines (Manterola et al. 1999). Consumption of this by-product by cows during pregnancy may be associated with the birth of calves with osseous malformation indicative of arthrogryposis and chondrodysplasia.

The conditions under which apple pomace can produce such malformations are still unknown. Arthrogryposis is characterized by permanent muscle contracture of the limbs in flexion or extension and by muscular atrophy (Schild 2007), and it is generally bilateral and symmetrical (Zachary 2018). Chondrodysplasia (dwarfism) is a congenital malformation hereditarily transmitted in most cases, characterized by the abnormal development of interstitial growth cartilage (endochondral ossification) of the long bones, resulting in abnormal and diminished longitudinal growth of the bones (Latter et al. 2006, Thompson 2007). Some authors refer to these syndromes as part of a set of abnormalities named

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² Laboratório de Patologia Animal, Departamento de Medicina Veterinária, Centro de Ciências Agroveterinárias, Universidade do Estado de Santa Catarina, Avenida Luiz de Camões 2090, Conta Dinheiro, Lages, SC 88520-000, Brazil.

*Corresponding author: aldo.gava@udesc.br

congenital chondrodystrophy of unknown origin (CCUO) (White et al. 2010a, 2010b, White & Windsor 2012).

This study aimed to report the occurrence of three outbreaks of osseous malformation in calves born of cows fed apple pomace and describe their epidemiological, clinical and pathological factors.

MATERIALS AND METHODS

An epidemiological survey was conducted in three farms, with a history of malformation in calves born of cows fed apple pomace during pregnancy, located in the municipalities of Lages, Santa Catarina state, and Ipê, Rio Grande do Sul state. Three affected calves were necropsied and had tissue samples collected from the central nervous system, heart, lung, kidney, liver, spleen, rumen, reticulum, omasum, abomasum, intestine, skeletal muscle, and bone, and fixed in 10% formalin for histological examination. The material was routinely processed, stained using the hematoxylin-eosin (HE) technique (Prophet et al. 1992), and assessed by optical microscopy.

RESULTS

The outbreaks occurred in three rural properties: two located in the municipality of Lages, Santa Catarina state (Properties 1 and 2) and one in the municipality of Ipê, Rio Grande do Sul state (Property 3). In the three outbreaks, the cows were raised for beef and were of the following breeds: crossbred cows x Charolais bull in Property 1, crossbred cows x Limousin bull in Property 2, and Red Angus cows and bull in Property 3. In the three farms, the cows grazed on native pasture and were supplemented with apple pomace at a dose of 20-30kg/animal/day. In Property 1, apple pomace was first fed to cows in 2007 and, according to the owner, since it began to be used for a herd of 30 cows, 15-20% of the calves born each year presented malformations. In 2012, 19 calves were born with malformations, five died, and one was necropsied. In Property 2, apple pomace was provided to six cows throughout pregnancy. Of the six calves born of these cows, three showed serious osseous malformations. Two of these calves died and one was euthanized for necropsy. In Property 3, in 2011, of the 13 cows that received apple pomace during pregnancy, 9 calves were born with osseous malformation, 5 of which were born dead. In 2012, nine calves with malformation were born of 17 cows. At the same property, a second herd of cows that did not receive apple pomace in the diet, but were kept in a nearby area, bred normal calves. According to reports obtained in the three properties, the disease appeared after introduction of apple pomace in the feed. The apple pomace was stored in silo and, after fermenting for a week, was gradually fed to the cattle in the trough until reaching the dose of 30kg/animal/day. The owners of Properties 1 and 3 reported that they exchanged the mating bull of the cows, but the problem persisted.

The main changes observed in the calves were similar in the three properties. They were characterized by enlarged humeral-radio-ulnar joints (Fig.1). Some calves, in addition to changes in the limbs, had a disproportionate skull and short muzzle (Fig.2). Some calves could not move and generally died a few days after birth and/or were euthanized. Those born with discrete lesions, although bending of the anterior and posterior limbs disappeared, showed proportional dwarfism after a few months. Necropsy findings were restricted to

the bone system, which presented curved forelimb bones of enlarged diameter (Fig.3A,B). Histological examination showed disorganized, irregular and misaligned hypertrophic zone, thin bone trabeculae, and closed epiphyseal plate (Fig.4).



Fig.1. Natural poisoning with apple pomace. Calf with shortened and curved forelimbs and enlarged joints.



Fig.2. Natural poisoning with apple pomace (Property 3). Calf with head disproportionate to the body (larger) and short muzzle. Curved and shortened anterior and posterior limbs and enlarged joints.

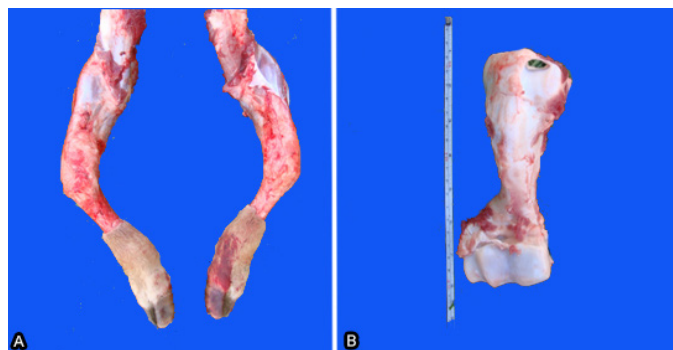


Fig.3. Natural poisoning with apple pomace. Calf necropsy (Property 2). (A) Curved bones of the forelimbs and (B) humerus with enlarged diameter.

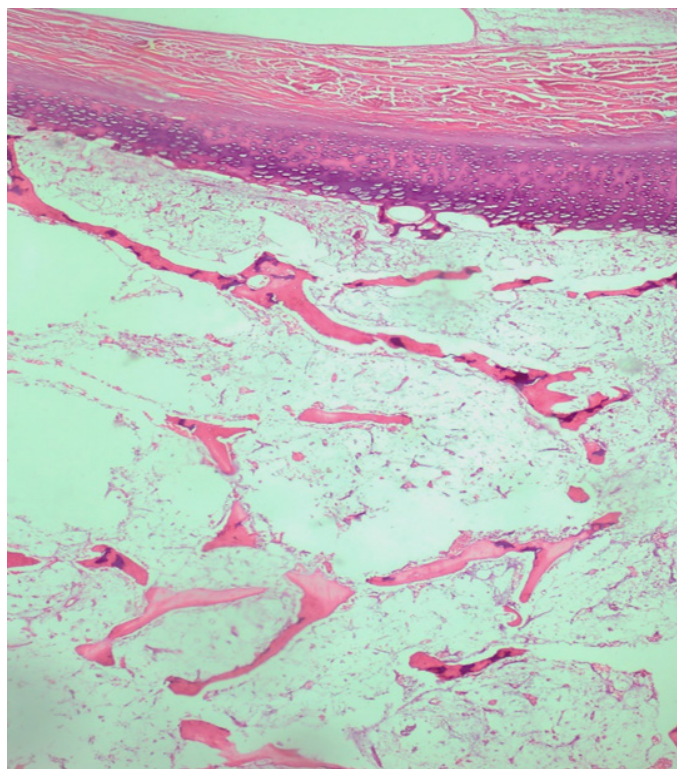


Fig.4. Natural poisoning with apple pomace. Disorganized, irregular and misaligned hypertrophic zone, thin bone trabeculae, and closed epiphyseal plate in humerus. HE, obj.10x.

DISCUSSION

Diagnosis of osseous malformations in calves associated with ingestion of apple pomace by pregnant cows was based on epidemiological and macro- and microscopic clinical findings. In the present study, after introduction of apple pomace in feed, most of the cows bred calves with malformations. In the assessed properties, occurrence of the disease coincided with the use of apple pomace at expressive doses (25-30kg/animal/day). Absence of abortions and persistence of the disease after the exchange of the mating bull reinforces the hypothesis that apple pomace was the cause of osseous malformation. The use of apple pomace was interrupted in all of the farms, and the disease did not occur within the following two years.

Most of the times, cases of osseous malformation are sporadic and of difficult etiology. They may be of hereditary origin, transmitted by autosomal, recessive gene in Holstein Friesian cattle (Marcolongo Pereira et al. 2010), or associated with infectious agents such as the bluetongue (BTV) (Riet-Correa 2007) and bovine viral diarrhea (BVDV) (Pavarini et al. 2008) viruses. The Border Disease, Cache Valley (Radostits et al. 2000), and Akabane (Konno & Nakagawa 1982) viruses have not been detected in Brazil so far. These diseases can be discarded in the present study since pregnant cows that were in the same properties and did not ingest apple pomace bred healthy calves. There are no reports of osseous malformation associated with ingestion of apple pomace in Brazil. In other countries, this condition has been observed in calves born of cows that fed on this by-product, corn silage, and non-protein nitrogen (NNP) (Bovard et al. 1977, Rumsey et al. 1977). Although the etiology of these malformations has not

been defined, Valero et al. 1990 suggested that manganese deficiency may produce osseous malformation in calves, and the low concentration of manganese in apple pulp may be the cause of these malformations. According to Rumsey et al. 1977, these malformations may also be associated with agrochemicals frequently used in apple culture, which may remain as residue in the bagasse. The National Agency of Sanitary Surveillance (Anvisa 2016) lists over 60 different types of pesticides for pest control in apple crops, including carbamates and organophosphates, which are cholinesterase inhibitors and lipophilic insecticides widely used in the control of pests in animals and plants. Their solubility characteristics facilitate their excretion through milk and passage through the placental barrier. Exposure of neonates to these insecticides at concentrations that do not show clinical signs of maternal systemic intoxication can cause harm to the developing individual (Grecco et al. 2009, Oliveira-Filho et al. 2010). Another product often used in apple trees is abamectin, which is also used as parasiticide in animals and, at doses higher than indicated, can produce neurological changes and death in cattle (Seixas et al. 2006).

Congenital defects may also occur as a result of consumption of plants. According to Dantas et al. (2010), *Mimosa tenuiflora* leads to facial malformation with bragnatia, cleft lip and palate, corneal opacity, arthrogryposis, scoliosis, and lordosis in ruminants in northeast Brazil. Other malformations might occur in calves born to cows that ingest *Lupinus* sp., *Nicotiana glauca*, and *Nicotiana tabacum*, and fetuses may show signs of torticollis, arthrogryposis, scoliosis, excessive flexion of the carpus, lordosis, cleft palate, and head deformity (Kellerman et al. 1990, Panter et al. 1998). Ingestion of *Veratrum californicum* by sheep during the 14th day of gestation will result in lambs with cyclopia, holoprosencephaly, and absence of the pituitary gland, after the 14th day of gestation, results in several skeletal anomalies and palatine clefts (Zachary 2018); ingestion of *Conium maculatum* causes arthrogryposis, among other clinical signs (Tokarnia et al. 1985, 2012). None of these teratogenic plants were found in the properties where the outbreaks occurred.

CONCLUSION

Pregnant cows fed apple pomace at the dose of 20-30kg/animal/day during the gestational period may breed calves with osseous malformations. When these changes are less severe, calves can evolve to improved clinical conditions, but reach adult age with reduced size.

Conflict of interest statement. - The authors declare that there are no conflicts of interest.

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***Staphylococcus aureus* and *Streptococcus agalactiae*: prevalence, resistance to antimicrobials, and their relationship with the milk quality of dairy cattle herds in Minas Gerais state, Brazil¹**

Alan A. Mesquita² , Christiane M.B.M. Rocha³, Fabio R.P. Bruhn⁴,
Dircéia A.C. Custódio³, Mirian S. Braz³, Sandra M. Pinto⁴, Délcio B. Silva⁶
and Geraldo M. Costa^{3*} 

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Bovine mastitis is the most frequent disease worldwide in dairy herds, causing high economic losses to producers and industry, as well as having implications for public health due to the zoonotic potential of some agents involved in its etiology and the increased risk of antimicrobial residues in milk and its derivatives. Considering the multifactorial aspect of this disease, knowledge of the agents involved in its etiology and their antimicrobial susceptibility profiles is very important. This study was conducted with 306 dairy herds from the Campo das Vertentes region, located in the south of Minas Gerais state, whose owners were milk suppliers to a dairy in the same region. The study involved approximately 34,000 dairy cows and covered an area of approximately 12,564 km². In these herds, prevalence rates of *Staphylococcus aureus* and *Streptococcus agalactiae* and their relationship with bulk milk somatic cell counts (BMSCC), total bacterial counts (TBC), and daily production were evaluated. In addition, analyses of resistance of these pathogens to the antimicrobials most commonly used in the treatment of mastitis in dairy herds were performed. Microbiological analyses of milk samples from collect from bulk milk tanks were performed aiming to evaluate the prevalence of *S. aureus* and *S. agalactiae*. For these proposes, the modified Baird-Parker Agar medium was used for detection of *S. aureus* and the modified Edwards Agar medium, enriched with 5% defibrinated sheep blood, was used for detection of *S. agalactiae*. The disc diffusion technique was applied to evaluate antimicrobial resistance. Results show high prevalence rates of *S. aureus* (70.3%) and *S. agalactiae* (67.0%) in the dairy farms studied, with 47.71% of the herds showing both pathogens. Associations between BMSCC and the presence of pathogens *S. aureus* and *S. agalactiae* and between TBC and the presence of *S. agalactiae* were observed, demonstrating the influence of these pathogens in milk quality. No variation was observed in the distribution of *S. aureus* and *S. agalactiae* in the different strata of daily production. High levels of resistance and multi-resistance were observed among the pathogens *S. aureus*

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² Departamento de Zootecnia, Universidade Federal de Lavras (UFLA), Campus Universitário, Cx. Postal 3037, Lavras, MG 37200-000, Brazil.

³ Departamento de Medicina Veterinária, UFLA, Campus Universitário, Cx. Postal 3037, Lavras, MG 37200-000. *Corresponding author: marciocostavet@gmail.com

⁴ Faculdade de Veterinária, Universidade Federal de Pelotas (UFPEL), Cx. Postal 354, Campus Universitário, Capão Leão, RS 96010-900, Brazil.

⁵ Departamento de Ciências dos Alimentos, UFLA, Campus Universitário, Cx. Postal 3037, Lavras, MG 37200-000.

⁶ Departamento de Medicina Veterinária, Instituto Federal de Educação, Ciência e Tecnologia do Sul de Minas, Campus Muzambinho, Estrada de Muzambinho, Morro Preto, Muzambinho, MG 37890-000, Brazil.

and *S. agalactiae*. The results indicate the need for more effective control measures for mastitis caused by *S. aureus* and *S. agalactiae* in the dairy herds of the region studied and more judicious use of antimicrobials in order to reduce the problem of resistance to them.

INDEX TERMS: *Staphylococcus aureus*, *Streptococcus agalactiae*, mastitis, antimicrobial resistance, milk quality, dairy herds, Minas Gerais, Brazil, cattle, bacterial diseases, bacterioses, pathology.

RESUMO.- [Staphylococcus aureus e Streptococcus agalactiae: prevalência, resistência aos antimicrobianos e suas relações com a qualidade do leite em rebanhos leiteiros de Minas Gerais, Brasil.]

A mastite bovina é a doença de maior frequência em rebanhos leiteiros em nível mundial, acarretando grandes prejuízos econômicos aos produtores e à indústria. Além disso, esta enfermidade tem implicações na saúde pública, devido ao potencial zoonótico de alguns agentes envolvidos em sua etiologia e por aumentar os riscos de resíduos de antimicrobianos no leite e derivados. Considerando o aspecto multifatorial da mastite bovina, o conhecimento dos agentes envolvidos em sua etiologia e os perfis de suscetibilidade aos antibióticos é de suma importância. O estudo envolveu 306 fazendas de leite da região de Campo das Vertentes, localizada no sul de Minas Gerais, cujos proprietários eram fornecedores de leite para um laticínio da região, totalizando aproximadamente 34.000 animais e abrangendo uma área aproximada 12.564 km². Nestes rebanhos, avaliaram-se a prevalência de *Staphylococcus aureus* e *Streptococcus agalactiae* e a relação destes agentes com os índices de contagem de células somáticas do leite do tanque de expansão (CCSt), contagem bacteriana total (CBT) e produção diária. Analisou-se também a resistência destes patógenos aos antimicrobianos mais comumente utilizados no tratamento da mastite em rebanhos leiteiros. Análises microbiológicas de amostras de leite dos tanques de expansão foram realizadas para se determinar as prevalências dos patógenos *S. aureus* e *S. agalactiae*. Para a detecção de *S. aureus*, utilizou-se o meio seletivo Ágar Baird-Parker modificado e para a detecção de *S. agalactiae*, o meio seletivo Ágar Edwards modificado, enriquecido com 5% de sangue ovino desfibrinado. Foi utilizada a técnica de difusão em discos para a avaliação de resistência aos antimicrobianos. Os resultados apontaram altas prevalências de *S. aureus* (70,3%) e de *S. agalactiae* (67,0%), com 47,71% dos rebanhos examinados apresentando ambos os agentes. Verificaram-se associações entre a CCSt e a presença dos patógenos *S. aureus* e *S. agalactiae*, e também entre a CBT e a presença de *S. agalactiae*, demonstrando a interferência negativa destes patógenos nestes quesitos de qualidade. Não se observaram variações nas distribuições dos patógenos *S. aureus* e nem *S. agalactiae* em função da produção diária das propriedades estudadas. Níveis elevados de resistência e de multirresistência foram observados para ambos os agentes. Os resultados apontam a necessidade de medidas mais efetivas de controle para *S. aureus* e *S. agalactiae* nos rebanhos da região estudada e do uso mais criterioso dos antimicrobianos, visando minimizar o problema da resistência aos mesmos.

TERMOS DE INDEXAÇÃO: *Staphylococcus aureus*, *Streptococcus agalactiae*, mastite, resistência a antimicrobianos, qualidade do leite, rebanhos leiteiros, Minas Gerais, Brasil, bovinos, bacterioses, patologia.

INTRODUCTION

Bovine mastitis is considered the main disease in dairy cattle farming worldwide (Lopes et al. 2012, Ruegg 2012, Costa et al. 2013). It is an infectious process usually caused by the presence of pathogenic microorganisms in the mammary gland, affecting the production and composition of quarter milk, with reflexes mainly in somatic cell counts (SCC), total bacterial counts (TBC), and contents of casein and fat (Freitas et al. 2005, Santos & Fonseca 2007, Langoni et al. 2017). Bovine mastitis can also cause economic losses owing to expenses with veterinary medicines and services and, in some cases, to death or early discard of affected animals (Oviedo-Boyso et al. 2007, Lopes et al. 2011, 2012). In addition, agents involved in its etiology, which in many cases are multi-resistant, may pose a risk to public health because of their involvement in food poisoning, and these microorganisms may carry resistance genes that can be horizontally transferred to bacteria in the human microbiota (De Vliegher et al. 2012, Forsythe 2013).

Bovine mastitis can be caused by different agents, including bacteria, viruses, fungi, and algae (Ranjan et al. 2006, Reyher et al. 2012). Among the bacteria, *Staphylococcus aureus* and *Streptococcus agalactiae* are the most frequently isolated pathogens in intramammary infections in Brazilian cattle. Previous studies have reported that *S. agalactiae* was found in 60% of the rural properties in the Zona da Mata region in Minas Gerais state (Brito et al. 1999) and in 39.7% of the farms in different municipalities of this state (Elias et al. 2012). Oliveira et al. (2013) studied the etiology of mastitis in approximately 6,000 lactating dairy cows from 112 herds of farms located in the states of Minas Gerais and Rio de Janeiro, and they found prevalence of *S. aureus* and *S. agalactiae* in 93.0% and 41.0% of the herds assessed, respectively. Cunha et al. (2015), in a study conducted in the municipality of Viçosa, in the Zona da Mata region of Minas Gerais state, observed prevalence of subclinical mastitis of 55.4% in the dairy herds evaluated, with *Corynebacterium* sp. (32.99%), *S. aureus* (28.35%), and *S. agalactiae* (13.66%) as the most frequent pathogens.

Combating bovine mastitis presents a high demand for the use of antibiotics, which are administered mainly for the treatment of clinical cases and in the dry cow therapy (Erskine et al. 2004); however, when antibiotic therapy is conducted indiscriminately, it becomes a determining factor for the selection of resistant strains. Antimicrobial resistance associated with indiscriminate use of antibiotics and management failures are the major obstacles to the success of antibiotic therapy in combating mastitis (Medeiros 2015); this combination can disseminate resistant strains in the environment and cause serious public health problems (Santos et al. 2006, OIE 2010). Therefore, it is important to emphasize that these medicines should be used more judiciously and antibiogram tests should be performed to choose the most appropriate drugs for the treatment and

prevention of bovine mastitis (Diniz et al. 2010, Jain et al. 2012, Costa et al. 2013).

Considering the economic relevance of bovine mastitis and its implications on human and animal health, it is essential to monitor the agents most commonly involved in its etiology, as well as the resistance indices of these pathogens to the antimicrobials used to combat them, thus enabling monitoring of their resistance dynamics and adoption of more effective therapeutic protocols for their control. In this context, the present study aimed to evaluate the prevalence rates of *Staphylococcus aureus* and *Streptococcus agalactiae* in dairy herds from Campo das Vertentes region, located in the south of Minas Gerais state, whose owners were milk suppliers to a dairy in the same region, and their relationship with bulk milk somatic cell counts (BMSCC), total bacterial counts (TBC), and daily production, as well as evaluate the resistance of these pathogens to the antimicrobials most commonly used in the treatment of mastitis in dairy cattle.

MATERIALS AND METHODS

This study was conducted with 306 dairy herds from the Campo das Vertentes region, located in the south of Minas Gerais state, involving approximately 34,000 dairy cows and covering an area of approximately 12,564 km² (FAEMG 2006). The owners of these herds were milk suppliers to a dairy in the region and expressed an interest in participating in the study. Thus, study sampling for choosing the participating properties was conducted for convenience, and the inclusion criteria comprised the previous relationship of the owners with the dairy and the research unit.

A non-probabilistic, descriptive study was conducted to determine the prevalence rates of pathogens *Staphylococcus aureus* and *Streptococcus agalactiae* in all properties linked to dairy (306). To this end, milk samples of approximately 40mL were aseptically collected from the expansion tanks after homogenization by trained technicians of the dairy and they were stored in sterile vials at -20°C until analysis. Milk samples were collected directly from the bulk milk tanks of the farms, with a small time variation (<30 days).

For detection of *S. aureus*, a 50µL aliquot of each milk sample was cultured on Petri plates containing modified Baird-Parker Agar selective medium and incubated at 37°C for 24-48h. The samples that, after incubation, showed 1-5mm diameter, black colonies with glossy appearance, convex shape, and formation of two halos in their contour, indicating lipolytic and proteolytic activity, were considered positive for *S. aureus* (Siqueira 1995). For isolation of *S. agalactiae*, a 50µL aliquot of each milk sample was cultured on Petri plates containing modified Edwards Agar selective medium enriched with 5% defibrinated sheep blood. Samples that showed growth of 1-2mm diameter, bluish colonies with bright appearance and convex shape, with or without formation of opaque zone at their contour were considered positive. All dishes were incubated at 37°C for 24-48h. All cultures were performed in duplicate. From the cultures that tested positive, five colonies suggestive of each of the agents of interest (*S. aureus* and *S. agalactiae*) were randomly selected and submitted to confirmatory tests according to the National Mastitis Council (NMC 2004). After phenotypic characterization, the isolates were stored in Brain-heart infusion (BHI) broth containing glycerol (15% v/v) at -20°C until performance of the other assays.

The disc diffusion technique was applied to evaluate *in vitro* antimicrobial resistance according to the specifications of the Clinical and Laboratory Standards Institute (CLSI 2013). To this end, we used three isolates of *S. aureus* from 99 different properties randomly

selected among those that tested positive for this bacterium and three isolates of *S. agalactiae* from 93 different farms randomly selected among those that tested positive to this bacterium. The three isolates of each agent from each farm were used to compose a pool that was used to perform the antimicrobial susceptibility tests. The following antimicrobials were tested: ampicillin, cephalothin, cefoperazone, ceftiofur, enrofloxacin, florfenicol, gentamicin, lincomycin, neomycin, nitrofurantoin, novobiocin, polymyxin B, penicillin G, oxacillin, sulfamethoxazole-trimethoprim, tetracycline, streptomycin and ciprofloxacin. Isolates of *S. aureus* (ATCC-25923) and *Escherichia coli* (ATCC 25992) were used as control in the antibiogram tests. Based on the standards previously defined by the CLSI (2013), isolates were classified as susceptible, moderately susceptible, or resistant. Isolates classified as moderately susceptible were considered resistant for statistical calculations. The multiple antibiotic resistance (MAR) index was calculated from these results. The index MAR was determined by the percentage ratio between the number of antimicrobials for which the isolate tested resistant and the total number of antimicrobials tested. MAR ≥0.2 characterizes multi-resistance according to Krumperman (1983).

Data on total bacterial counts (TBC) and bulk milk somatic cell counts (BMSCC) were obtained at the dairy from the biweekly evaluations conducted in laboratories of the Brazilian Milk Quality Network (RBQL). Results of the five most recent TBC and BMSCC analyses of each property were used to obtain the arithmetic means of these factors, from which the existence of association with the microbiological data (presence/absence of pathogens *S. aureus* and *S. agalactiae*) of the farms was assessed.

Aiming to identify whether the presence of pathogens *S. aureus* and *S. agalactiae* was associated with the daily production of the farms and the impact of these agents on the TBC and BMSCC indices of the properties, we assessed the existence of a correlation between presence of pathogens *S. aureus* and *S. agalactiae* in different strata of daily milk production (≤100 L/day; >100 and ≤500 L/day; >500 and ≤1000 L/day; ≥1000 L/day), as well as in different mean scores of BMSCC (≤200 cell/mL; >200 and ≤500 cell/mL; >500 and ≤1,000 cell/mL; >1,000 cell/mL) and TBC (≤10 CFU/mL; >10 and ≤50 CFU/mL; >50 and ≤100 CFU/mL; >100 CFU/mL).

Statistical analyses were processed using the Statistical Package for the Social Sciences (SPSS, Chicago, USA) software, version 20.0 for Windows®. Correlations between the presence/absence of agents *S. aureus* and *S. agalactiae* and the BMSCC and TBC indices and the daily milk production of the farms were assessed using the Pearson's chi-squared test (χ^2) at 5.0% significance level. Since application of the Kolmogorov-Smirnov test showed that data distribution was not normal, the non-parametric Mann-Whitney test was used to verify the existence of difference in the distribution of the quantitative variables (daily milk production, BMSCC, and TBC) and between the qualitative variables (presence of *S. aureus* and *S. agalactiae*).

RESULTS AND DISCUSSION

Results of this study showed high prevalence rates of pathogens *Staphylococcus aureus* (70.3%) and *Streptococcus agalactiae* (67.0%) among the assessed dairy herds (306), with 47.71% showing presence of both agents, 89.54% showing presence of at least one agent, and only 10.45% farms showing absence of both agents (Table 1). These results corroborate the findings of previous studies in the literature. Brito et al. (1999) verified prevalence rates of 77.80% for *S. aureus* and 60% for *S. agalactiae* when analyzing 6,315 milk samples from 48 dairy herds in the regions of Zona da Mata and Campo das

Vertentes in Minas Gerais state. Arcuri et al. (2006) evaluated the microbiological quality of milk in 24 cattle herds in the southeast of Minas Gerais state and north of Rio de Janeiro state and observed prevalence rates of 91.66% for *S. aureus* and 50% for *S. agalactiae*. In a study conducted with 112 dairy herds in the states of Minas Gerais and Rio de Janeiro involving approximately 6,000 dairy cows, Oliveira et al. (2013) found prevalence rates of 93.0% and 41.0% for *S. aureus* and *S. agalactiae*, respectively.

The high prevalence rates observed in the present survey for pathogens *S. aureus* and *S. agalactiae* (Table 1) suggest that the control measures for infectious mastitis are not being correctly applied, a finding that corroborates the results reported by Rodrigues et al. (2017). This fact emphasizes the need to adopt specific measures for the control of these contagious pathogens that affect the daily production of herds and the milk quality, especially the BMSCC index.

Analyses of the BMSCC and TBC indices as a function of the daily milk production of the properties are presented in Tables 2 and 3. The legislation actual in Brazil - Normative

Instruction no. 07 (Brasil 2016) - establishes a limit up to 400,000 cell/mL for BMSCC. However, BMSCC scores >200,000 cell/mL are indicative of losses in milk production and quality, indicating subclinical mastitis at high levels in the herd (Barbosa et al. 2002, Coldebella et al. 2004, Magalhães et al. 2006).

Findings of the present study showed that 50% of the herds presented BMSCC $\geq 400,000$ cell/mL, with mean of 483,000 cell/mL. These results are in agreement with those reported by Rodrigues et al. (2017), who verified a geometric annual BMSCC mean in herds in southeastern Brazil of 518,000 cell/mL, with a confidence interval from 491,000 to 546,000 cell/mL; these results are similar to those found in the present study - 483,000 cell/mL. High levels of BMSCC can cause numerous losses to the dairy industry, because they directly affect the composition of milk and, consequently, reduce its shelf life and that of its derivatives (Silva & Nogueira 2010).

The results presented in Table 4 show that, among the farms with lower BMSCC scores, the prevalence rates of

Table 1. Prevalence of pathogens *Staphylococcus aureus* and *Streptococcus agalactiae* in the bulk milk tanks of dairy herds in the region of Campo das Vertentes/MG, 2017

Factor	Unit	Values	%	CI
<i>S. aureus</i>	Present	215	70.3	(24.9-35.1)
	Absent	91	29.7	
<i>S. agalactiae</i>	Present	205	67.0	(28.0-38.5)
	Absent	101	33.0	
Presence of both agents	Present	146	47.71	
Absence of both agents	Absent	32	10.45	
Presence of one or both agents	Present	274	89.54	

Table 2. Bulk milk somatic cell counts (BMSCC) of dairy herds in the region of Campo das Vertentes/MG, 2017

Daily production	BMSCC x 1,000 (cell/mL)				Herd total
	≤ 200	201-500	501-1,000	$\geq 1,000$	
≤ 100	10 (16.67%)	33 (55.00%)	12 (20.00%)	5 (8.33%)	60
100-500	18 (11.11%)	81 (50.00%)	55 (33.95%)	8 (4.94%)	162
500-1000	3 (6.98%)	28 (65.12%)	8 (18.60%)	4 (9.30%)	43
≥ 1000	3 (7.32%)	22 (53.66%)	13 (31.71%)	3 (7.32%)	41
Total	34 (11.11%)	164 (53.59%)	88 (28.76%)	20 (6.54%)	306

Table 3. Total bacterial counts (TBC) in bulk milk tanks of dairy herds in the region of Campo das Vertentes/MG, 2017

Daily production	TBC x 1,000 (CFU/mL)				Herd total
	≤ 10	11-50	51-100	≥ 101	
≤ 100	18 (30.00%)	29 (48.33%)	7 (11.29%)	6 (9.68%)	60
100-500	45 (27.77%)	86 (53.08%)	17 (10.56%)	14 (8.70%)	162
500-1000	14 (32.56%)	24 (55.81%)	3 (6.98%)	2 (4.65%)	43
≥ 1000	11 (27.50%)	23 (56.09%)	5 (12.50%)	2 (5.00%)	41
Total	89 (29.08%)	161 (52.61%)	32 (10.46%)	24 (7.84%)	306

Table 4. Prevalence of pathogens *Staphylococcus aureus* and *Streptococcus agalactiae* as a function of bulk milk somatic cell counts (BMSCC) of dairy herds in the region of Campo das Vertentes/MG, 2017

Variable	BMSCC x 1,000 (cell/mL)				Total
	≤ 200	201-500	501-1000	> 1000	
Presence of <i>S. aureus</i>	19 (55.88%)	114 (69.51%)	66 (75.00%)	17 (85.00%)	216 (70.30%)
Presence of <i>S. agalactiae</i>	15 (44.12%)	112 (68.29%)	65 (73.86%)	13 (65.00%)	205 (66.99%)
Presence of both agents	8 (23.53%)	76 (46.34%)	50 (56.82%)	12 (60.00%)	146 (47.71%)
Absence of both agents	8 (23.53%)	15 (9.15%)	7 (7.95%)	2 (10.00%)	32 (10.46%)

pathogens *S. aureus* and *S. agalactiae* are generally lower, and it can be observed that 23.53% of the properties with BMSCC <200,000 cell/mL showed absence of both agents, whereas only 10% of the farms with BMSCC >1,000,000 cell/mL had the same result. In the latter, the prevalence rates verified for pathogens *S. aureus* and *S. agalactiae* were 85% and 65%, respectively.

The BMSCC indices obtained in the present study suggest that improvements need to be made in the control of mastitis, especially regarding contagious pathogens, because the BMSCC scores observed in most properties are above those accepted in the international market, especially in the European Union, where the limit is <400,000 cell/mL, but with a decreasing trend in the coming years, since the national BMSCC scores in the Scandinavian countries ranged from 200,000 to 250,000 cell/mL (More 2009).

Current legislation (Brasil 2016) establishes a TBC limit of 100,000 CFU/mL for milk from bulk milk tanks. Among the properties assessed in the present study, 92.16% showed TBC ≤100,000 CFU/mL, with only 7.84% of them presenting scores >100,000 CFU/mL. Increased TBC indices can be caused by failures in the washing of the utensils that come in contact with the milk (milking machine and expansion tank), and faults in cleaning and antiseptics of udder prior to milking and in post-milking cooling, as well as by the presence of intramammary infection, especially when infected

with streptococci that cause subclinical mastitis (Santos & Fonseca 2007, Vargas et al. 2013). This fact may explain the increased TBC indices found in the herds affected by the *S. agalactiae* pathogen, as it can be verified in Table 5. Such finding corroborates the study by Zadoks et al. (2004), who reported increased TBC of milk from bulk milk tanks in dairy herds affected by *S. agalactiae*.

Prevalence of *S. aureus* was greater than that of *S. agalactiae* in all daily production strata, except for the 500-1,000 L/day stratum, in which a lower prevalence rate was observed for *S. aureus* (60.47%), with a prevalence rate of *S. agalactiae* of 67.44% (Table 6). The smallest prevalence rate for *S. agalactiae* was found in the daily production stratum of >1,000 L/day; however, no significant difference was verified with respect to any of the daily milk production strata ($p>0.05$). Also, no significant correlation was observed between the pathogens *S. aureus* and *S. agalactiae* ($p=0.601$), that is, they may be present in the herds regardless of each other.

No significant correlation was found between daily milk production and presence of pathogens (*S. aureus* and *S. agalactiae*) ($p>0.05$), which shows that they are equally distributed among the herds, regardless of the production stratum (Table 7). No association was verified between daily production and TBC ($p>0.05$) and between daily production and BMSCC ($p>0.05$), demonstrating that the daily production of the properties did not influence these variables (Table 7).

Table 5. Prevalence of pathogens *Staphylococcus aureus* and *Streptococcus agalactiae* in the different mean scores of total bacterial counts (TBC) in bulk milk tanks of dairy herds in the region of Campo das Vertentes/MG, 2017

Variable	TBC x 1,000 (CFU/mL)				Total
	≤10	11-50	51-100	≥101	
Presence of <i>S. aureus</i>	59 (66.29%)	120 (75.00%)	22 (40.00%)	15 (62.50%)	215 (70.59%)
Presence of <i>S. agalactiae</i>	51 (57.30%)	111 (69.38%)	26 (47.27%)	17 (70.83%)	205 (66.99%)
Presence of both agents	34 (38.20%)	85 (53.13%)	33 (60.00%)	21 (87.50%)	173 (56.54%)
Absence of both agents	13 (14.61%)	15 (9.38%)	2 (3.64%)	2 (8.33%)	32 (10.46%)

Table 6. Variation in the prevalence rates of *Staphylococcus aureus* and *Streptococcus agalactiae* and inadequacies in bulk milk somatic cell counts (BMSCC) and total bacterial counts (TBC), as a function of daily production, in bulk milk tanks of dairy herds in the region of Campo das Vertentes/MG, 2017

Daily production (L)	N(%)	Presence of <i>S. aureus</i> N(%)	Presence of <i>S. agalactiae</i> N(%)	BMSCC >500,000 cell/mL N(%)	TBC >100,000 CFU/mL N(%)
≤100	62 (20.26%)	46 (73.02%)	43 (68.25%)	19 (30.16%)	1 (1.59%)
100-500	163 (52.94%)	115 (70.55%)	111 (68.10%)	64 (53.27%)	5 (1.63%)
500-1000	42 (14.05%)	26 (60.47%)	29 (67.44%)	12 (27.91%)	1 (2.33%)
>1000	39 (12.75%)	28 (71.79%)	22 (56.41%)	14 (35.90%)	1 (2.56%)
All farms	306 (100%)	215 (70.30%)	205 (67.00%)	109 (35.62%)	8 (2.61%)

Table 7. Correlation between quantitative variables: daily production, bulk milk somatic cell counts (BMSCC), and total bacterial counts (TBC) of dairy herds in the region of Campo das Vertentes/MG that tested positive for *Staphylococcus aureus* and *Streptococcus agalactiae* in 2017

Variable	<i>S. aureus</i>		p value	<i>S. agalactiae</i>		p value
	Positive	Negative		Positive	Negative	
Prevalence N (%)	215 (70.3)	91 (29.7)		205 (67.0)	101 (33.00)	
Average daily production (L)	467	528	0.223	460	538	0.216
Mean BMSCC (cell/mL)	503,000	434,000	0.003*	503,000	442,000	0.002*
Mean TBC (CFU/mL)	40,000	38,000	0.867	46,000	38,000	0.009*

*Significant by the Mann-Whitney test at 5%.

Significant associations were observed between BMSCC and presence of *S. aureus* ($p=0.003$), BMSCC and presence of *S. agalactiae* ($p=0.002$), and TBC and presence of *S. agalactiae* ($p=0.009$) in the dairy herds investigated, which suggests that the presence of these agents in the herds negatively influence the BMSCC and that the presence of *S. agalactiae* is associated with increased TBC. In this study, herds affected by *S. agalactiae* showed mean TBC of 46,000 CFU/mL, whereas herds affected by *S. aureus* presented mean TBC of 40,000 CFU/mL (Table 7). Table 5 evidences that the properties with TBC >100,000 CFU/mL had higher levels of infection caused by *S. aureus* and *S. agalactiae* (87.50%), whereas presence of both agents was observed in only 38.20% of the farms with TBC <10,000 CFU/mL. These results corroborate the findings by Lopes Júnior et al. (2012), who verified correlation between BMSCC, TBC, and mastitis pathogens, and found correlation between BMSCC and TBC greater than 0.60 for the agents assessed, in which the coefficient of linear regressions for *S. agalactiae* (0.542) was higher compared with those for the other mastitis pathogens, followed by that of *S. aureus* (0.503), suggesting that the intensity of the inflammatory process measured by the BMSCC was associated with the bacterial burden present in the milk of animals affected by mastitis.

Tables 8 and 9 show the results of the antimicrobial susceptibility tests for pathogens *S. aureus* and *S. agalactiae*. It can be observed that the resistance indices of *S. aureus* varied from 2% to 72%, with the following most efficient drugs and percentages of susceptible isolates: sulfamethoxazole-trimethoprim (98%), florfenicol (97%), nitrofurantoin (94%), and ciprofloxacin (91%). The highest resistance levels were observed for polymyxin B (72%), penicillin G (69%), ampicillin (60%), streptomycin (58%), and tetracycline (49%) (Table 8). The Multiple Antibiotic Resistance (MAR) indices for *S. aureus* ranged from 0.00 to 0.84 - mean of 0.33, with 62.6% of the isolates presenting multi-resistance (Table 9).

The antimicrobial susceptibility profiles verified for *S. aureus* in the present study are in agreement with the findings of previous surveys. Cardoso et al. (2000) isolated and subjected to antibiogram tests 127 strains of *S. aureus* in 23 municipalities in Minas Gerais state from 1994 to 1997. The most effective *in vitro* antimicrobials were cefotaxime (100%), enrofloxacin (98.4%), gentamicin (98.4%), rifampicin (96.1%), chloramphenicol (90.4%), sulfamethoxazole-trimethoprim (86.6%), and novobiocin (85.8%). The following medicines showed the highest resistance levels: polymyxin B (8.7%), ampicillin (28.6%), and penicillin G (29.1%).

Table 8. Results of antimicrobial susceptibility tests on strains of *Staphylococcus aureus* and *Streptococcus agalactiae* isolated from milk bulk tanks of dairy herds in the region of Campo das Vertentes/MG, 2017

Antibiotic	<i>Staphylococcus aureus</i>			<i>Streptococcus agalactiae</i>		
	N of resistant strains	% of resistant strains	CI 95% (Lower limit - Upper limit)	N of resistant strains	% of resistant strains	CI 95% (lower limit - upper limit)
Ampicillin	59	60	(49.8-68.7)	34	37	(27.5-46.7)
Cephalothin	24	24	(16.9-33.5)	62	67	(56.6-75.4)
Cefoperazone	28	28	(20.4-37.8)	79	85	(76.3-90.8)
Cefotaxime	35	35	(26.6-45.2)	82	88	(80.1-93.3)
Ceftiofur	25	25	(17.7-34.6)	73	78	(69.1-85.6)
Ciprofloxacin	9	9	(4.9-16.4)	23	25	(17.1-34.4)
Chloramphenicol	5	5	(2.2-11.3)	41	44	(34.4-54.2)
Enrofloxacin	17	17	(11.0-25.8)	44	47	(37.5-57.4)
Streptomycin	57	58	(47.7-66.3)	89	96	(89.5-98.3)
Florfenicol	3	3	(1.0-8.5)	1	1	(0.0-4.0)
Gentamicin	21	21	(14.3-30.3)	47	51	(40.6-60.5)
Lincomycin	34	34	(25.7-44.1)	88	95	(88.0-97.3)
Neomycin	31	31	(23.0-41.0)	76	82	(72.6-88.3)
Nitrofurantoin	6	6	(2.8-12.6)	22	24	(16.2-33.2)
Novobiocin	45	45	(36.0-55.3)	90	97	(96.0-100)
Oxacillin	38	38	(29.4-48.2)	90	97	(90.9-98.9)
Penicillin G	68	69	(57.9-76.1)	80	86	(77.5-91.7)
Polymyxin B	71	72	(62.2-79.7)	89	96	(89.5-98.3)
sulfamethoxazole-trimethoprim	2	2	(0.6-7.1)	14	15	(9.2-23.7)
Tetracycline	49	49	(39.9-59.2)	72	77	(67.9-84.7)

Table 9. Cumulative frequency of resistance to antimicrobials of *Staphylococcus aureus* and *Streptococcus agalactiae* isolated from bulk milk tanks of dairy herds in the region of Campo das Vertentes/MG, 2017

Pathogen	Number of antimicrobials cumulatively tested				
	0	1	2	3	>4*
<i>S. aureus</i>	7	7	15	8	62
<i>S. agalactiae</i>	0	0	2	0	91

*Resistance to four or more antimicrobials characterizes multi-resistance in this study.

Donatele et al. (2002) analyzed antimicrobial susceptibility in 180 strains of *S. aureus* isolated from subclinical mastitis in herds in Rio de Janeiro state and found high resistance rates for β -lactam antibiotics (82.9%) and tetracyclines (24.4%), with the highest susceptibility indices obtained for sulfamethoxazole-trimethoprim and gentamicin. Coelho et al. (2007) reported high rates of resistance to penicillin (67.7%) and ampicillin (64.4%) for *S. aureus*; however, differently from the findings of the present research, gentamicin was more effective against the tested isolates, with only 12.5% resistance. Nunes et al. (2007) evaluated antimicrobial resistance in 234 pathogens isolated from cases of mastitis in dairy properties in Portugal and observed high levels of resistance to penicillin (78.7%) and ampicillin (65.6%) in *S. aureus* isolates; results similar to those found in the present study. Costa et al. (2013), assessed the antimicrobial resistance profile in 352 *S. aureus* isolates in 35 dairy herds in the south of Minas Gerais state and observed that the polymyxin B presented the highest resistance index (82%), followed by the β -lactam antibiotics ampicillin and penicillin, which showed resistance indices of 80.92 and 80.45%, respectively. The same authors also found multi-resistance with MAR index, varying from 0.2 to 0.5, differently from what was verified in the present survey, which showed MAR values ranging from 0.00 to 0.76.

Results in disagreement with those of our study were also reported by Freitas et al. (2005), who verified resistance indices >50% for gentamicin, lincomycin, tetracycline, and oxacillin in *S. aureus* isolates in dairy herds in Pernambuco state, and by Fontana et al. (2010), who evaluated the resistance of *S. aureus* isolates of 174 dairy cows in nine different farms in the municipality of Jataí, Goiás state, and observed 100% resistance to oxacillin, penicillin and ampicillin - indices higher than those found in the present study.

Antimicrobial susceptibility tests for the *S. agalactiae* demonstrated that the overall resistance of the isolates varied from 1% to 97%. The most efficient *in vitro* medicines with their respective percentages of susceptible isolates are shown ahead: florfenicol (99%), sulfamethoxazole-trimethoprim (85%), nitrofurantoin (76%), ciprofloxacin (75%), and ampicillin (63%). The antimicrobials that showed the highest resistance rates were novobiocin (97%), oxacillin (97%), polymyxin B (96%), streptomycin (96%), and lincomycin (95%) (Table 8). Among the isolates of *S. agalactiae*, 97.8% of strains showed multi-resistance, with MAR indices varying from 0.11 to 0.95, and mean of 0.65 (Table 9).

Silva et al. (2017) analyzed the antimicrobial susceptibility profiles of *S. agalactiae* isolates in cattle herds in different Brazilian regions. Their study showed the following drug resistance rates: erythromycin (26.23%), tetracycline (47.54%), gentamycin (3.28%), sulfonamide (98.36%), and clindamycin (29.51%). All isolates assessed were susceptible to penicillin, ceftiofur, and cephalothin. The results of the aforementioned survey differed from those of the present research especially with respect to penicillin and gentamicin resistance rates, which were lower than those observed herein, with values of 86% and 51%, respectively. Jain et al. (2012) assessed the antimicrobial resistance profiles in *S. agalactiae* isolates from cases of subclinical mastitis and observed resistance indices, in general, lower than those obtained in the present study. Our results show higher overall indices of resistance and multi-resistance for *S. agalactiae*

compared with *S. aureus* in the farms investigated. Table 9 shows that 48.48% of the *S. aureus* isolates were resistant to five or more different antimicrobials, whereas this index was 95.70% for *S. agalactiae* isolates. According to Nunes et al. (2007) and Fontana et al. (2010), poorly directed antibiotic therapy and indiscriminate use of antimicrobials, resulting in increased selective pressure on resistant strains, contribute to increased antimicrobial resistance rates. Different authors have referred to the difficulty in treating intramammary infections caused by *S. aureus*, with low cure rates (Costa et al. 2012, 2013, Silva et al. 2014). This fact is justified not only by the resistance phenomenon, but also by its escape mechanisms of the immune system and of the antimicrobials in the infected gland, such as intracellular invasion and biofilm formation (Novick et al. 2001, DeLeo et al. 2009).

As for intramammary infections caused by *S. agalactiae*, increasing difficulty has been observed with regards to treatment, including failure of the classic blitz therapy protocols, which in part may be associated with the high resistance rates observed in the present study. The differences between the results of the susceptibility tests verified in this research for pathogens *S. aureus* and *S. agalactiae* and those of previous studies in the literature can be justified by spatial and temporal variations in the origin of the isolates, as well as by the different methodologies used in the antibiotic susceptibility tests (minimal inhibitory concentration (MIC); disc diffusion). Differences in the origin of the isolates (whether from clinical or subclinical mastitis, if from individual mammary quarters or expansion tanks, or from the environment) and in selection pressure due to the selective and/or indiscriminate use of antimicrobials may also justify this variation in the resistance indices.

Results of the present study demonstrated high resistance levels among *S. aureus* and *S. agalactiae* isolates for the main medicines used in the treatment of mastitis, which highlights the need for constant monitoring of susceptibility profiles with a view to a more careful selection of antimicrobials in order to improve high cure rates and minimize the increase in drug resistance. In addition, it is fundamental to periodically and permanently monitor the pathogens involved in the etiology of bovine mastitis and their resistance indices, because these parameters are very dynamic and present spatial and temporal variations. According to Myllys et al. (1994), resistance to antimicrobials is an important factor in the establishment and dissemination of bacterial clones in a herd, and shows close association with changes in management, such as the use of systematic antibiotic treatment, cattle stabling, and introduction of mechanical milking machines - factors that impose selective pressure on the pathogens that cause mastitis.

CONCLUSIONS

Results show high prevalence rates of pathogens *Staphylococcus aureus* and *Streptococcus agalactiae* in dairy farms of the study region and association between presence of these agents in the herd with total bacterial counts (TBC) and bulk milk somatic cell counts (BMSCC), demonstrating that the presence of these pathogens negatively influence milk quality.

No difference in the presence of pathogens *S. aureus* and *S. agalactiae* were observed as a function of the daily production of the properties, demonstrating homogeneous distribution of these agents in the herds regardless of the daily production.

High resistance and multi-resistance levels were observed among the pathogens *S. aureus* and *S. agalactiae*, emphasizing the need for more judicious use of antimicrobials used in mastitis control.

Conflict of interest statement. - The authors declare that there are no conflicts of interest.

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Clinical biochemistry profile of American Quarter Horse broodmares fed Tifton-85 (*Cynodon* spp.) hay and haylage¹

Taciano C. Guimarães², Adalgiza S.C. Rezende², Maria L.L. Costa³,
Marina G. Ferreira², Ana. L.S. Miranda², Ana F.M. Botelho², Benito Soto-Blanco²
and Marília M. Melo^{2*} 

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The conservation of haylage (a pre-dried feed) can be challenging, since there is an increased risk of mould growth, which can contaminate this foodstuff with mycotoxins. However, when the hygienic quality is secured, haylage enhances grass palatability and provide enough supply of dry matter throughout the year. Due to the lack of information regarding its effect on blood parameters in horses fed exclusively with this foodstuff, the aim of this study is to provide information regarding its use in comparison to hay and ensure that it does not affect horses' biochemical profile. Twelve Quarter Horse broodmares were distributed into two groups, each fed with Tifton-85 (*Cynodon* spp.) hay or haylage for a period of 28 days, and the biochemical profile was done in five different times (T0 before the experiment started and, chronologically, seven days apart - T1, T2, T3 and T4). It was analyzed total protein (TP) and its fractioning; enzymes alanine aminotransferase, aspartate aminotransferase and γ -glutamyl-transferase; endogenous catabolism products urea and creatinine; and ions calcium and phosphorus. Mycotoxins in haylage were also investigated and remained below the legislation thresholds. Only TP was higher in the last sampling (T4) of the haylage group, which may be related to the foodstuff's higher protein digestibility. No differences were observed between serum enzymes, urea, creatinine and Ca/P from both experimental groups. Haylage has proven to be safe, when well prepared for horses, without causing impairing side effects, as shown by the normal serum biochemistry parameters presented in this study.

INDEX TERMS: Biochemistry, clinics, American Quarter Horse, Tifton-85, *Cynodon* spp., hay, haylage, equine, forage, clinical chemistry, horses, nutrition.

RESUMO.- [Perfil bioquímico clínico de matrizes Quarto de Milha alimentadas com feno e *haylage* Tifton 85 (*Cynodon* spp.)] A conservação do *haylage* (alimento pré-seco) pode ser desafiadora, considerando o aumento do risco de crescimento de fungos, com consequente produção

de micotoxinas. Entretanto, quando a qualidade da higiene e armazenamento é assegurada, o *haylage* aumenta a palatabilidade da forragem e fornece suplemento de matéria seca suficiente ao longo do ano. Devido à falta de informação relativa aos efeitos dessa alimentação nos parâmetros sanguíneos de equinos alimentados exclusivamente com essa dieta, o objetivo do presente estudo é avaliar o perfil bioquímico sanguíneo dos equinos após administração da *haylage* em comparação com feno. Doze matrizes Quarto de Milha foram distribuídas em dois grupos, cada um recebendo feno ou *haylage* de Tifton 85 (*Cynodon* spp.) por um período de 28 dias. O perfil bioquímico foi realizado em cinco tempos (T) diferentes (T0, antes do início do experimento e cronologicamente, a cada

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² Escola de Veterinária, Universidade Federal de Minas Gerais (UFMG), Avenida Presidente Antônio Carlos 6627, Belo Horizonte, MG, 31275-013, Brazil. *Corresponding author: mariliamartinsmelovet@hotmail.com

³ Departamento de Zootecnia, Centro de Ciências Agrárias, Universidade Federal da Paraíba (UFPB), Campus II, Rodovia PB-079 s/n, Areia, PB 58397-000, Brazil.

sete dias após o fornecimento das dietas - T1, T2, T3 e T4) para análise de proteína total (PT) e seu perfil fracionado, das enzimas alanina aminotransferase, aspartato aminotransferase, γ -glutamyl-transferase, dos produtos de catabolismo creatinina e ureia e, dos íons cálcio e fósforo. Micotoxinas na *haylage* foram investigadas e mantiveram-se abaixo dos limites determinados pela legislação brasileira. O perfil bioquímico revelou, somente, elevação da PT em T4 no grupo que recebeu *haylage*, o que pode estar relacionado à sua maior digestibilidade proteica. Nenhuma diferença foi observada nos outros parâmetros estudados em ambos os grupos experimentais. Conclui-se que *Haylage* é comprovadamente seguro, quando bem preparado para equinos, sem causar efeitos na saúde geral, conforme demonstrado pelos exames bioquímicos no presente estudo.

TERMOS DE INDEXAÇÃO: Bioquímica, clínica, matrizes, Quarto de Milha, feno, *haylage*, Tifton 85, *Cynodon* spp., cavalos, equinos, forragem, hematologia, nutrição.

INTRODUCTION

Tifton-85 (*Cynodon* spp.) is a sterile hybrid resulting from crossings between Tifton-68 and Tifton-292. It was developed by the United States Department of Agriculture as a collaborative project with the University of Georgia, and registered in October 1992 (Burton et al. 1993). Tifton-85 is largely employed in hay-making due to its favorable structural characteristics, such as fast dehydration, primary presence of thin stems, and a high leaf/thatch ratio (Domingues 2009).

In recent years, haylage has been studied around the world (Müller & Udén 2007, Müller 2011, Guimarães et al. 2016, Harris et al. 2017). Haylage is a pre-dried feed, and an intermediate between hay and silage. Its processing is based on partial drying and fermentation of forages in aerobic conditions. Such a processing of forages can provide various benefits, especially in the intensive farming systems, such as the enhancement of its palatability and certification, that enough dry matter (DM) will be offered to the animal despite seasonal variations. In addition, replacement of high starch containing concentrate with energy dense fibrous feeds (silage and haylage) is a potential solution for several clinical problems in horses and ruminants, such as colic, laminitis and acidosis. Energy dense fibrous feeds have been shown to enhance volatile fatty acid production in the hindgut, and present high palatability (Richardson & Murray 2016).

Considering that haylage contains higher water content than hay, its conservation is challenging by the considerable risk of mould growth, which can contaminate the foodstuff with mycotoxins, accounted for respiratory diseases, mycotoxicosis, skin disorders and reproductive failure in horses (Müller et al. 2011).

The main toxigenic fungi proliferations include *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus parasiticus* and *Fusarium moniliforme* (Kamphues 2013, Wambacq et al. 2016).

Employing haylage in the equine farming system is only welcomed by horse owners if its quality of conservation and nutritional values are established. Nutritional values of fermented forages are often better than those of dry hay, because of the growth stage of the harvested grass and the smaller losses of leaves during haylage processing (Bergero et al. 2002, Schenck & Müller 2014).

There is a lack of information regarding the effect of haylage on blood parameters in horses fed exclusively with this foodstuff. Blood biochemical paneling might provide information regarding animal's health status (Rezapour et al. 2016). Therefore, the aim of this study is to provide comparative biochemical blood values of broodmares fed with hay and haylage of Tifton-85 (*Cynodon* spp.) to ensure that haylage is capable of maintaining horses' normal blood parameters.

MATERIALS AND METHODS

Ethics statement. This study was approved by the Committee of Ethics and Use of Animals (Protocol 552010), at the Federal University of Minas Gerais (UFMG), in compliance with the Ethics Principles in Animal Experimentation.

Feed production. The experiment was conducted in farms of the Itaúna district, in the State of Minas Gerais, Brazil. The two evaluated treatments comprised haylage or hay of Tifton-85 (*Cynodon* spp.) diets, both supplemented with mineral salt, water *ad libitum*, and concentrate. Concentrate was manufactured on the farm, with a 94.82 DM content, 70% corn, 15% whit bran, 5% soy bean, 3% calcitic limestone, 2% mineral salt, 1% dicalcium phosphate and 4% soy oil. All animals were weighed weekly and received the daily diet considering 2% of their body weight, in the proportion of concentrate: hay or haylage of 50% each. Both hay and haylage were administrated 3 times a day, at 7am, 13pm and 17pm. Concentrate was provided twice a day, at 9am and 15pm.

For both feedstuffs, grass was harvested at 30 days of growth, wilted and tilled on the field until a 70% DM content was achieved. For haylage production, a biological additive (Silobac®) was added, according to the manufacturer's recommendations, consisting of diluting 2g of the product in 2L of water to inoculate each ton of forage intended for haylage-making. This product supplied *Lactobacillus plantarum* and *Pediococcus pentosaceus* in a concentration of 2.5×10^5 CFU per gram of forage, for each bacterium species. The purpose of this addition was to balance the microbial population in the material for haylage production, improving anaerobic fermentation. Approximately 1.3kg of green mass was wrapped in polyethylene bags (40 x 60cm) and vacuum sealed according to the technique described earlier (Kung Junior et al. 2010). Diet composition is described in Table 1 and haylage composition is established in Table 2. To verify possible fungi development and consequent mycotoxin production, visual inspection of hay and haylage was performed, along with mycotoxin investigation, since visual inspection alone is considered insufficient to make such a determination (Raymond et al. 2000).

Table 1. Chemical composition of the diet supplied for the horses

Diet (concentrate + forage)/Compounds		
	Hay	Haylage
Dry matter - DM (%)	94.54	95.71
Organic matter (% DM)	91.20	90.85
Ash (% DM)	8.80	9.15
Crude protein (% DM)	11.44	12.71
Neutral detergent fiber corrected for ash (% DM)	55.16	54.77
Acid detergent fiber corrected for ash (% DM)	20.41	22.58
Hemicellulose (% DM)	34.74	32.29
Gross energy (Mcal/kg DM)	4.72	4.85

Data based on dry matter.

Table 2. Chemical composition Tifton-85 haylage (*Cynodon* spp.) in different opening times (days)

Parameters	0d	1d	3d	7d	14d	28d	56d
Dry matter	26.78	71.70	71.98	73.20	70.62	70.45	72.28
IVDDM (%)	38.01	38.42	37.51	36.98	35.00	37.18	36.05
Crude protein (%)	17.63	19.06	18.91	19.65	17.18	17.09	17.71
% N-NH ₃ /3 total	0.39	0.41	0.67	0.73	1.04	1.24	1.10
pH	5.48	6.08	5.19	5.53	5.37	5.42	5.21
NDF (%)	74.16	74.31	72.23	74.25	73.21	73.24	73.33
ADF (%)	32.33	31.03	31.58	31.87	32.15	31.31	31.96
HEM (%)	41.83	43.27	40.64	42.38	41.56	40.93	41.27
LIG (%)	3.28	3.56	3.36	3.59	3.79	3.56	3.39
Acetic acid (g/kg MS)	0.91	1.17	0.60	2.02	2.14	1.85	2.79
Propionic acid (g/kg/MS)	0.27	0.10	0.03	0.11	0.08	0.10	0.08
Butyric acid (g/kg/MS)	0.77	-0.70	0.18	0.36	0.42	0.23	0.20
Lactic acid (g/kg/MS)	2.26	0.35	3.01	10.24	11.44	16.15	26.06

d = days, IVDDM = *In vitro* digestibility of dry matter, NDF = neutral detergent fiber, ADF = acid detergent fiber, HEM = hemicelluloses, LIG = lignin.

Haylage underwent mycotoxin evaluation to discard the possibility of any effect on the blood results. Veratox kits (Neogen Food Safety, Lansing/MI, USA) were used to quantify aflatoxins, fumonisins and zearalenone. A spectrophotometric reading was performed (ELISA plate reader in 650nm) on the grass after harvesting and on haylage 56 days after its production in order to assess the fermentation potential (Costa 2012). Analysis took place in the Laboratory of Veterinary Toxicology housed in the Veterinary College of UFMG.

Animals and groups. Twelve healthy Quarter Horse broodmares, with mean weight of 452±46.80kg and ranging in age from eight to 12 years, were used. Horses were randomly distributed into two groups (n=6): Group 1 underwent the hay-based diet, and Group 2, the haylage-based diet. Animals were kept in 9m² stalls and were allowed a one-hour stroll within a known enclosure. Animals were weighed weekly for the calculation of the feedstuffs for maintenance requirements (NRC 2007). Haylage and hay were supplied for the first time to the animals during the experimental period, since their prior diet consisted of Tifton-85 (*Cynodon* spp.), mineral salt and water *ad libitum*. Hay and haylage were provided three times a day, and concentrate, twice a day. Before feeding the horses, haylage was visually inspected for mould.

Blood samples and biochemistry profile. Blood sampling was performed once a week within the 28- day experimental period through venipuncture of the jugular vein, using vacuum tubes without anticoagulant. The first sample was collected on time zero (T0) before the animals were introduced to their new experimental diets, and then after 7 (T1), 14 (T2), 21 (T3) and 28 (T4) days. All samples were collected in the morning, before the concentrate was fed.

Alanine aminotransferase (ALT), aspartate aminotransferase (AST), γ -glutamyl-transferase (GGT), alkaline phosphatase (AP), urea and creatinine, calcium (Ca) and phosphorus (P) were determined in sera samples using commercial kits (Bioclin, Belo Horizonte/MG, Brazil) and a semi-automated analyzer (TP Analyzer Basic, Thermoplate, São Paulo/SP, Brazil). Total protein (TP) was estimated through refractometry, and protein profile (serum protein electrophoresis) was accomplished using horizontal electrophoresis in agarose gel at 12% and TRIS buffer. Gels were stained with Amido black (Sigma-Aldrich, St. Louis/MO, USA) and discolored in a series of ethanol and acetic acid, with a 35-minute gel run. Reading was made through a scanner with a software (Celm CE-250, São Paulo/SP, Brazil). The concentration of protein in each fraction (g/dL) was determined through percentage multiplication of each fraction obtained in the total protein concentration.

Statistical analysis. Experimental design was completely randomized, with an arrangement of subdivided parcels in which a parcel was the randomized groups (1 or 2) and the subdivided parcels were moments of blood sampling, with six repetitions (animals in each group). Variables were tested for normality of distribution. AST, ALT, GGT and creatinine were transformed by log¹⁰ (x+1). Urea, Ca and AP presented non-parametric distribution and were analyzed by Mann-Whitney test, (for group comparison), and by Friedman test (for moments' comparison). All other variables were analyzed by ANOVA with Tukey test. A P<0.05 value was accepted as a statistically significant difference for all the tests.

RESULTS AND DISCUSSION

This study provides the effects on blood parameters of horses fed with haylage compared to hay diet. Haylage is a pre-dried feed processed with partial drying and aerobic fermentation. Since it has a greater water content than hay, its conservation has a higher risk of mould growth and mycotoxins production that may impair animal health (Müller et al. 2011).

The levels of mycotoxins in the present study were equivalent between grass and haylage (Table 3), which allows inferring that haylage production conditions were appropriate. These concentrations are lower than the tolerance levels estimated for horses, which are 5mg/kg for fumonisin (Diener 1996), 10µg/kg for aflatoxins (Basalan et al. 2004) and 1000µg/kg for zearalenone (Juhász et al. 2001). Mufatto et al. (2016) observed that pre-drying Tifton-85, while adopting at least a 29-day fermentation period is directly related to improvement of microbiological quality in the foodstuff.

Blood biochemical panel was used to define the health status of animals, providing information regarding tissue injury, organ malfunction, adaptation to nutritional and physiological challenges, and during specific metabolic dysfunctions. Therefore, blood biochemistry can provide grounds for the interpretation of liver, kidney, bone and muscular adjustments. Total protein, AST, GGT, ALT provide information of liver function, whereas urea and creatinine are associated with kidney lesions, Ca and P are related to basic cellular function (González & Silva 2006).

Serum enzymes are presented in Table 4. Discrete differences were observed amongst evaluated times, but GGT, ALT and AST, remained close to the established parameters for the species as

Table 3. Mycotoxins concentrations found in the Tifton-85 and haylage at day 56 compared with established tolerance levels for horses

Micotoxins	Tifton-85	Haylage (56d)	Tolerance levels for horses
Aflatoxin	0.95 µg/kg	0.95 µg/kg	10µg/kg*
Fumonisin	0.60 mg/kg	0.60mg/kg	5mg/kg**
Zearalenone	0.71 µg/kg	0.71 µg/kg	1000 µg/kg***

* Basalan et al. 2004, ** Diener 1996, *** Juhasz et al. 2001.

Table 4. Mean values and standard deviation of GGT (UI/L), AST (UI/L), ALT (UI/L) of horses after hay administration (Group1-G1) and haylage of Tifton-85 (Group 2-G2)

Time	GGT		AST		ALT	
	G1	G2	G1	G2	G1	G2
T0	12.65 ± 3.19 ^B	12.90 ± 4.71	262.27 ± 123.72	177.41 ± 11.48 ^{AB}	7.06 ± 3.31	8.14 ± 9.11
T1	13.50 ± 6.82 ^B	14.27 ± 9.28	227.18 ± 101.30	138.39 ± 39.06 ^B	8.95 ± 6.61	9.11 ± 12.01
T2	17.36 ± 4.52 ^{AB}	16.65 ± 4.17	200.43 ± 97.81	145.17 ± 37.74 ^B	9.74 ± 6.63	15.24 ± 11.43
T3	20.73 ± 6.35 ^{AB}	17.81 ± 6.67	231.89 ± 121.64	158.50 ± 69.04 ^B	5.72 ± 3.09	6.77 ± 2.79
T4	22.66 ± 3.14 ^A	19.24 ± 9.10	259.97 ± 96.02	230.23 ± 83.20 ^A	9.23 ± 3.10	10.83 ± 4.10

^{A, B, AB} Means followed by the same uppercase letters do not differ between sample times (columns); means followed by the lowercase letters do not differ between groups (lines) (Friedman, P<0.05).

slight variations were observed. Only the hay group presented significant rise (P<0.05) of GGT (12.6±3.19 to 22.7±3.14U/L) (Fig.1). Haylage group showed similar values, 12.9±4.71U/L (T0) e 19.2±9.10U/L (T4), but without statistic increase. Although discrete alterations occurred, all values remained within the normal limit for equine (9 to 26U/L) according to Padilha et al. (2017), demonstrating that no cholangitis or cholestasis occurred.

In regards to AST, no alterations were observed in the group that was fed with hay. The groups treated with haylage presented a discrete decrease that persisted for three weeks (T1, T2 and T3). Mean values observed in both G1 and G2 ranged respectively from 200.43U/L to 259.97U/L and 138.39U/L to 230.23U/L, similarly to those reported by Souza et al. (2016) of 140U/L and Padilha et al. (2017) with means of 246.34U/L. According to Kaneko & Harvey (1997) all values remained within the normal range (226-366 U/L).

ALT remained unchanged in both groups and times. G1 mean levels were from 5.72 to 9.23U/L, while G2 from 6.77 to 15.24U/L. Previous reports indicate mean values between 5.13 U/L (Souza et al. 2016) and 12.6U/L (Ural et al. 2009). According to Kaneko & Harvey (1997) all values remained within the normal range (3-23U/L). Both GGT and ALT are mostly present in liver cells, and the fact that both serum levels were considered normal is an indicator of the animals' health. Hay and haylage did not alter the physiology of the liver.

ALT activity in horses' liver is considered to be low, and as so it can't be used separately from other serum biochemistry evaluations, only in association is enzymes, such as AST and GGT. It is important to highlight that AST, although not organ-specific, is frequently used in routine exams for the detection of liver injury. After careful analysis of hepatic enzymes, it is clear that haylage supply for 28 days is not able to cause any type of injury or lesion to the hepatic tissue, or even stimulation or production of enzymes.

Ca mean values observed in both G1 and G2 ranged respectively from 2.76 to 3.84mmol/L and 2.77 to 3.84mmol/L,

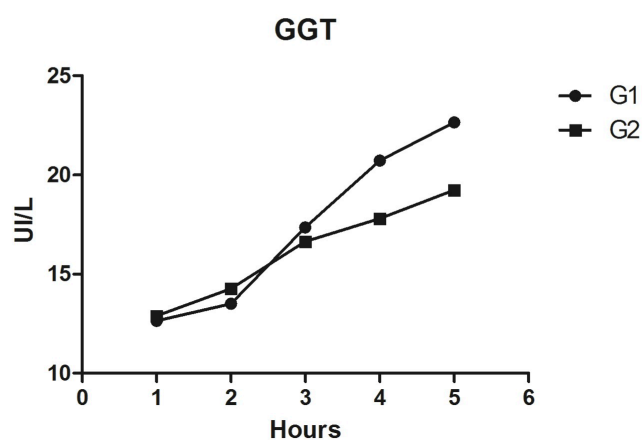


Fig.1. GGT (UI/L) serum relationship in horses that received hay (G1) and haylage of Tifton-85 (G2).

very similar to 2.43 to 3.10mmol/L described by Kaneko & Harvey (1997) and did not change between time and/or experimental groups (Table 5).

P mean values observed in both G1 and G2 ranged respectively from 3.38 to 4.28mg/dL and 3.51 to 3.80mg/dL inside the normal limits (3.1-5.6mmol/L) described by Kaneko & Harvey (1997).

These findings represent that both hay and haylage provide sufficient diet content to maintain ionic balance and energetic metabolism. Such a result is yet another confirmation of the haylage proper nutritional balance for horses.

Urea and creatinine levels had discrete variations between analyzed times (Table 6). Urea had a significant decrease in T3 and, highest levels of creatinine were seen in T3 for both groups (1.60±0.22, hay group) and (1.54±0.38, haylage group). All the creatinine values remained in the normal range for the species (creatinine 1.0 to 2.0mg/dL); however, the urea values presented variations and are above the values

described by Kaneko & Harvey (1997) (10 to 24mg/dL). These variations were expected, due to protein intake and test methodology, with no clinical significance (Souza et al. 2009, Padilha et al. 2017). These results indicate that the

feeding and protein intake caused minor variations in urea and creatinine levels but hay and haylage consumption did not result in kidney lesions.

Protein fractions were similar between groups as described in Tables 7 and 8; however, protein distribution was different from previously reported. Protein fractioning revealed nine distinct bands, denominated albumin, α 1b-globulin, α 1b-globulin, α 2a-globulin, α 2b-globulin, β 1-globulin, β 2a-globulin, β 2b-globulin and γ -globulin. In the present work, albumin represented the highest percentage of serum proteins, followed by γ -globulin, β 2a-globulin, β 1-globulin, β 2b-globulin and α 1 and α 2 fractions. In a study using 126 horses, the highest levels were also found in albumin and γ -globulin, followed however by α 2, β 1 and β 2 globulin, and α 1 (Riond et al. 2009). These differences may be associated with different breeds, sexes, ages and diet between the studies, revealing the importance of future work to determine normal range values for Quarter horses.

Total protein concentration (Table 8) presented no statistical difference between experimental groups and time of analysis and ranged from 5.57 to 6.67g/dL in G1 and from 6.10 to 7.70g/dL in G2. All levels remained within normal parameters for the equine species. Nonetheless, Moore-Colyer & Longland (2000) highlighted a higher digestibility of haylage in comparison to hay, which may elicit the rise of TP in the haylage group, not seen in the current study. Since there was no significant difference between the groups regarding protein concentrations, it may be inferred that the experimental diets were not able to alter their pattern.

The relation between albumin and globulin is presented in Figure 2. This is an important measure that grants data regarding dysproteinemias and the systematic classification of electrophoretic profile (Riond et al. 2009). In the present work, no differences were found between groups, showing that protein profile is unchanged by haylage. This methodology is

Table 5. Mean values of calcium (Ca) (mmol/L) and phosphorus (P) (mmol/L) of horses after hay administration (Group 1-G1) and haylage of Tifton-85 (Group 2-G2)

Time	Ca		P	
	G1	G2	G1	G2
T0	2.76 $\pm$ 0.002	2.77 $\pm$ 0.002	3.38 $\pm$ 0.86	3.52 $\pm$ 0.47
T1	3.83 $\pm$ 0.024	3.82 $\pm$ 0.002	4.28 $\pm$ 0.48	3.51 $\pm$ 0.57
T2	3.85 $\pm$ 0.004	3.58 $\pm$ 0.02	3.77 $\pm$ 0.36	3.63 $\pm$ 0.7
T3	3.84 $\pm$ 0.015	3.84 $\pm$ 0.02	3.88 $\pm$ 0.50	3.71 $\pm$ 0.7
T4	3.82 $\pm$ 0.055	3.84 $\pm$ 0.019	4.21 $\pm$ 0.45	3.80 $\pm$ 0.54

Friedman, P<0.05.

Table 6. Mean values and standard deviation of urea (mg/dL) and creatinine (mg/dL) of horses after hay administration (Group 1-G1) and haylage of Tifton-85 (Group 2-G2)

Time	Urea		Creatinine	
	G1	G2	G1	G2
T0	42.82 $\pm$ 4.96 ^A	41.54 $\pm$ 7.36 ^{AB}	1.37 $\pm$ 0.21 ^{ABC}	1.26 $\pm$ 0.22 ^{AB}
T1	62.80 $\pm$ 3.47 ^A	60.28 $\pm$ 6.96 ^A	1.54 $\pm$ 0.33 ^{AB}	1.45 $\pm$ 0.27 ^{AB}
T2	30.91 $\pm$ 2.85 ^B	35.73 $\pm$ 7.31 ^{BC}	1.18 $\pm$ 0.09 ^{BC}	1.13 $\pm$ 0.05 ^B
T3	32.61 $\pm$ 4.97 ^B	34.31 $\pm$ 1.11 ^{BC}	1.60 $\pm$ 0.22 ^A	1.54 $\pm$ 0.38 ^A
T4	31.80 $\pm$ 3.97 ^B	31.80 $\pm$ 1.34 ^C	1.10 $\pm$ 0.08 ^C	1.21 $\pm$ 0.14 ^{AB}

A, B, C, AB, BC, ABC Means followed by the same uppercase letters do not differ between sample times (columns); means followed by the lowercase letters do not differ between groups (lines) (Friedman, P<0.05; Urea Tukey, P<0.05).

Table 7. Mean values and standard deviation for albumin (Alb) (g/dL), α 1a (g/dL), α 1b (g/dL), α 2a (g/dL) and α 2b (g/dL) from horses serum, after administration of hay (Group 1-G1) and haylage (Tifton-85) (Group 2-G2)

Time	Albumin		α 1a		α 1b		α 2a		α 2b	
	G1	G2	G1	G2	G1	G2	G1	G2	G1	G2
T0	2.23 $\pm$ 0.38 ^A	2.25 $\pm$ 0.71	0.27 $\pm$ 0.09 ^{AB}	0.27 $\pm$ 0.09	0.12 $\pm$ 0.05	0.15 $\pm$ 0.11	0.13 $\pm$ 0.08 ^B	0.11 $\pm$ 0.07	0.27 $\pm$ 0.20	0.25 $\pm$ 0.25
T1	1.28 $\pm$ 0.36 ^B	1.74 $\pm$ 0.42	0.17 $\pm$ 0.12 ^B	0.20 $\pm$ 0.10	0.12 $\pm$ 0.08	0.12 $\pm$ 0.10	0.21 $\pm$ 0.07 ^{AB}	0.21 $\pm$ 0.05	0.24 $\pm$ 0.15	0.25 $\pm$ 0.13
T2	1.78 $\pm$ 0.62 ^{ABb}	2.40 $\pm$ 0.53 ^a	0.27 $\pm$ 0.05 ^{AB}	0.23 $\pm$ 0.06	0.14 $\pm$ 0.07	0.16 $\pm$ 0.04	0.17 $\pm$ 0.11 ^{AB}	0.22 $\pm$ 0.12	0.28 $\pm$ 0.11 ^a	0.22 $\pm$ 0.14
T3	1.87 $\pm$ 0.48 ^{AB}	2.06 $\pm$ 0.51	0.28 $\pm$ 0.06 ^A	0.28 $\pm$ 0.09	0.19 $\pm$ 0.10	0.17 $\pm$ 0.02	0.25 $\pm$ 0.15 ^{AB}	0.23 $\pm$ 0.11	0.31 $\pm$ 0.18 ^a	0.34 $\pm$ 0.21
T4	2.11 $\pm$ 0.27 ^A	2.34 $\pm$ 0.28	0.21 $\pm$ 0.11 ^{AB}	0.26 $\pm$ 0.06	0.13 $\pm$ 0.04 ^b	0.21 $\pm$ 0.08 ^a	0.38 $\pm$ 0.23 ^A	0.27 $\pm$ 0.11	0.16 $\pm$ 0.13 ^a	0.31 $\pm$ 0.14

Means followed by the same uppercase letters do not differ between sample times (columns); means followed by the lowercase letters do not differ between groups (lines) (Tukey, P<0.05).

Table 8. Mean values and standard deviation for β 1 (g/dL), β 2a (g/dL), β 2b (g/dL), γ globulins (g/dL) and total protein (TP) (g/dL) from horses serum, after administration of hay (Group 1-G1) and haylage (Tifton-85) (Group 2-G2)

Time	β 1		β 2a		β 2b		γ globulinas		Total protein	
	G1	G2	G1	G2	G1	G2	G1	G2	G1	G2
T0	0.63 $\pm$ 0.26	0.74 $\pm$ 0.23 ^B	1.40 $\pm$ 0.49 ^{Aa}	0.93 $\pm$ 0.42 ^b	0.86 $\pm$ 0.42 ^{Ab}	1.22 $\pm$ 0.26 ^{Aa}	0.86 $\pm$ 0.21	1.11 $\pm$ 0.39	6.67 $\pm$ 0.82	7.70 $\pm$ 0.80
T1	0.73 $\pm$ 0.30 ^b	1.29 $\pm$ 0.51 ^{Aa}	1.00 $\pm$ 0.30 ^{AB}	1.14 $\pm$ 0.47	0.51 $\pm$ 0.24 ^{AB}	0.59 $\pm$ 0.11 ^B	1.15 $\pm$ 0.49	1.22 $\pm$ 0.23	5.57 $\pm$ 1.40	6.10 $\pm$ 0.84
T2	0.61 $\pm$ 0.19	0.84 $\pm$ 0.30 ^B	0.89 $\pm$ 0.24 ^B	0.90 $\pm$ 0.34	0.56 $\pm$ 0.35 ^{AB}	0.53 $\pm$ 0.28 ^B	1.11 $\pm$ 0.25	1.07 $\pm$ 0.31	5.80 $\pm$ 0.9	6.60 $\pm$ 1.47
T3	0.84 $\pm$ 0.24	0.92 $\pm$ 0.26 ^{AB}	1.28 $\pm$ 0.26 ^{AB}	1.18 $\pm$ 0.45	0.45 $\pm$ 0.09 ^B	0.64 $\pm$ 0.25 ^B	1.27 $\pm$ 0.5	1.25 $\pm$ 0.27	6.57 $\pm$ 1.8	6.90 $\pm$ 1.29
T4	0.77 $\pm$ 0.11	0.92 $\pm$ 0.10 ^{AB}	1.01 $\pm$ 0.19 ^{AB}	1.06 $\pm$ 0.20	0.37 $\pm$ 0.07 ^{Bb}	0.76 $\pm$ 0.25 ^{Ba}	1.07 $\pm$ 0.15	1.29 $\pm$ 0.23	6.20 $\pm$ 0.5	7.53 $\pm$ 0.62

A, B, AB Means followed by the same uppercase letters do not differ between sample times (columns); ^{a, b} means followed by the lowercase letters do not differ between groups (lines) (Tukey, P<0.05).

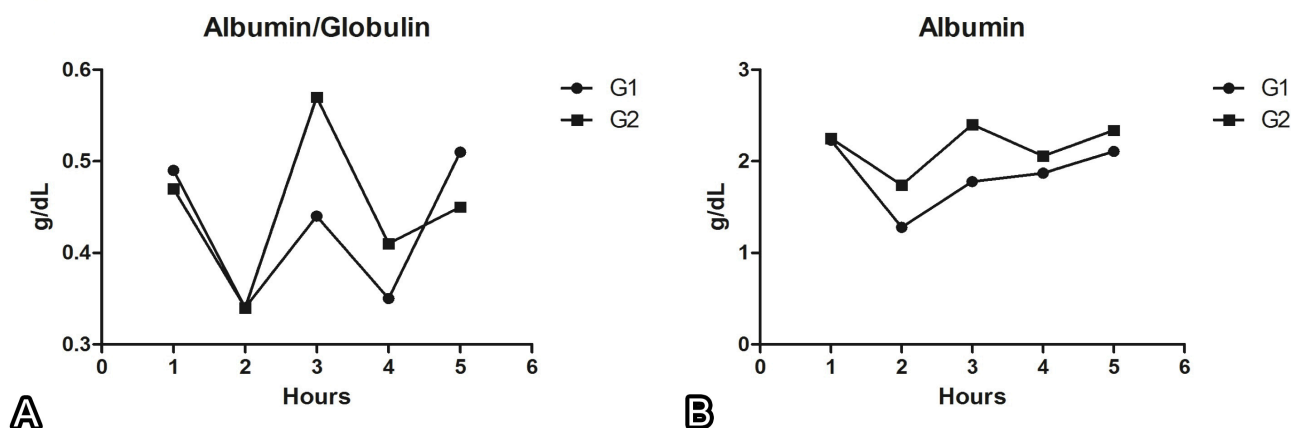


Fig.2. (A) Albumin/Globulin ratio from horses that received hay (G1) and haylage of Tifton-85 (G2). (B) Serum albumin relationship in g/dL from horses that received hay (G1) and haylage (G2).

simple and useful as a screening test for the species, informing the clinician of valuable information in changes of such fractions that may help characterize dysproteinemias in the equine patient (Riond et al. 2009).

CONCLUSION

The use of haylage of Tifton-85 (*Cynodon* spp.) with 70% of DM for 28 days is a safe and adequate alternative feeding for Quarter Horse broodmares as it did not cause alterations in horses' biochemical profiles when compared to hay.

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

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Effect of eCG on the follicular dynamics and vascularization of crossbred cows with different circulating progesterone concentrations during synchronization of ovulation in an FTAI protocol¹

Priscila A. Ferraz^{2*} , Mariana A.A. Silva², Bia S.S. Carôso², Endrigo A.B. Araujo²,
Thereza C.B.S.C. Bittencourt², Marcos Chalhoub², Rodrigo F. Bittencourt²
and Antonio L. Ribeiro Filho²

ABSTRACT.- Ferraz P.A., Silva M.A.A., Carôso B.S.S., Araujo E.A.B., Bittencourt T.C.B.S.C., Chalhoub M., Bittencourt R.F. & Ribeiro Filho A.L. 2019. **Effect of eCG on the follicular dynamics and vascularization of crossbred cows with different circulating progesterone concentrations during synchronization of ovulation in an FTAI protocol.** *Pesquisa Veterinária Brasileira* 39(5):324-331. Departamento de Anatomia, Patologia e Clínicas Veterinárias, Universidade Federal da Bahia, Rua Ademar de Barros 500, Ondina, Salvador, BA 40170-110, Brazil. E-mail: prisferraz@gmail.com

This study aimed to evaluate the effect of treatment with equine chorionic gonadotrophin (eCG) on the follicular dynamics and function of crossbred cows with different circulating progesterone (P4) concentrations during synchronization of ovulation in a fixed-time artificial insemination (FTAI) protocol. To this end, 30 crossbred cows were submitted to a pre-synchronization protocol to ensure that all of them presented corpus luteum (CL) at the beginning of the protocol, and were evaluated by transrectal ultrasonography (TRUS) to verify the presence of CL. After that, the animals underwent an ovulation synchronization protocol and evaluation of follicular dynamics and vascularization by B-mode and power-Doppler ultrasound (US). High plasma P4 concentrations at the time of ovulation synchronization negatively influenced follicle diameter on day 10 (D10), preovulatory follicle diameter, and preovulatory follicle wall vascularization area ($p < 0.05$). Cows with high P4 concentration at the time of ovulation synchronization that were treated with eCG showed follicle diameter on D10 and preovulatory follicle diameter and wall vascularization area ($p > 0.05$) similar to those of animals with low P4 concentration at the time of ovulation synchronization. Therefore, high P4 concentrations at the time of ovulation synchronization negatively influence follicular diameter and vascularization, and eCG can be used as a strategy to favor better follicular and luteal response in crossbred cows with high P4 concentrations submitted to an FTAI protocol.

INDEX TERMS: Effect of eCG, follicular vascularization, crossbred cows, progesterone, ovulation, FTAI protocol, cattle, equine chorionic gonadotrophin, synchronization, Doppler, clinics.

RESUMO.- [Efeito do eCG na dinâmica e na vascularização folicular de vacas mestiças com diferentes concentrações circulantes de progesterona durante a sincronização de ovulação em um protocolo IATF.] Objetivou-se neste estudo avaliar o efeito do tratamento com gonadotrofina coriônica equina (eCG) sobre a dinâmica e função folicular

em fêmeas mestiças com diferentes concentrações circulantes de P4 durante a sincronização da ovulação em um protocolo de IATF. Para tanto, foram utilizadas 30 fêmeas mestiças e submetidas a um protocolo de pré-sincronização para garantir que todos os animais apresentassem corpo lúteo (CL) no início do protocolo, sendo avaliadas por ultrassonografia (US) transretal para a verificação da presença ou não de CL. Em seguida foram submetidas a um protocolo de sincronização da ovulação e avaliação da dinâmica e vascularização folicular por ultrassonografia (US) em modo B e Doppler colorido. Altas concentrações de P4 no momento da sincronização da ovulação impactaram negativamente no diâmetro do

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² Departamento de Anatomia, Patologia e Clínicas Veterinárias, Universidade Federal da Bahia (UFBA), Rua Ademar de Barros 500, Ondina, Salvador, BA 40170-110, Brazil. *Corresponding author: prisferraz@gmail.com

folículo no D10, o diâmetro do folículo pré-ovulatório e na área de vascularização da parede do folículo pré-ovulatório ($P < 0,05$). As vacas com alta concentração de P4 no momento da sincronização da ovulação e que foram tratadas com eCG apresentaram diâmetro do folículo no D10 e no diâmetro e a área de vascularização da parede do folículo pré-ovulatório ($P > 0,05$), semelhantes às vacas que com baixa concentração de P4 no momento da sincronização da ovulação. Conclui-se que elevadas concentrações de P4 no momento da sincronização da ovulação impactam negativamente no diâmetro e vascularização folicular e que o eCG pode ser utilizado como uma estratégia para favorecer uma melhor resposta folicular e luteal em fêmeas mestiças com altas concentrações de P4 submetidas a um protocolo de IATF.

TERMOS DE INDEXAÇÃO: Efeito do eCG, vascularização folicular, vacas mestiças, progesterona, ovulação, protocolo IATF, bovinos, gonadotrofina coriônica equina, sincronização, doppler, clínica.

INTRODUCTION

Brazilian cattle raising has greatly advanced throughout the years with enormous quantitative and qualitative gain to its herd, and the incorporation of biotechnology has contributed to the prominent position the Country currently occupies in the global livestock sector (Baruselli et al. 2012). In this context, animal breeding biotechnologies play an important role in the dissemination of high quality genetics, increase of reproductive indexes, and improvement in the reproductive efficiency of cattle herds (Bó et al. 2013).

Fixed-time artificial insemination (FTAI) stand out among the most widely used biotechnologies in Brazil (Baruselli et al. 2012). This protocol allows insemination procedures to be conducted at predetermined times, eliminating the need for estrus detection (Sá Filho et al. 2010). Currently, FTAI is a quite widespread biotechnology, showing an advanced level of development; however, its reproductive rates range from 35 to 55%, and these results can be improved by enhancing the synchronization protocols (Bó et al. 2013).

Several hormonal treatments have been developed to support FTAI (Sá Filho et al. 2010). In Brazil, the most commonly used protocols are based on use of progesterone (P4) intravaginal devices associated with administration of estradiol benzoate (EB) at the beginning of treatment, day 0 (D0), on a random day of the oestrous cycle. Upon removal of the P4 intravaginal device, day 8 (D8), prostaglandin (PGF2 α) is applied together with estradiol cypionate (EC) or with administration of EB after 24h, and FTAI is usually performed up to 56h after device removal (Baruselli et al. 2012).

It has been reported that circulating P4 concentrations during the protocol of ovulation synchronization for FTAI influence the probability of conception through changes in the follicular and luteal functions (Wiltbank et al. 2014). In addition, the breed and level of milk production of animals are of major importance (Carvalho et al. 2008). Nevertheless, there is still lack of scientific evidence regarding the impact of P4 concentration on ovulation synchronization in crossbred cows.

Strategies have been developed to favor follicular development in the synchronization protocols and the positive effect of eCG on stimulating follicular growth, consequently increasing ovulation rate in FTAI protocols (Sá Filho et al. 2010). The eCG

is a long half-life glycoprotein produced in the endometrial calyces of the pregnant mare that binds to the receptors of follicle-stimulating (FSH) and luteinizing (LH) hormones of the follicles and to the LH receptors of the corpus luteum (CL) (Murphy 2012). Thus, some studies indicate that the positive effect of eCG on conception rates is due to the ability of this hormone to promote an increase in the absolute steroidogenic capacity of the CL (Rigoglio et al. 2013) and induce changes at the tissue, cellular and molecular levels that are associated with increase in functional capacity and progesterone synthesis of this structure (Fátima et al. 2012), and this is essential to favor better results for synchronization protocols.

Inclusion of eCG in the FTAI protocol may improve the follicular function of bovine females, which are challenged with high circulating P4 concentrations. However, the effects of this gonadotrophin on crossbred cows with different circulating P4 concentrations in an FTAI protocol are still scarce. In this context, this study aimed to assess the effect of eCG on the follicular dynamics and vascularization of crossbred cows with different plasma P4 concentrations during the ovulation synchronization in an FTAI protocol.

MATERIALS AND METHODS

Location and management of the animals. The study was conducted from May to August, 2016 at the Entre Rios experimental farm, "Escola de Medicina Veterinária e Zootecnia", "Universidade Federal da Bahia" (UFBA), located in the municipality of Entre Rios, in the northeast region of Bahia state, Brazil (11° 56'31" S, 38°05'04" W), 162m a.s.l.. Climate in the study region is hot and semi-humid and mean annual rainfall is 1,251mm. Animals in the farm were kept under extensive rearing, grazing on *Brachiaria decumbens* pasture with mineralized salt supplementation and access to water *ad libitum*. Thirty multiparous, Holstein-Friesian x Gir crossbred cows were used in this study. These animals were at 77.34 $\pm$ 22.13 day post-partum, showed lactation duration of 279.67 $\pm$ 58.67 days, average daily milk production of 4.85 $\pm$ 0.83kg, and average body condition score (BCS) of 3.07 $\pm$ 0.38 in a 1-5 scale (Houghton et al. 1990). The experimental farm was in compliance with the official immunization schedule. Prior to study commencement, the animals were submitted to clinical gynecological examination and TRUS using a 7.5MHz frequency linear transducer (Mindray z5, Shenzhen, China), and only the cows with absence of abnormality in the reproductive tract at the time of assessment were included in the study.

Synchronization protocol. The 30 multiparous, Holstein-Friesian x Gir crossbred cows were submitted to the same pre-synchronization protocol to ensure that all animals presented corpus luteum (CL) at the beginning of the protocol as described ahead: on a random day of the oestrous cycle, named day minus 16 (D-16), all animals received a new intravaginal P4 device (CIDR®, Zoetis, São Paulo, Brasil) containing 1.9g progesterone (P4) plus 2mg estradiol benzoate (EB) (Gonadiol®, Zoetis, São Paulo, Brasil) intramuscularly (IM). On day minus 8 (D-8), the intravaginal P4 device was removed and 12.5mg of Dinoprost tromethamine (PGF2 α) (Lutalyse®, Zoetis, São Paulo, Brasil) were administered IM, followed by 0.6mg estradiol cypionate (EC) (ECP®, Zoetis, São Paulo, Brasil) IM.

Eight days after removal of the P4 devices, on day 0 (D0), all animals were evaluated by B-mode TRUS using a 7.5MHz frequency linear transducer (Mindray z5, Shenzhen, China) to verify the presence of CL. After the ultrasound (US) assessment on D0, only 26 cows were submitted to a synchronization protocol and received a new intravaginal P4 device (CIDR®, Zoetis, São Paulo, Brasil) containing

1.9g P4 plus 2mg EB (Gonadiol®, Zoetis, São Paulo, Brasil) IM; on the same day, half of the animals were treated with 12.5mg PGF2 α IM (Lutalyse®, Zoetis, São Paulo, Brasil), whereas the other half was treated with 1.5mL saline solution at 0.9% IM, and thus the following study groups were formed: High P4 concentration (cows that did not receive PGF2 α at the beginning of the synchronization protocol (D0); n=13) and Low P4 concentration (animals that received PGF2 α at the beginning of the synchronization protocol (D0); n=13). On day 8 (D8), the intravaginal P4 devices were removed and 12.5mg PGF2 α (Lutalyse®, Zoetis, São Paulo, Brasil) plus 0.6mg EC (ECP®, Zoetis, São Paulo, Brasil) were administered IM to all cows.

At this time, the crossbred cows were randomly separated to be treated with 300UI eCG (FOLLIGON®, MSD Saúde Animal, São Paulo, Brasil) or 1.5mL saline solution at 0.9% IM. Thus, of the 13 cows that were not treated with PGF2 α on D0, six were treated with saline solution (High P4-S/eCG; n=6) and seven were treated with eCG (High P4-C/eCG; n=7), whereas of the 13 animals treated with PGF2 α on D0, six received saline solution (Low P4-S/eCG; n=6) and seven received eCG (Low P4-C/eCG; n=7). No intravaginal P4 device losses were observed in this study.

Ultrasound (US) assessment. US assessments were performed using a portable device equipped with the B-mode and power-Doppler functions (Mindray z5, Shenzhen, China): a 7.5MHz frequency transrectal transducer was used for the B-mode examinations, whereas for the power-Doppler assessments, the device was adjusted to 6cm/s velocity range in order to detect movement of blood cells in small vessels; to this end, the power-flow mode was used at a frequency of 8.5Mhz, 70% color gain standards, 0.7KHz pulse repetition frequency (PRF), and 6cm depth of evaluation.

Follicular dynamics were performed every 12h through B-mode and power-Doppler US, starting on D8 until ovulation, or up to 96h after removal of the intravaginal P4 device. Ovulation was defined by the non-observance of the largest follicle present in the previous examination, which was defined as preovulatory follicle, and with consequent CL formation. The preovulatory follicle growth rate (PFGR) was obtained by the difference between the preovulatory follicle diameter (PFD) and the dominant follicle diameter in the first assessment on D8 (DFD8) divided by the growth period and expressed as millimeters per day (mm/d). The total area of the dominant follicle of each animal was also calculated, and then the inner wall follicle area was subtracted from the outer wall follicle area, so that the total dominant follicle wall area on D8 (DFWA8) and D10 (DFWA10) of the synchronization protocol could be determined,

as well as the preovulatory follicle wall area (PFWA). Subsequently, the power-Doppler US images were stored in order to calculate the dominant follicle wall vascularization area on D8 (DFWVA8) and D10 (DFWVA10) of the synchronization protocol, as well as the total preovulatory follicle wall vascularization area (PFWVA). To this end, the total follicle wall vascularization area of each cow was calculated using a function of the US device. The percentages of dominant follicle wall vascularization area were evaluated on D8 (%DFWVA8) and D10 (%DFWVA10) of the synchronization protocol, as well as the percentage of preovulatory follicle wall vascularization area (%PFWVA), which was obtained by dividing the follicle wall vascularization area by the total follicle wall area according to the methodology proposed by Rodrigues (2016), as illustrated in Figure 1.

Blood collection and hormone dosing. Blood samples for plasma P4 content dosing were collected at different times of the protocol: days 0, 8, 10, and 24, through puncture of coccygeal vessels, into 10mL vacuum tubes without anticoagulant (VACUTAINERR, Becton, Dickinson and Company, Franklin Lakes/NJ, USA) and immediately stored in styrofoam containers with ice at 4°C. Subsequently, the blood samples were centrifuged at 3,000G for 15min for serum separation, transferred to labeled polyethylene microtubes, and stored at -20°C until analysis. Analyses of P4 concentration were conducted according to the chemiluminescence method using the Access Immunoassay System (Beckman Coulter, Fullerton/CA, USA) at the Laboratório de Imunologia do Instituto de Ciências da Saúde (ICS) of UFBA. Inter-assay coefficient of variation (CV) was 2.6% and assay sensitivity was 0.006ng/mL. The highest and lowest inter-assay CVs were 6.4% and 0.7%, respectively.

Experimental design and statistical procedures. The four study groups were arranged in a 2x2 completely randomized factorial design: first factor, application of PGF2 α on protocol D0 (levels of 0 and 12.5mg); second factor, application of eCG on protocol D8 (levels of 0 and 300 UI). Data were processed using the Statistical Package for Social Science 19.0 (SPSS 2012) software. Statistical analysis was as follows: 1) Mean and standard deviation of the variables of interest to the study were obtained through descriptive analysis; 2) Differences between the variables of interest: dominant and preovulatory follicle diameters (DFD8, DFD10, and PFD), preovulatory follicle growth rate (PFGR), dominant and preovulatory follicle wall areas (DFWA8, DFWA10, and PFWA), dominant and preovulatory follicle wall vascularization area (DFWVA8, DFWVA10, and PFWVA), percentages of dominant and preovulatory follicle wall

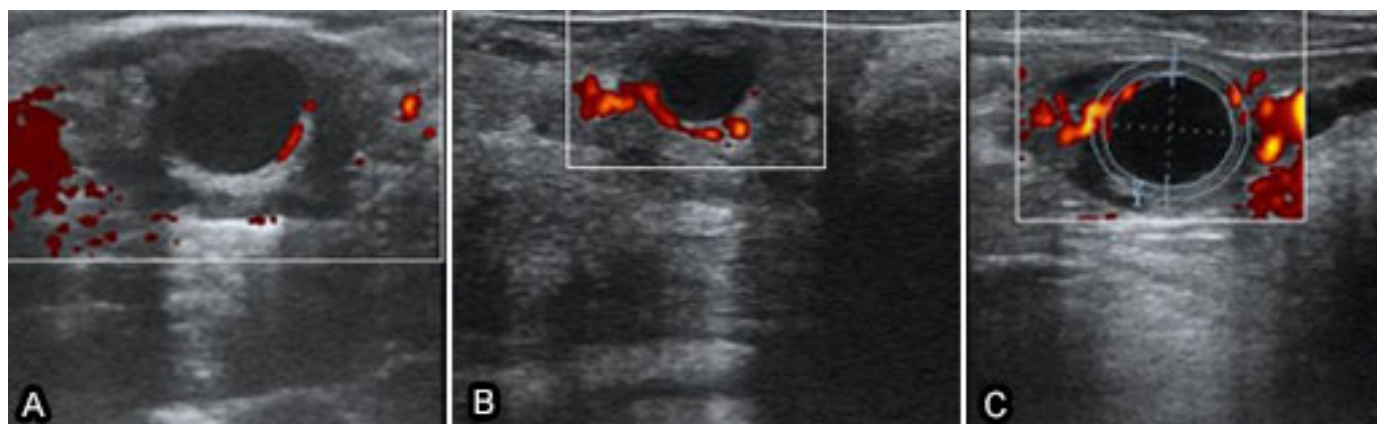


Fig.1. Power-Doppler US images of (A) compact corpus luteum, (B) cavitory corpus luteum, and (C) corpus luteum with delimited irrigated area.

vascularization areas (%DFWVA8, %DFWVA10, and %PFWVA), and analyses of circulating progesterone concentrations (P4D0, P4D8, and P4D10) for the different study groups were assessed by analysis of variance (ANOVA) with subsequent application of the Tukey's test. Comparison between the aforementioned variables of interest was performed using repeated measures ANOVA. A significance level of 5% was adopted for all statistical analyses. This study was approved by the Ethics Committee on Animal Use (CEUA) of EMEVZ-UFBA under protocol no. 41/2016.

RESULTS

Of the 30 crossbred cows initially included in the study, four did not ovulate to the pre-synchronization protocol and were excluded from this phase of the experiment. Therefore, 26 cows remained for the analysis of follicular dynamics and function and were distributed into study groups as follows: High P4-S/eCG (n=6), High P4-C/eCG (n=7), Low P4-S/eCG (n=6), and Low P4-C/eCG (n=7). No interaction was observed between the treatments regarding the variables of interest. In this study, the presence of a corpus luteum (CL) at the beginning of the synchronization protocol for FTAI significantly increased the circulating progesterone (P4) concentration at the time of ovulation synchronization. This hormonal scenario negatively influenced the dominant follicle diameter on day 10 (D10)

($p=0.01$), the preovulatory follicle diameter ($p=0.02$), and the preovulatory follicle wall vascularization area ($p=0.04$) compared with those of cows with low P4 concentrations (Table 1 and Fig.2). In the comparison between treatments over time, the variables total dominant and preovulatory follicle wall area and diameter were significantly affected ($p<0.05$); however, in the variables dominant and preovulatory follicle wall vascularization areas ($p=0.26$) and percentages of dominant and preovulatory follicle wall vascularization areas ($p=0.79$), no statistically significant differences were observed between the study groups over time, suggesting that all synchronization protocols equitably stimulated follicular vascularization.

In this study, administration of eCG upon removal of the intravaginal P4 device in cows with high plasma P4 concentration at the time of ovulation synchronization was able to equate the dominant follicle diameter on D10 ($p>0.05$), the preovulatory follicle diameter ($p>0.05$), and the preovulatory follicle wall vascularization area ($p>0.05$) of these cows with those of animals with low circulating P4 concentration (Table 1 and Fig.2).

With respect to the time of ovulation, cows with high circulating P4 concentration had a longer interval between removal of intravaginal P4 device and ovulation compared with those with low plasma P4 concentration ($p<0.05$).

Table 1. Mean and standard deviation of the parameters follicular dynamics (mm and cm²), follicular growth rate (mm/d), follicular vascularization (cm² and %), and time of ovulation (h) assessed by B-mode and power-Doppler US and plasma progesterone (P4) concentration (ng/mL) on days 0, 8, and 10 of the synchronization protocol for the different study groups in crossbred cows

Variables	Study groups				p-value	Overall (n=26)
	High P4-S/eCG (n=6)	High P4-C/eCG (n=7)	Low P4-S/eCG (n=6)	Low P4-C/eCG (n=7)		
DFD8 (mm)	8.70±2.10	9.37±1.40	10.78±2.31	11.67±2.55	0.06	10.19±2.35
DFD10 (mm)	8.78±2.04a	10.67±1.65ab	13.12±3.52b	13.37±2.34b	0.01	11.46±2.93
PFD (mm)	9.48±2.12a	11.82±2.64ab	13.66±2.58b	14.17±2.22b	0.02	12.36±2.91
PFGR (mm/dia)	0.03±0.02	0.05±0.01	0.07±0.02	0.07±0.03	0.52	0.06±0.02
DFWA8 (cm ²)	0.33±0.13	0.42±0.12	0.51±0.17	0.56±0.17	0.53	0.46±0.17
DFWA10 (cm ²)	0.25±0.14a	0.52±0.25ab	0.58±0.24ab	0.67±0.12b	0.01	0.51±0.23
PFWA (cm ²)	0.33±0.12a	0.47±0.13ab	0.54±0.25ab	0.64±0.17b	0.02	0.50±0.20
DFWVA8 (cm ²)	0.07±0.04	0.08±0.04	0.13±0.06	0.14±0.01	0.23	0.10±0.06
DFWVA10 (cm ²)	0.11±0.04a	0.18±0.10b	0.17±0.04ab	0.17±0.08ab	0.34	0.16±0.07
PFWVA (cm ²)	0.09±0.04a	0.15±0.05ab	0.18±0.09b	0.18±0.04b	0.04	0.15±0.06
%DFWVA8 (%)	16.67±12.37	18.86±6.33	29.00±12.88	21.20±3.49	0.12	21.92±10.68
%DFWVA10 (%)	20.29±10.49	24.83±15.14	22.00±7.98	32.80±2.96	0.59	24.27±5.95
%PFWVA (%)	42.17±15.31	37.29±21.53	25.29±6.10	32.80±12.27	0.26	34.20±15.89
TO (h)	96.00±13.14a	94.29±10.79a	74.40±21.46ab	61.50±11.89b	0.03	80.77±20.23
P4D0 (ng/mL)	4.58±1.06	5.94±3.49	6.54±3.21	5.48±3.40	0.38	5.60±2.91
P4D8 (ng/mL)	7.56±3.70a	7.95±2.90a	1.72±1.16b	1.55±0.74b	0.001	4.69±2.87
P4D10 (ng/mL)	0.25±0.51	0.52±0.31	0.36±0.18	0.41±0.16	0.48	0.51±0.47

Means followed by different lowercase letters on the same line differ from each other at $p<0.05$. ANOVA; Tukey's test. Groups: High P4-S/eCG = without application of PGF2 α on D0 and without administration of eCG on D8 of the synchronization protocol, High P4-C/eCG = without application of PGF2 α on D0 and with administration of eCG on D8 of the synchronization protocol, Low P4-S/eCG = with application of PGF2 α on D0 and without administration of eCG on D8 of the synchronization protocol, Low P4-C/eCG = with application of PGF2 α on D0 and with administration of eCG on D8 of the synchronization protocol; DFD8 = dominant follicle diameter on D8, DFD10 = dominant follicle diameter on D10, PFD = preovulatory follicle diameter, PFGR = preovulatory follicular growth rate, DFWA8 = dominant follicle wall area on D8, DFWA10 = dominant follicle wall area on D10, PFWA = preovulatory follicle wall area, DFWVA8 = dominant follicle wall vascularization area on D8, DFWVA10 = dominant follicle wall vascularization area on D10, PFWVA = preovulatory follicle wall vascularization area, %DFWVA8 = percentage of dominant follicle wall vascularization area on D8, %DFWVA10 = percentage of dominant follicle wall vascularization area on D10, %PFWVA = percentage of preovulatory follicle wall vascularization area, TO = time of ovulation, P4D0 = progesterone concentration on D0, P4D8 = progesterone concentration on D8, P4D10 = progesterone concentration on D10.

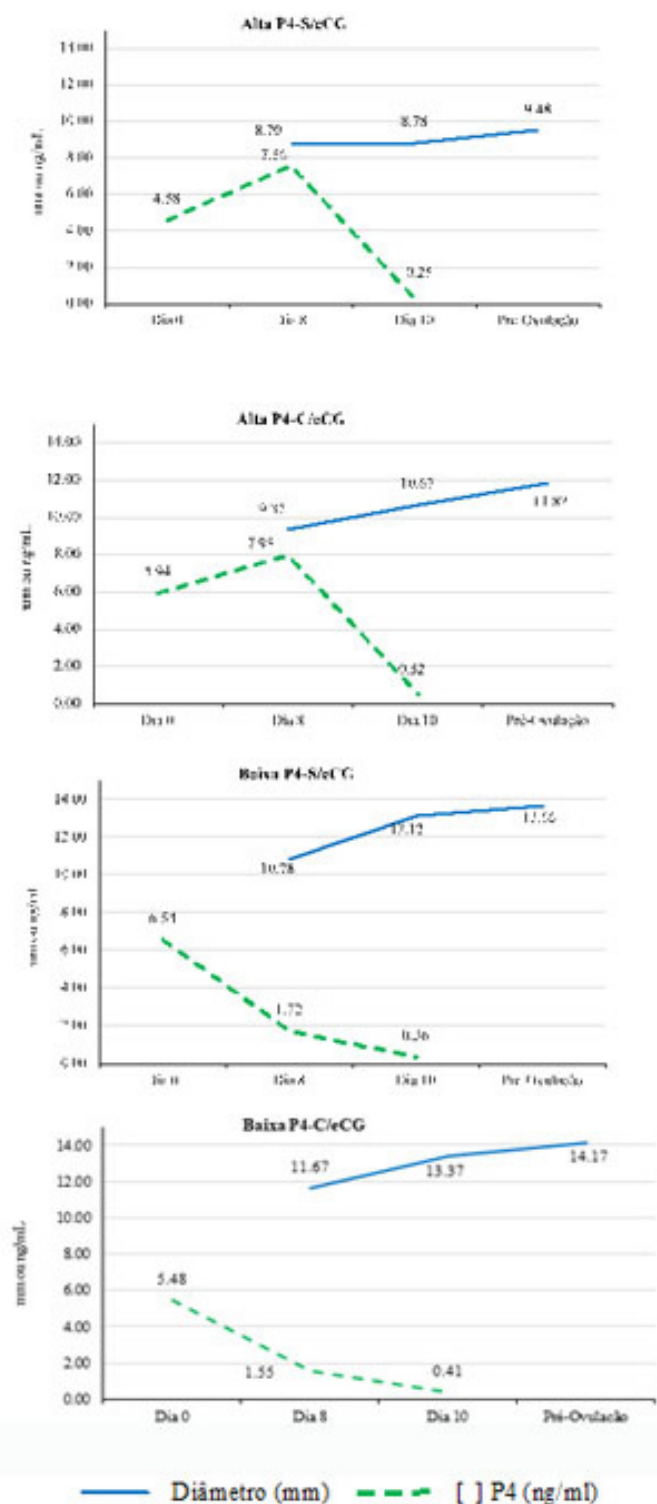


Fig.2. Dominant follicle diameter (mm) on days 8 and 10. Preovulatory follicle diameter (mm) and circulating progesterone concentration (ng/mL) on days 0, 8, and 10 of the synchronization protocol for the different study groups.

In addition, it was observed that cows with low circulating P4 concentration treated with eCG ovulated earlier than those with low serum P4 concentration at the time of ovulation synchronization that did not receive eCG ($p=0.03$, Table 1).

Cows that were not treated with PGF2 α at the beginning of the synchronization protocol showed higher circulating P4 concentration on D8 ($p=0.001$; Table 1) on average at this time, that is, at the end of the luteal phase and ovulation synchronization. These animals presented plasma P4 concentration of 7.76ng/mL; it is worth noting that this high concentration results from endogenous (CL) and exogenous (intravaginal device) P4 sources. Cows that received PGF2 α at the beginning of the synchronization protocol showed lower circulating P4 concentration on D8 ($p=0.00$, Table 1) on average at this time, that is, these animals presented plasma P4 concentration of 1.64ng/mL, and this concentration results only from an exogenous (intravaginal device) P4 source.

DISCUSSION

Findings of the present study emphasize the importance of an adequate endocrine environment during growth and maturation of ovulatory follicle in synchronization for FTAI protocols. Studies have reported that high preovulatory plasma P4 concentrations may decrease the efficiency of the synchronization protocols for FTAI (Pfeifer et al. 2009, Pergorer et al. 2011), and may be associated with reduction in the LH pulsatility, triggering a decrease in follicular development and, consequently, in the preovulatory follicle diameter (Sales et al. 2015). The mechanism by which high circulating P4 concentrations negatively influence FTAI results in beef cattle may be linked to reduction of the LH pulsatility and, therefore, to decreased dominant follicle diameter (Savio et al. 1993).

Results of this research corroborate those reported by Pergorer et al. (2011), who used administration or not of PGF2 α at the beginning of the synchronization protocol for FTAI in Nelore heifers aiming to provide a hormonal environment with different circulating P4 concentrations, and observed that low preovulatory plasma P4 concentrations increased the frequency of LH pulses, favoring follicular growth and increase in dominant follicle diameter (High P4: 10.3 ± 0.22 mm and Low P4: 9.3 ± 0.21 mm), thus allowing this follicle to reach a larger ovulatory diameter.

Similar results were found by Pfeifer et al. (2009) when studying the effect of circulating P4 concentration on a synchronization protocol for FTAI on follicular dynamics and hormonal profile in *Bos taurus taurus* beef cows. They observed that cows with low serum P4 concentration showed larger dominant (14.3 ± 0.51 mm) and ovulatory (16.5 ± 0.52 mm) follicles compared with those of cows in the high circulating P4 concentration group (dominant: 12.3 ± 0.34 and ovulatory: 14.2 ± 0.34 mm follicles). Carvalho et al. (2008) conducted a study with cyclic crossbred heifers submitted or not to application of PGF2 α on D0 of the synchronization protocol aiming to obtain animals with and without CL at the beginning of the protocol, and verified that high preovulatory circulating P4 concentrations compromised the growth of the dominant and preovulatory follicle diameters - animals with high plasma P4 concentration showed smaller dominant and preovulatory follicle diameters (10.5 ± 0.4 and 11.7 ± 0.5 mm) compared with those of cows with low circulating P4 concentration (11.9 ± 0.4 and 12.6 ± 0.4 mm). Corroborating the findings of the present survey, Sá Filho et al. (2010) conducted a study with *Bos indicus* (Nelore) heifers in the presence and absence of CL at the beginning of the synchronization protocol using

Norgestomet and EB, and verified that the preovulatory follicle diameter at the time of removal of the intravaginal P4 device was higher in animals that did not show CL at the start of treatment. In addition, they observed that these heifers had lower follicular growth rate and reduced CL diameter after the protocol, as well as lower conception rate.

Overall, the findings of the present study are in agreement with the literature, which describes that a scenario with low plasma P4 concentration stimulates a pattern of LH release of high frequency and low amplitude, promoting hormonal environment for follicular development and maturation; so with the increased frequency of LH pulses, the dominant follicle continues its growth and reaches an adequate diameter and high concentrations of estradiol, becoming responsive to the induction of the preovulatory peak of LH and to ovulation (Roberson et al. 1989).

Application of eCG is a strategy that can be used in animals that present limitation in LH pulsatility due to high preovulatory plasma P4 concentration. This glycoprotein has a positive effect on the development of the dominant follicle, increase of ovulatory follicle size, and improvement in ovulation rate (Dias et al. 2009, Peres et al. 2009). The results of the present research are in agreement with those observed by Dias et al. (2009) and Peres et al. (2009), who reported that application of eCG promotes a stimulus to the release of gonadotropins, and thus can provide adequate gonadotrophic support for the development of the ovulatory follicle. Therefore, eCG administration is a strategy to mitigate the negative effect of high circulating P4 concentrations at the time of ovulation synchronization in FTAI protocols.

Sá Filho et al. (2010) evaluated the influence of the presence or absence of CL at the beginning of the FTAI protocol using Norgestomet and BE in Nellore heifers that received or not 400UI of eCG at the time of ovulation synchronization. They observed a positive effect of eCG treatment on increasing the dominant follicle diameter on D8 and D10 of the protocol, follicular growth rate, and ovulation rate of the animals and, similarly to the findings of the present study, heifers with CL that did not receive eCG at the time of removal of the intravaginal P4 device showed smaller ovulatory follicle diameter at D10 ($9.4 \pm 0.3 \times 10.6 \pm 0.3$ mm) and reduced follicular growth rate ($0.68 \pm 0.1 \times 1.25 \pm 0.1$ mm/day) compared with those that showed CL at the beginning of the protocol and received eCG at the time of removal of the intravaginal P4 device. Moreover, they observed that heifers that did not have CL at the beginning of the protocol and received eCG showed larger ovulatory follicle diameter on D10 of the protocol ($10.8 \pm 0.4 \times 9.6 \pm 0.5$ mm) and increased follicular growth rate ($0.86 \pm 0.2 \times 0.53 \pm 0.1$ mm/day) than those with no CL at the beginning of the protocol that did not receive eCG. According to De Rensis & López-Gatius (2014), the positive effect of this glycoprotein can be attributed to the increase in steroidogenesis in the theca and follicular cells of the dominant and ovulatory follicles, fostering better follicular growth.

Contrary to the results of this study, Souza et al. (2009) investigated the follicular dynamics in Holstein-Friesian cows submitted to a synchronization protocol using P4 plus EB with or without application of eCG at the time of removal of the intravaginal P4 device and did not find a significant difference in the characteristics of the ovulatory follicle (without eCG: 14.3 ± 0.5 mm and with eCG: 13.6 ± 0.4 mm)

between cows that received gonadotrophin or not; however, Souza and collaborators did not consider the circulating P4 concentrations at the time of ovulation synchronization. Ferreira et al. (2013) developed an experiment to assess the effect of eCG on follicular and luteal parameters of high production dairy cows, and also did not observe effect on ovulatory follicle diameter for cows treated with eCG (15.4 ± 0.6 mm) or not (14.9 ± 0.7 mm) in a synchronization protocol for FTAI. They reported that the effect of eCG was evidenced only in cows with low BCS and in the post-partum anestrus period. According to Shephard (2013), animals in this physiological condition present insufficient LH support, inadequate dominant follicle growth, and subsequent failure in ovulation, or formation of CL with inefficient P4 production. Thus, treatment with eCG can increase gonadotrophin support to the follicle, improving follicular development and ovulation likelihood (Prata et al. 2017).

Although there are studies that associated follicular dynamics with blood flow of the follicles, and others that investigated the effect of different circulating P4 concentrations on the follicular and luteal parameters in cows submitted to an FTAI protocol, there is still lack of research characterizing follicular and luteal vascularization in animals with different hormonal scenarios and with or without administration of eCG in an ovulation synchronization protocol (Pfeifer et al. 2009).

According to Acosta et al. (2003), the follicle wall vascularization area is an indicator of follicular maturity prediction and ovulation proximity. Corroborating Acosta & Miyamoto (2004), adequate blood flow to the follicle wall near the time of ovulation is essential to promote blood support to the follicular cells of gonadotrophins, nutrients, and hormone substrates necessary for follicular maturation and ovulation.

In this study, the preovulatory follicle wall vascularization area in crossbred cows with high plasma P4 concentration at the time of ovulation synchronization that were treated with eCG on the day of removal of the intravaginal P4 device was similar to that of animals with low circulating P4 concentration that received or not eCG. Results of the present survey for the ovulatory follicle wall vascularization area are in agreement with the literature, which describes that an increase in the follicular blood flow occurs along with the increase of the ovulatory follicle diameter in the preovulatory period, resulting in significant increase in follicle wall vascularization area (Siddiqui et al. 2009). In this research, it was observed that application of eCG promoted improvement in follicular development and vascularization in cows that had high circulating P4 concentrations at the beginning of the protocol, reducing their negative impact at the time of ovulation synchronization. This increase can be assigned to the fact that this gonadotrophin acts on the theca and follicular cells of the follicle and activates angiogenic factors and vasodilatory molecules, fostering increased blood flow (Honnens et al. 2009).

In the present research, the preovulatory follicle wall vascularization area of animals with CL at the beginning of the protocol that were treated with eCG on the day of removal of the intravaginal P4 device was similar to that of cows that did not present CL at the beginning of the protocol. The results found for the preovulatory follicle wall vascularization area corroborate the literature, which describes the occurrence of increased blood flow in the follicle wall and follicular growth

in the peri-ovulatory period, resulting in significant increase in the follicle wall vascularization area (Acosta et al. 2003). These results may be explained by the fact that administration of eCG leads to increased follicular blood flow (Honnens et al. 2009). This increase can be attributed to activation of angiogenic factors and vasodilator molecules, as well as to the ability of this gonadotrophin to act in the theca and follicular cells of the follicle (Murphy 2012). Thus, eCG may have promoted improvement in follicular development and vascularization in the cows with CL at the beginning of the protocol, alleviating the negative effect of high preovulatory circulating P4 concentration.

These findings are in agreement with those reported by Acosta et al. (2003), who described the follicle wall vascularization area as a strategy to predict the maturity of this follicle and ovulation proximity. Siddiqui et al. (2009) observed positive and significant association of blood flow in the ovulatory follicle wall with oocyte maturity and embryonic development, and reported that follicles with higher vascularization percentage provide better supply of metabolites and increase in the action of several vasoactive factors essential for the process of follicular maturation. Increased blood flow to the follicle wall near the time of ovulation is essential to provide blood supply to follicular cells of gonadotrophins, nutrients, and hormone substrates required for follicle maturation and for ovulation (Acosta et al. 2003).

As for the time of ovulation, reduced circulating P4 concentration at the time of removal of the P4 intravaginal device is associated with the increased frequency of LH pulses, which stimulates dominant follicle growth and secretion of estradiol; this hormonal scenario allows this follicle to reach follicular maturity and ovulatory capacity, and thus the moment of ovulation occurs earlier (Dias et al. 2009, Peres et al. 2009). Similarly to the present study, Sales et al. (2015) assessed the effect of plasma P4 concentration during ovulation synchronization for an FTAI protocol in Nelore cows, and found that animals with low serum P4 concentration ovulated earlier (68.4 ± 2.3 h) compared with cows with high circulating P4 concentration (73.7 ± 2.9 h). This occurs because cows with low plasma P4 concentration present high concentrations of LH during dominant follicle growth, which leads to premature induction of LH receptors and ovulatory competence in these animals (Pfeifer et al. 2009).

Also corroborating the findings of the present study, Carvalho et al. (2016) reported that lower preovulatory circulating P4 concentration allowed the cows to ovulate more precociously compared with those with high plasma P4 concentration. These results can be evidenced by the fact that high circulating P4 concentrations reduce the LH pulsatility, triggering a negative feedback, which can diminish dominant follicle growth, causing it to present an inadequate development and a delay in reaching follicular maturity and, consequently, ovulation (Roberson et al. 1989). In this study, it was observed that smaller follicles demand more time for growth and development and thus reach the follicular maturity required for ovulation.

Macmillan et al. (1991) investigated the hormonal profile in ovariectomized *Bos taurus taurus* heifers treated with intravaginal devices containing 1.9g P4 for 12 days and observed that the mean plasma P4 concentration in this period was 5.6ng/mL. According to Rahe et al. (1980), low LH

pulsatility can be observed when plasma P4 concentrations are high, from 5 to 8ng/mL. Therefore, the high circulating P4 concentrations at the time of ovulation synchronization observed in the animals of the High-P4 group are close to the values found for pregnant cows (10.45ng/mL) (Rodrigues et al. 2018), and this hormonal scenario prevents follicular maturation and, consequently, ovulation from occurring. When cows are in a hormonal environment with high preovulatory circulating P4 concentrations, a negative feedback occurs in the secretion of GnRH by the hypothalamus, as well as decreased LH pulsatility, and this condition is determining for the development of smaller diameter follicles (Dias et al. 2009).

CONCLUSIONS

An endocrine environment with high preovulatory circulating progesterone (P4) concentration in a fixed-time artificial insemination (FTAI) synchronization protocol negatively influences follicular diameter and vascularization in crossbred cows.

Administration of equine chorionic gonadotrophin (eCG) at the time of removal of the intravaginal P4 device can be used as a strategy to favor better follicular response in cows with high plasma P4 levels at the time of ovulation synchronization due to the characteristic of this hormone in stimulating follicular growth and to its steroidogenic capacity, and it is an alternative to overcome the negative effects of high circulating P4 concentrations at the beginning of the synchronization protocol for FTAI.

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

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Use of the ACP® and BTS extenders for cooling at 15°C white-lipped peccary (*Tayassu pecari*) semen¹

Celso H.S.C. Barros^{2*} , William M. Machado², Renan L.A. Vieira², Ivan B. Allaman³, Sérgio L.G. Nogueira-Filho², Rodrigo F. Bittencourt⁴ and Paola P.N. Snoeck²

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Knowledge about reproduction of white-lipped peccary is of great importance to assist with the conservation of this species and enable its rational use in captivity. This study aimed to evaluate the effect of ACP-103®, ACP-116® and BTS semen extenders on sperm viability during cooling of *Tayassu pecari* semen. Five ejaculates from four adult males were chilled. The animals were submitted to the protocols of sedation and anesthesia for semen collection by the electroejaculation method. After collection, the semen was macro- and microscopically assessed and diluted to reach 35x10⁶ spermatozoa/mL in each of the three different extenders tested. The fresh-extended semen was packed in a BotuFLEX® thermal box to keep samples at 15°C for 24 hours. After cooling, the following semen parameters were analyzed: sperm motility, functional and structural integrity of sperm membranes, mitochondrial activity, chromatin condensation, and the thermoresistance test was performed. The parameters sperm motility, structural and functional integrity of sperm membranes, mitochondrial activity, and chromatin condensation were preserved after use of the extenders tested, and were similar to those of *in natura* semen ($p>0.05$). Curvilinear velocity (VCL) ($p<0.05$) was the only parameter with reduced values after cooling regardless of the extender used. The percentage of sperm with normal morphology was greater in samples cooled using the BTS extender ($p<0.05$). The ACP-103®, ACP-116® and BTS extenders can be used for the cooling and preservation of white-lipped peccary semen at 15°C for 24 hours.

INDEX TERMS: ACP® extenders, BTS extender, white-lipped peccary, *Tayassu pecari*, semen, coconut water, biobank, wild animals.

RESUMO.- [Uso dos diluidores ACP® e BTS para refrigeração a 15°C de sêmen de queixada (*Tayassu pecari*).] Para auxiliar na conservação da espécie e permitir o uso racional do queixada em cativeiro é de grande importância o conhecimento sobre a reprodução da espécie. Objetivou-se avaliar o efeito dos diluidores de sêmen ACP-103®, ACP-116® e BTS na viabilidade

espermática durante a refrigeração do sêmen do *Tayassu pecari*. Foram refrigerados cinco ejaculados provenientes de quatro machos adultos. Os animais foram contidos com auxílio de puçá e submetidos ao protocolo de sedação e anestesia para realização da coleta de sêmen pelo método da eletroejaculação. Depois da coleta, o sêmen foi avaliado macro e microscopicamente e diluído para atingir 35x10⁶ espermatozoides/mL em cada um dos três diferentes diluidores testados. O sêmen diluído foi acondicionado em caixa térmica BotuFLEX® para manter as amostras a 15°C por um período de 24 horas. Depois da refrigeração, os espermatozoides foram avaliados quanto aos parâmetros de movimento espermático, integridade funcional e estrutural das membranas espermáticas, atividade mitocondrial, condensação da cromatina e teste de termorresistência. Os diluidores testados preservaram as

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² Departamento de Ciências Agrárias e Ambientais, Universidade Estadual de Santa Cruz (UESC), Rodovia Ilhéus-Itabuna Km 16, Salobrinho, Ilhéus, BA 45662-000, Brazil. *Corresponding author: celso_barrosmv@hotmail.com

³ Departamento de Ciências Exatas e Tecnológicas, UESC, Rodovia Ilhéus-Itabuna Km 16, Salobrinho, Ilhéus, BA 45662-000.

⁴ Escola de Medicina Veterinária e Zootecnia, Universidade Federal da Bahia (UFBA), Av. Adhemar de Barros 500, Ondina, Salvador, BA 40170-110, Brazil.

características cinéticas, a integridade estrutural e funcional das membranas espermáticas, a atividade mitocondrial e a condensação da cromatina semelhante ao sêmen *in natura* ($P>0,05$). O único parâmetro que reduziu com o processo de refrigeração independente do diluidor utilizado foi a Velocidade Curvilínea (VCL) ($P<0,05$). Foi observado aumento do percentual de espermatozoides morfologicamente normais nas amostras refrigeradas em BTS ($P<0,05$). Os diluidores ACP-103®, ACP-116® e BTS podem refrigerar e conservar o sêmen de queixada a 15°C por 24 horas.

TERMOS DE INDEXAÇÃO: Diluidores ACP®, diluidor BTS, refrigeração, sêmen, queixada, *Tayassu pecari*, água de coco, biobanco, animais silvestres.

INTRODUCTION

White-lipped peccary (*Tayassu pecari*), a species commonly known as wild pig, is classified under the vulnerable category (VU) in the Red List of the International Union for the Conservation of Nature (IUCN) as a consequence of hunting and loss of habitat in its entire range of occurrence (Keuroghlian et al. 2013). This situation is more critical in some regions, such as in the significant remnants of Atlantic Forest in southeastern Brazil, where presence of peccaries was verified in only 31.4% of the area (SOS Mata Atlântica & INPE 2010). Owing to this sharp population decline, peccaries are considered as critically endangered in the regions of Atlantic Forest (Azevedo & Conforti 2008, Galetti et al. 2009). This species, however, plays a crucial role in the maintenance of forests through both dispersal and predation of seeds and seedlings (Keuroghlian & Eaton 2009, Altrichter et al. 2011). Because white-lipped peccary is a social ungulate that forms cohesive, large groups of hundreds of individuals (Fragoso 2004), they significantly influence biotic communities. For instance, a group of 400 peccaries represents the displacement of 12,000-20,000 kg of biomass, moving in synchrony, trampling the soil when consuming roots, seeds, and seedlings (Fragoso 2004, Beck 2006).

In contrast, this species is an important source of food for Neotropical indigenous populations and a source of income for rural populations (Fragoso 2004). In Brazil, commercial hunting is prohibited whereas subsistence hunting is simply ignored by the authorities, which has led to the disappearance of this and other native species (Nogueira & Nogueira Filho 2011). A captive breeding program could be one way to reverse the decline in white-lipped peccary population, enabling a more sustainable exploitation of the species in isolated communities that practice subsistence hunting (Figueira et al. 2003). In this context, studies addressing the behavior, nutrition and management of this species have been conducted (Nogueira Filho et al. 1999, 2014, Figueira et al. 2003, Nogueira et al. 2015, 2016), and although white-lipped peccary reproduce in captivity, there are reports that some males present low fertility (Sowls 1997).

When small, isolated populations are formed, considering linear social structure of white-lipped peccary, inbreeding can occur, reducing heterosis and compromising the reproductive efficiency and survival of the species (Biondo et al. 2011). The negative effect of inbreeding can be mitigated by the introduction of new animals or the use of fresh-extended or cooled and frozen semen from other breeding males and

the exchange of biological material to increase the genetic variability and sustainability of these captive populations. The use of semen processing technologies may be an alternative for future use in assisted reproduction, such as in artificial insemination (AI) and *in vitro* fertilization of breeding females (Garcia et al. 2012, Souza et al. 2016, Campos et al. 2017).

Utilization of cooled semen is a possibility for the best use of ejaculates, facilitating the dissemination of genetic material in different regions (Silva et al. 2012), contributing to biodiversity conservation. However, semen needs to be processed into appropriate extenders prior to cooling to ensure sperm viability and cell survival during processing (Dziekońska et al. 2009). Nevertheless, spermatozoa are particularly sensitive to thermal shock, which induces changes in plasma membranes, resulting in reduction of their biological properties, mainly in the *Suidae* species (Johnson et al. 2000). Effect of thermal shock on spermatozoa depends on cooling rate, final storage temperature, and type of extender used (Paulenz et al. 2002). Because of this sensitivity to reduced temperature, swine semen has been extensively processed with dilution and storage at 15-20°C for 1-5 days (Johnson et al. 2000, Tonioli et al. 2010). In view of the general similarities with the domestic pig (*Sus scrofa*), the semen processing protocols used in white-lipped peccary were extrapolated from this species (Mercado et al. 2010). Recent studies conducted with collared peccary (*Pecari tajacu*) have demonstrated that semen cooled at 5°C and 17°C can remain viable for up to 36 h and 48 h, respectively (Garcia et al. 2012), however, no reports have been found in the literature on the behavior of cooled semen from white-lipped peccary.

Coconut water-based extenders have been described to conserve semen from different domestic and wild species at low temperature, namely, the ACP-103® extender was reported cooling swine semen (Silva et al. 2015) and the ACP-116® extender cryopreserving collared peccary semen (Silva et al. 2012). These extenders are composed of standardized *in natura* coconut water and sugar, proteins, vitamins, amino acids, minerals, fat, and pH regulators (glycine and sodium citrate). Another extender widely used to cool swine semen is BTS (Beltsville Thawing Solution), which does not contain coconut water in its formulation, and is composed of sodium citrate, sodium bicarbonate, EDTA, potassium chloride, streptomycin, and glucose, and has been used in the cooling of semen of other species (Garcia et al. 2016, Zorinkimi et al. 2017).

Therefore, aiming to preserve the species for the possibility of facilitating dissemination of genetic material and due to lack of reports in the literature on the cooling of white-lipped peccary semen for use in assisted reproduction, the present study aimed to evaluate the effect of three different semen extenders (ACP-103®, ACP-116®, and BTS) on storage of semen of this species at 15°C and maintenance of sperm viability characteristics compatible with fertility.

MATERIALS AND METHODS

The experimental protocols applied in this study were approved by the Ethics Committee on Animal Use of the aforementioned Institution (CEUA-UESC) under protocol no. 031/16.

During the study period (March to June 2017), the adult, male white-lipped peccary presented an average weight of 36kg. Animals from Experimental Captive Breeding Center of the State University of Santa Cruz (UESC), municipality of Ilhéus, Bahia state,

Brazil (14° 47' 47" S; 39°10' 20" W), were individually allocated in individual pens prior to semen collection. In these enclosures, the animals were fed a diet composed of corn mixture, soybean meal, and mineral supplements twice daily, providing 120 g/kg of crude protein and 14.5 MJ/kg of digestible energy, based on dry matter, following the recommendations by Nogueira-Filho et al. (2014), and water *ad libitum*.

At semen collection, the white-lipped peccary were restrained using a net and submitted to anesthetic protocol, with an association of acepromazine and ketamine, after feed fast of 12 h and water fast of 6 h. Drug administration was as follows: acepromazine (0.2 mg/kg, IM) and ketamine (5 mg/kg IM), as described by Kahwage et al. (2010), who performed similar procedures for collecting semen from collared peccary (*Tayassu tajacu*), a peccary of the same family (*Tayassuidae*) and with morphological and physiological characteristics similar to those of white-lipped peccary (Sowls 1997).

After anesthesia, 5 UI of oxytocin was administered in the saphenous vein 5 min before semen collection (Ungerfeld et al. 2016). Semen was collected using an electroejaculator (Eletrogen®) equipped with a probe with two linear electrodes connected to a 12 V source according to the protocol for collared peccary (Castelo et al. 2010).

After collection, the samples were immersed in dry bath at 37°C and the following macro- and microscopic parameters of *in natura* semen were assessed: volume, color, odor, appearance, pH, motility, spermatic concentration and morphology (CBRA 2013), as well as functional and structural integrity of the plasma membrane.

Volume of the ejaculates was measured using an adjustable volume micropipette. Spermatic concentration was evaluated by counting the spermatozoa in Neubauer chamber and pH was measured using urine strips (UROFITA®). For evaluation of sperm morphology, an aliquot of fresh semen of all ejaculates was fixed in 4% sodium citrate solution and 200 spermatozoa were analyzed under phase contrast microscopy (obj.1000x) in wet mount between slide and coverslip, and the spermatic defects were classified according to the region of the morphological change found.

Sperm Class Analyser® (SCA®; Microptics S.L, v.5.2, Barcelona, Spain) software was used for evaluation of the sperm motility parameters, according to the manufacturer's recommendations for swine spermatozoa, as follows: 25 images/sec with 25 Hz; particle size captured between 10 and 80 µm/m²; velocity of spermatozoa: static <10 µm/s, slow <25 µm/s, medium >25 µm/s and <45 µm/s, and rapid >45 µm/s, as described by Silva et al. (2012) when performing the same analysis in collared peccary semen, and according to Vieira et al. (unpublished data), who investigated the sperm morphometry of white-lipped peccary and reported that it was within the particle size captured by the swine setup. One 5 µL drop of semen was placed between slide and coverslip preheated on warming platen at 37°C, and an average of five different fields were digitized. The following parameters were assessed: total motility (TM); progressive motility (PM); linearity (LIN); straightness (STR): rapid, medium, slow and hyperactive in percentage (%); curvilinear velocity (VCL); straight-line velocity (VSL); average path velocity (VAP); wobble (WOB), expressed in micrometers per second (µm/s); amplitude of lateral head displacement (ALH), expressed in micrometers (µm); beat cross frequency (BCF), expressed in Hertz (Hz).

Assessment of the plasma membrane functional integrity was performed by the hyposmotic swelling test (HOST) by adding 10 µL of semen to 90 µL distilled water (0 mOsmol/L) followed by immersion in dry bath at 37°C for 40 min (Santos et al. 2013). After incubation, reading of percentage of HOST reactive sperm was performed under optical microscopy (Olympus® CX 21), using immersion objective (obj.1000x) in wet mount between slide and

coverslip stained with 3% rose bengal. A total of 200 spermatozoa were evaluated and classified as reactive or non-reactive to HOST based on the presence or absence of bended and/or coiled tails, respectively (Jeyendran et al. 1984). For calculation of the percentage of spermatozoa with functionally intact membrane, the formula described by Melo & Henry (1999) was used, in which the percentage of spermatozoa with tail defects (based on morphology evaluation) was subtracted from the percentage of spermatozoa reactive to HOST with bended and coiled tails.

Structural integrity of the plasma and acrosomal membranes was evaluated under fluorescence microscopy (Olympus® CX 31; obj.400x) after staining of spermatozoa with fluorochrome carboxyfluorescein diacetate (CFDA) and propidium iodide (PI) according to the method of Harrison & Vickers (1990). CFDA staining was evaluated using a standard fluorescein filter set, whereas PI staining was assessed using a standard rhodamine filter set. Two hundred spermatozoa were analyzed per sample (Silva et al. 2006).

Five ejaculates from four different white-lipped peccary were used in the present study. Only ejaculates with minimum volume of 0.2 mL, minimum concentration of 35x10⁶ spermatozoa/mL, and total motility >60% were used, which were considered compatible with the experimental objective. The ejaculates were analyzed *in natura* and divided into three fractions to be processed with three different extenders, namely, ACP-103® (ACP Biotecnologia, Fortaleza, CE, Brazil); ACP-116® (ACP Biotecnologia, Fortaleza/CE, Brazil), and Beltsville Thawing Solution (BTS)- Prolimax®BTSE (Vet life, Nova Odessa, SP, Brazil). Semen was diluted according to Barros et al. (2016) to show 35x10⁶ spermatozoa/mL. The sperm motility parameters were assessed 5 min after completion of the dilution process (SCA®). The semen diluted using the ACP-103®, ACP-116® and BTS extenders was cooled in semen transport thermal box (BotuFLEX®, Botupharma Botucatu/SP, Brazil) soon after sperm motility analysis and verification of interaction between extender and semen. Final storage temperature of 15°C and cooling rate of 0.05°C/min were used. The samples remained chilled in BotuFLEX® thermal box for 24 h. After this period, the box was opened, the semen was thawed in dry bath at 37°C for 5 min, and the following sperm motility parameters were analyzed to verify the effect of the cooling process and the extenders tested on the behavior of the spermatozoa: sperm morphology, functional and structural integrity of the membranes, mitochondrial activity, and chromatin condensation.

Sperm mitochondrial activity was analyzed by 3,3'-diaminobenzidine (DAB) staining (Hrudka 1987) after incubation of 20 µL of the sample in 20 µL of DAB (1mg/mL of PBS) at 37°C for 60 min in the dark. After incubation, smears were fixed in 10% formalin for 10 min, washed in distilled water, and air dried under light protection. Two hundred cells were analyzed under phase contrast microscopy (Olympus® CX 31; obj.1000x). The cells were classified according to the amount of stain deposited on the mid-piece (MP). In class I, the spermatozoa presented the MP completely stained (all mitochondria active); in class II, the spermatozoa showed >50% of the MP stained (most mitochondria active); in class III, the spermatozoa had <50% of the MP stained (most mitochondria inactive); in class IV, the spermatozoa were not stained (all mitochondria inactive).

Spermatic chromatin condensation was evaluated using the metachromasia technique induced by toluidine blue (Naves et al. 2004). Smears were prepared with an aliquot of 10 µL of the cooled sample, dried at room temperature, and fixed for 1 min in Carnoy's solution (3:1; 75mL 100% alcohol + 25mL acetic acid) and then in 70% alcohol for 3 min. Subsequently, the smears were hydrolyzed with 4N hydrochloric acid for 15 min, washed with distilled water, and

dried at room temperature. For smear staining, 20µL of 0.025% toluidine blue solution was deposited (0.00125g toluidine blue in 5mL McIlveine solution, at pH 4.0) between slide and coverslip, and 500 cells were analyzed under phase contrast microscopy (Olympus® CX 31; obj.1000x). Spermatozoa were classified as follows: with condensed chromatin (head region stained in light blue) and with fragmented chromatin (head region stained in dark blue or violet).

Sperm longevity after 24 h of cooling was assessed by the slow thermoresistance (STT) test after the samples were incubated at 37°C for 240 min. Sperm motility characteristics were evaluated using the SCA® software, as previously described.

For statistical analysis, a completely randomized block design was used, considering each individual as a block. All assumptions of normality and homoscedasticity were tested and found adequate. Results were submitted to analysis of variance (ANOVA). Comparison of the means of the sperm motility parameters between five ejaculates was performed by the Tukey test. Comparison of the means of the sperm motility parameters between the two ejaculates that showed resistance to cooling was conducted using the Scott-Knott test. The Friedman's non-parametric test was applied to compare the means of sperm morphology and structural and functional integrity of the membranes. The Tukey test was also used to compare the means of mitochondrial activity. Means were considered statistically different when $p < 0.05$. All analyses were performed using the R Core Team (2016) software.

RESULTS

Macroscopic characteristics of *in natura* semen of five ejaculates from four white-lipped peccary evaluated were as follows: mean volume of 0.34 mL, white in color, *sui generis* odor, milky appearance, and pH values from 7.0 to 7.5. The microscopic characteristics of sperm motility are presented in Table 1. The mean concentration of spermatozoa in the ejaculate was 947.3×10^6 /mL.

Dilution process of the five ejaculates using the ACP-103®, ACP-116® and BTS extenders did not influence the sperm motility parameters, except for VCL and VAP ($p < 0.05$).

VCL and VAP were significantly reduced when fresh semen was diluted using the ACP-103® and ACP-116® extenders. The BTS extender preserved these parameters, which were similar to those of *in natura* semen (Table 1).

Table 2 shows the sperm motility parameters of *in natura* semen after dilution with the three extenders tested and after 24 h of cooling at 15°C. It is worth noting that only the semen of two peccaries showed resistance to the cooling process; the spermatozoa of the three samples from the two other animals submitted to cooling were dead after 24 h of storage at 15°C.

It was possible to observe that the percentage of spermatozoa with medium velocity increased after semen cooling using the ACP-103® and BTS extenders ($p < 0.05$). VCL was reduced in the diluted and cooled samples ($p < 0.05$). STR was increased in semen extended with ACP-116® and BTS ($p < 0.05$). Increased percentage of morphologically normal spermatozoa was observed in the cooled samples using BTS ($p < 0.05$), whereas decreased percentage of spermatozoa with distal cytoplasmic droplets (DCD) was observed in all chilled samples regardless of the extender used ($p < 0.05$). Statistically significant increase in the percentage of spermatozoa with general tail defects (GTD) involving the mid piece, principal piece and end piece was observed in all cooled samples using the ACP extenders. It should be noted that there was a statistically significant reduction in the percentage of spermatozoa with most mitochondria inactive in the chilled samples using the BTS extender. It is worth emphasizing that the dilution and cooling process did not significantly affect the other parameters: sperm motility, structural and functional integrity of the membranes, mitochondrial activity, and chromatin condensation; although the predilection of white-lipped peccary sperm for the BTS extender is evidenced by analysis of the standard deviation of the means of the characteristics studied.

Figure 1 illustrates the sperm behavior of the ejaculates from two peccaries during the STT to evaluate sperm longevity using the SCA® software after 24 h of cooling. It was not possible to assess the data statistically, but it was observed that the spermatozoa of white-lipped peccary 1, despite showing sperm

Table 1. Sperm motility parameters of *in natura* and ACP-103®, ACP-116® and BTS fresh-extended semen of five ejaculates from four white-lipped peccary

Parameters	<i>In natura</i>	ACP-103®	ACP-116®	BTS
Total motility (%)	85.7± 9.3	72.7± 18.9	70.2± 17.7	84.3± 14.5
Progressive motility (%)	46.7 ±13.7	33.3± 19.9	32.8± 21.4	42.7± 17.8
Rapid (%)	48.8± 24.0	30.4 ± 23.9	28.3± 24.3	38.6 ± 22.4
Medium (%)	19.4± 7.8	19.2 ± 5.4	17.4± 5.7	24.1±4.1
Slow (%)	17.4± 10.6	23.0± 8.6	24.7± 10.2	21.4±9.5
Hyperactive (%)	5.2± 5.5	3.1± 3.7	2.2±1.9	3.8± 3.5
VCL (µm/s)	51.2± 13.8 ^a	39.1 ±10.8 ^b	38.8± 11.1 ^b	43.6± 9.3 ^{ab}
VSL (µm/s)	19.8 ± 6.1	13.4± 5.5	14.2± 7.8	14.6± 5.6
VAP (µm/s)	34.2± 12.8 ^a	22.9± 8.4 ^b	23.8± 11.3 ^b	24.6± 8.4 ^{ab}
LIN (%)	38.8± 8.1	33.8 ± 10.1	34.7± 10.2	32.7± 7.1
STR (%)	59.4± 8.6	57.4± 5.7	57.9± 5.1	58.2± 3.9
WOB (%)	65.4± 10.9	58.1 ± 11.9	59.1± 12.4	55.6± 8.7
ALH (µm)	2.3± 0.4	2.2± 0.3	2.2± 0.3	2.4± 0.1
BCF (Hz)	6.6± 0.9	6.2± 1.4	5.9± 1.3	6.0± 0.7

^{a,b} Letters in superscript on the line indicate significant statistical difference ($p < 0.05$), assessment of the dilution effect after 5min of addition of the extenders to *in natura* semen; VCL = curvilinear velocity, VSL = straight-line velocity, VAP = average path velocity, LIN = linearity, STR = straightness, WOB = wobble, ALH = amplitude of lateral head displacement, BCF = beat cross frequency.

Table 2. Parameters of sperm motility and morphology, functional and structural integrity of sperm membranes, mitochondrial activity, and chromatin condensation in semen *in natura* and ACP-103®, ACP-116® and BTS fresh-extended, and cold-stored for 24h at 15°C - data from two white-lipped peccary

Parameters	<i>In natura</i>	Fresh-extended semen			Cold-stored semen		
		ACP-103®	ACP-116®	BTS	ACP-103®	ACP-116®	BTS
MT (%)	91.0±11.3	92.5±5.4	87.9±3.9	96.3±0.8	93.5± 5.5	78.4± 24.3	97.0± 0.3
MP (%)	51.0±12.1	53.8±10.4	55.8±8.3	61.5±2.0	45.5± 16.6	34.8± 23.6	51.6±1.3
Rapid (%)	66.3±6.5	56.4±10.1	52.6±19.1	62.0±7.8	39.8±20.2	33.9± 25.4	46.5± 4.2
Medium (%)	16.1±6.8 ^b	22.2±6.7 ^b	19.6±10.0 ^b	23.3±4.2 ^b	31.5±2.8 ^a	21.7± 7.4 ^b	33.3± 0.3 ^a
Slow (%)	8.7± 2.1	13.9±3.5	16.2±5.9	11.1±2.8	21.9±12.2	22.8± 8.4	17.2± 3.5
Hyperactive (%)	7.7± 8.1	5.4±5.6	3.6±1.8	6.7±3.7	4.6±5.3	3.5± 4.5	5.3± 5.3
VCL (µm/s)	63.7± 1.4 ^a	50.9±0.6 ^b	49.1±9.3 ^b	53.0±2.7 ^b	44.0±10.1 ^b	42.5±11.3 ^b	47.3± 1.4 ^b
VSL (µm/s)	23.0± 2.6	18.0±4.1	22.5±4.5	20.8±0.0	14.4±6.1	14.0± 6.2	14.2±1.0
VAP (µm/s)	44.2± 9.5	31.0±4.6	35.6±6.4	33.9±0.3	25.8±8.4	25.5±8.8	25.7± 1.4
LIN (%)	36.0± 3.2	35.5±8.4	45.7±0.4	39.4±2.1	32.1±6.5	32.2± 6.2	30.2± 3.1
STR (%)	52.6± 5.6 ^b	57.8±4.5 ^b	63.1±1.3 ^a	61.4±0.6 ^a	55.1±5.4 ^b	53.9± 6.0 ^b	55.5± 0.9 ^b
WOB (%)	69.1±13.4	61.0±9.7	72.5±0.9	64.1±2.7	57.9±6.0	59.4± 4.9	54.4± 4.7
ALH (µm)	2.7±0.7	2.5±0.1	2.1±0.1	2.6±0.1	2.4±0.2	2.3± 0.3	2.6± 0.1
BCF (Hz)	6.4±1.2	7.5±1.7	7.2±1.0	6.7±0.7	5.8±0.2	5.7± 0.1	6.1± 0.9
Normal (%)	45.0±38.1 ^b	-	-	-	53.8±35.0 ^{ab}	60.5±29.0 ^{ab}	71.0±18.4 ^a
PCD (%)	4.3±5.3	-	-	-	4.3±1.1	2.0±2.8	3.5±3.5
DCD (%)	32.8±26.5 ^a	-	-	-	0.0±0.0 ^b	6.0±8.5 ^{ab}	0.0±0.0 ^b
MPD (%)	5.8±6.7	-	-	-	4.0±5.7	0.0±0.0	2.5±3.5
GTD (%)	8.3±10.2 ^b	-	-	-	32.3±28.6 ^a	31.5±23.3 ^a	23.0±18.4 ^{ab}
Intact (%)	40.5±11.3	-	-	-	56.0±14.1	62.3±11.7	51.5±19.1
HOST-r (%)	51.0±22.6	-	-	-	46.5±24.7	56.5±19.1	60.0±8.5
DAB I (%)	-	-	-	-	90.8±5.3	91.5±6.4	97.3±1.8
DAB II (%)	-	-	-	-	0.0±0.0	1.0±0.7	0.5±0.7
DAB III (%)	-	-	-	-	2.8±1.1 ^a	1.8±1.1 ^{ab}	1.0±0.7 ^b
DAB IV (%)	-	-	-	-	6.5±4.2	5.8±4.6	1.3± 1.8
DNA-c (%)	-	-	-	-	93.5±7.8	94.8±6.0	94.5± 6.4
DNA-f (%)	-	-	-	-	6.5±7.8	5.3±6.0	5.5±6.4

^{a, b} Letters in superscript on the line indicate significant statistical difference ($p<0.05$), assessment of the dilution effect after 5min of addition of the extenders to *in natura* semen; (-) sperm motility parameters not assessed; VCL = curvilinear velocity, VSL = straight-line velocity, VAP = average path velocity, LIN = linearity, STR = straightness, WOB = wobble, ALH = amplitude of lateral head displacement, BCF = beat cross frequency, Normal = morphologically normal; Morphological changes in spermatozoa: PCD = proximal cytoplasmic droplets, DCD = distal cytoplasmic droplets, MPD = mid-piece defects, GTD = general tail defects, involving mid piece, principal piece and end piece; Intact = spermatozoa with integral structural membrane (CFDA+), HOST-r = spermatozoa reactive to the hypotonic swelling test, with functionally intact plasma membrane, DAB I = all mitochondria active, DAB II = most mitochondria active, DAB III = most mitochondria inactive, DAB IV = all mitochondria inactive, DNA-c = condensed chromatin, DNA-f = fragmented chromatin.

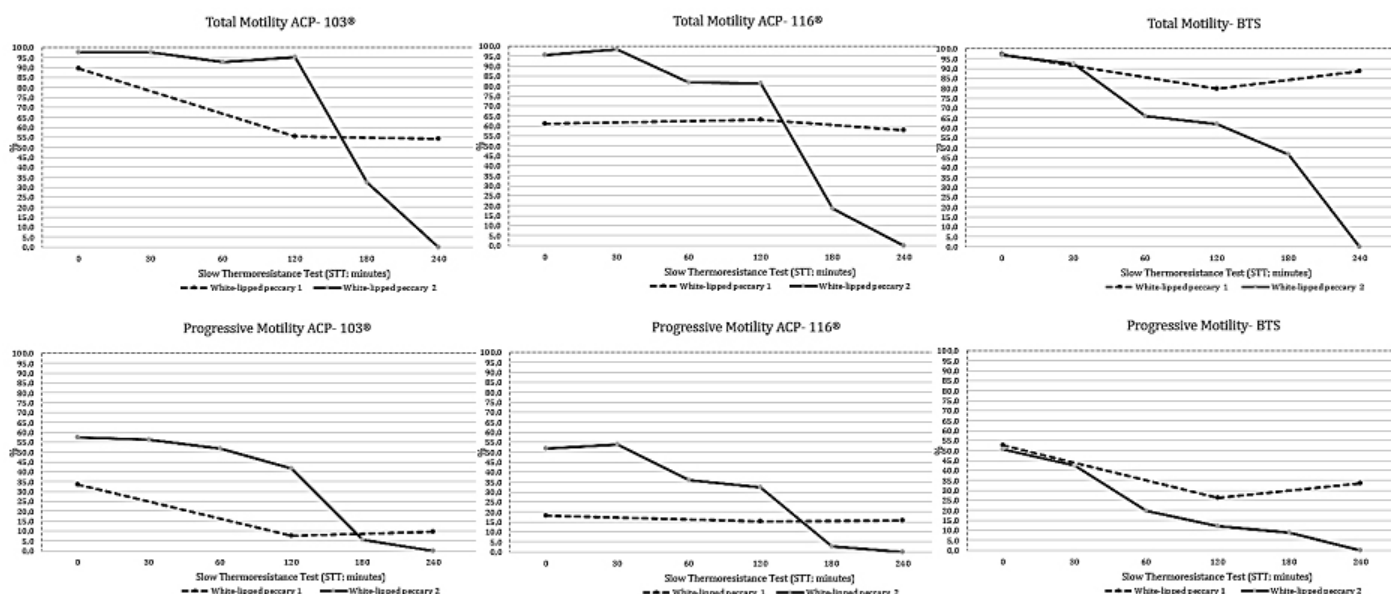


Fig.1. Total (TM) and progressive (PM) motility of the semen of two white-lipped peccary diluted using the ACP-103®, ACP-116® and BTS extenders and assessed with regards to sperm longevity for up to 240 min by the slow thermoresistance test (STT).

motility parameters slightly lower than those of white-lipped peccary 2, presented greater thermoresistance, since there was no significant variation in these parameters between the beginning and the end of the STT, as it was found in the second animal. Moreover, the spermatozoa showed motility for up to 240 min in the STT.

DISCUSSION

Dilution process of the five ejaculates from white-lipped peccary using the ACP and BTS extenders, at the spermatic concentration used, did not negatively influence the motility characteristics of the spermatozoa. However, it was observed that the ACP extenders were not as efficient in preserving VCL and VAP as the BTS extender, which maintained these parameters with values similar to those found in *in natura* semen. The ability of the BTS extender to preserve VCL and VAP from spermatozoa after dilution of *in natura* semen may be associated with the medium constitution, which contains a small amount of potassium compared with that of ACP-based extenders. According to Alvarez & Storey (1982), this characteristic favors the Na/K pump, maintaining the intracellular concentration of this ion at physiological levels, fostering sperm motility. In contrast, reduction in sperm kinetics after use of ACP extenders had already been described by Rondon et al. (2008) when using this extender for the conservation of collared peccary (*Numida meleagris*) semen at 15°C for 24 h.

It was possible to verify that the semen of two white-lipped peccary was very resistant to the cooling process at 15°C for 24 h, because no effects of dilution with the tested extenders and of temperature decrease were verified on most of the parameters studied, namely, sperm motility, structural and functional integrity of membranes, mitochondrial activity, and chromatin condensation. The results of sperm quality after cooling found in the present research were higher than those reported by Garcia et al. (2012), who conserved the semen of collared peccary, a peccary of the same family of white-lipped peccary, which suggests that spermatozoa of the first may be slightly more resistant to metabolism reduction by cold than those of the latter.

Cooling of semen resulted in increased percentage of spermatozoa with medium velocity in the samples processed with the ACP-103® and BTS extenders compared with that *in natura*, diluted regardless of the medium used, and processed with the ACP-116® extender. In addition, VCL was reduced in all diluted samples and after cooling regardless of the extender used. The increase in medium velocity occurred because of reduction in the percentage of spermatozoa with rapid velocity and increase in the percentage of spermatozoa with slow velocity and, according to Campos et al. (2017), the rapid and medium velocities and the VCL are not kinetic parameters positively and significantly correlated with the capacity of spermatozoa to bind to oocytes or perivitelline membrane in *in vitro* binding tests. Nevertheless, the parameter of slow velocity presents statistically significant negative correlation with binding to perivitelline membrane ($r=-80.5$). It is worth mentioning that preservation of motility after 24 h of cooling is indicative of sperm quality, considering that these authors reported that this parameter is a good predictor of fertility in the perivitelline membrane binding assays in collared peccary semen.

STR was increased in the samples diluted with the ACP-116® and BTS extenders, although this parameter remained with the same percentage verified in *in natura* semen after the cooling process. Campos et al. (2017) also demonstrated that STR is a marker of sperm progression, and that it was positively correlated with the capacity of collared peccary sperm to interact with heterologous swine oocytes ($r=66.5$).

It is necessary that studies addressing the sperm motility parameters that influence sperm binding capacity be conducted with white-lipped peccary, because the success of fertilization is dependent on the meeting of gametes and, to this end, the spermatozoa must move along the genital system of the female and overcome anatomical and physiological barriers (Yaniz et al. 2014). Furthermore, knowledge of the correlations between the sperm viability and kinetics parameters and binding tests could provide relevant information about the *in vitro* fertilizing potential, enabling identification of which sperm quality parameters can effectively impair *in vivo* fertilization capacity (Campos et al. 2017).

There was significant increase in the percentage of morphologically normal spermatozoa after cooling with the BTS extenders and significant decrease in the percentage of cytoplasmic droplets after refrigeration with the ACP-103® and BTS extenders, because of the DCD were somehow released by the flagellum beat during semen processing. Leidl et al. (1999) and Saravia et al. (2007) reported that cytoplasmic droplets are not considered of great importance in swine, considering that they do not show significant correlation with change in fertility in this species. It is possible that this also occurs in peccaries.

Percentage of GTD, bending and coiling, increased in ACP-cooled samples, indicating that coconut water-based extender were not as effective in controlling the lesions caused by thermal shock. The extender tested were efficient in preserving more than 90% of the spermatozoa that showed all mitochondria active. Mitochondrial metabolism plays a crucial role in the regulation of several sperm functions (Flores et al. 2009), especially those associated with displacement and projection of the cell to the fertilization site (Halang et al. 1985, Ruiz-Pesini et al. 1998). According to Cummins et al. (1994), the effect of oxidation on mitochondrial activity and on the spermatic membrane can be considered the main causing factor of sperm motility reduction, thus evaluation of mitochondrial activity can be a good indicator of its integrity and functionality. However, it should be noted that the ACP-103® extender developed for swine resulted in increased percentage of spermatozoa with most mitochondria inactive.

In the present study, it was possible to demonstrate that the white-lipped peccary semen can be cooled at 15°C for 24 h using the ACP-103®, ACP-116® and BTS extenders. Nevertheless, it was also possible to observe that there was preference of the spermatozoa for the BTS extender, because the standard deviation of the means of the parameters sperm motility; percentage of rapid, medium, slow spermatozoa; VCL; VSL; VAP; LIN; STR; percentage of spermatozoa morphologically normal, functionally intact, and with all mitochondria active showed lower values compared with the results of samples processed using the ACP extenders. Coconut water-based extenders did not show a composition of substances capable of ensuring more homogeneous semen quality responses. This variation observed in the present research can also be attributed to the question of animal individuality in the semen

cooling process that has already been observed by other authors (Roca et al. 2006, Garcia et al. 2012). Garcia et al. (2012) reported significant difference in resistance of cooled spermatozoa stored at 17°C for up to 48 h between the collared peccary investigated and between the ejaculates of the same individual. It is important to note that the white-lipped peccary in this study did not undergo reproductive selection and nevertheless produced spermatozoa with potential to be chilled and stored for 24h with the possibility of use in assisted reproduction techniques such as AI.

Of the five ejaculates diluted and refrigerated, only two ejaculates from two white-lipped peccary remained with viable spermatozoa after 24 h of cooling. Three ejaculates from two white-lipped peccary, which were in sexual rest, presented spermatozoa with no resistance to the cooling process, since 100% of the cells were dead when the BotuFLEX® thermal box was opened. There are no reports in the literature on the behavior of white-lipped peccary chilled spermatozoa. Garcia et al. (2012) studied the spermatozoa of collared peccary and observed reduction in motility, vigor, integrity of the plasma membrane, and increase of total sperm defects compared with samples of *in natura* semen and cooled for 24 and 48h at 17°C, which evidences that collared peccary semen is less resistant to the cooling process than that of white-lipped peccary. It is also known that swine spermatozoa are very sensitive to temperature oscillations, because it is common the occurrence of thermal shock that causes irreversible lesions in cell structure, and loss of sperm motility, selective permeability and plasma membrane integrity, which can lead to cell death (Watson 1996). It is possible that the spermatozoa of white-lipped peccary that have not resisted the cooling process have suffered the effect of thermal shock, since it is known that spermatozoa stored for a long time in the tail of the epididymis are less resistant to semen manipulation and processing (Pellestor et al. 1994).

Reduction in the quality of cooled spermatozoa is expected, because the process does not interrupt cellular metabolism, it only reduces the mechanical activity of the cells. Spermatozoa consume the nutrients available in the extracellular environment and excrete toxic products through constant production of substances reactive to the oxygen resulting from this metabolism (Johnson et al. 2000). In addition, sperm viability and cell osmotic resistance are also reduced by increasing the number of abnormal cells in semen (Johnson et al. 2000, Peña & Linde-Forsberg 2000). Additionally, it is known that spermatozoa undergo successive changes in the plasmatic membrane fluidity and functionality during cooling (Holt 2000), resulting in reduced selective permeability, leading to calcium influx (Maxwell & Johnson 1997), as observed in samples diluted in ACP extenders (Garcia et al. 2012, Souza et al. 2016). Therefore, sperm stored for a long time in animals with long sexual rest may show greater sensitivity to the aforementioned factors.

Aiming to minimize the effects of thermal shock, a slow curve with a mean cooling rate of approximately 0.05°C/min and final storage temperature of 15°C in the BotuFLEX® thermal box was used to cool white-lipped peccary semen in this study. The cooling rate and storage container used were different from those described in the literature for cooling swine and collared peccary semen, namely, cooling rate of -0.38°C/min to decrease the temperature from 35°C

to 25°C and cooling rate of -0.11°C/min with temperature reduction from 24.9°C to 17°C, respectively (Nascimento et al. 1998, Garcia et al. 2012). The BotuFLEX® thermal box does not allow adjustment of the cooling rate, only selection of final storage temperature to 15°C or 5°C. In spite of that, it was observed that the semen of the two peccaries behaved quite well during cooling when the sperm viability data were compared with the findings by Garcia et al. (2012), who reported mean motility of approximately 22.5%, mean percentage of spermatozoa with intact membrane of 30.4%, and percentage of total defects of 39.4% after 24 h of cooling at 17°C in semen from collared peccary. Those authors used the cooling protocol for swine in collared peccary, and reported that the spermatozoa of the latter showed sensitivity to cold very similar to that of the first, which is considered one of the most sensitive to the cooling and freezing protocols compared with spermatozoa of other domestic species (Watson 2000) such as cattle, goats, and canines.

The low resistance of sperm to thermal shock observed in some species of wild and domestic mammals and between ejaculates of the same individual is associated with the chemical and molecular composition of the plasma and mitochondrial membranes, especially with the amount of fatty acids and phospholipids and of seminal plasma (Holt et al. 2005). Regarding swine spermatozoa, this sensitivity is closely associated with the cholesterol:phospholipid ratio (0.12) present in the plasma membrane, and it is lower than those observed in bulls (0.38) or rams (0.36). This characteristic predisposes spermatozoa to several modifications: changes in membrane fluidity and selective permeability, and it can lead to cell death when semen is chilled (Leeuw et al. 1991). No reports on the composition of semen plasma and plasma membrane of white-lipped peccary spermatozoa have been found in the literature.

During the STT, a sharp decline was observed in the sperm motility parameters of white-lipped peccary 2 - an individual that presented better parameters of sperm quality after cooling and had better viability results regardless of the extender tested. However, the spermatozoa of this animal did not show resistance at 37°C for up to 240 min, and somehow the cooling process contributed to reduction in sperm longevity. The sperms of white-lipped peccary 1 showed greater resistance in the STT and were longer lived regardless of the extenders used, although when conditioned with the BTS extender - a medium containing glucose, sodium citrate, sodium bicarbonate, potassium chloride, EDTA and streptomycin, there was no sharp decrease in the kinetic parameters of spermatozoa, as observed in samples diluted in ACP extenders. Indole-3-acetic acid, a molecule of the auxin group, a plant hormone present in coconut water and ACP extenders (Nunes & Salgueiro 1999), may somehow not have guaranteed sperm motility in white-lipped peccary for a long time, even though it is already known that ACP extenders are excellent for the sperm quality parameters of swine (Toniolli et al. 1996).

Sperms of the white-lipped peccary of the present research showed motility results after STT higher than those of swine in the study by Mendez et al. (2013), which suggests that they may have greater longevity in the female reproductive tract.

CONCLUSION

ACP-103®, ACP-116® and BTS extenders can be used for the cooling and preservation of white-lipped peccary semen at 15°C for 24h.

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

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Serum proteinogram and biochemistry of Holstein cows in the peripartum period¹

Thaís G. Rocha^{2*} , Camila Bortoletto², Daniela G. Silva², Kalina M.M.G. Simplício²,
Luiz F. Zafalon³ and José J. Fagliari²

ABSTRACT.- Rocha T.G., Bortoletto C., Silva D.G., Simplício K.M.M.G., Zafalon L.F. & Fagliari J.J. 2019. **Serum proteinogram and biochemistry of Holstein cows in the peripartum period.** *Pesquisa Veterinária Brasileira* 39(5):342-347. Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista “Julio de Mesquita Filho”, Via de Acesso Prof. Paulo Donato Castellane s/n, Jaboticabal, SP 14884-900, Brazil. E-mail: thaisgrocha@yahoo.com.br

In face of the few reports found in national literature analyzing the potential influence of parturition number in serum proteinogram and biochemical profile in the peripartum period of high yielding dairy cows, the aim of the present study was to comparatively evaluate the dynamics of these serum constituents' concentrations in blood samples obtained from primiparous and multiparous Holstein cows, 60 and 30 days prepartum and in the day of parturition. Data were analyzed by repeated measures variance analysis (ANOVA) and differences between groups and moments were analyzed by Tukey's test. Results were considered significant when $P < 0.05$. Parity influenced levels of total protein, albumin, globulins, magnesium, cholesterol, which were higher in multiparous cows, as well as concentrations of ceruloplasmin, total calcium, chloride and alkaline phosphatase activity, which were higher in primiparous cows. Parturition influenced serum concentrations of ceruloplasmin (+58%), transferrin (-25%), haptoglobin (+33%), total protein (-17%), globulins (-25%), immunoglobulin A (-43%), immunoglobulin G (-24%), total calcium (-12%), inorganic phosphorus (-10%), chloride (+5%), sodium (+4%), cholesterol (-23%), triglycerides (-38.6%), as well as activities of aspartate aminotransferase (+14%) and alkaline phosphatase (+28%). A decrease in serum levels of total calcium, inorganic phosphorus, cholesterol and triglycerides was more pronounced in multiparous than in primiparous cows. These results demonstrate that the interpretation of proteinogram and serum constituents should take into consideration lactation number and the moment of parturition as relevant factors in high yielding dairy cows in the transition period.

INDEX TERMS: Proteinogram, biochemistry, Holstein cows, peripartum period, transition period, multiparous, primiparous, acute phase proteins, cattle, morphology.

RESUMO.- [Proteinograma e bioquímica séricas de vacas da raça Holandesa no período periparto.] Diante da escassez de relatos encontrados na literatura nacional quanto à potencial influência do número de parições sobre o proteinograma sérico e perfil bioquímico no período periparto de vacas leiteiras de alta produção, o objetivo do

presente estudo foi avaliar comparativamente a dinâmica de constituintes séricos em amostras de sangue obtidas de vacas da raça Holandesa primíparas e plúriparas, 60 e 30 dias pré-parto e no dia do parto. Os resultados foram avaliados por análise de variância (ANOVA) com medidas repetidas no tempo e as diferenças entre grupos e entre momentos foram analisadas pelo teste de Tukey, sendo os resultados considerados significativos quando $P < 0,05$. O número de parições influenciou os teores de proteína total, albumina, globulinas, magnésio e colesterol, que foram maiores em vacas plúriparas, bem como as concentrações de ceruloplasmina, cálcio total, cloreto e atividade de fosfatase alcalina, que foram maiores em vacas primíparas. O número de parições influenciou as concentrações séricas de ceruloplasmina

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² Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista “Julio de Mesquita Filho” (Unesp), Via de Acesso Prof. Paulo Donato Castellane s/n, Jaboticabal, SP 14884-900, Brazil. *Corresponding author: thaisgrocha@yahoo.com.br

³ Embrapa Pecuária Sudeste, Rod. Washington Luiz Km 234 s/n, Fazenda Canchim, São Carlos, SP 13560-970, Brazil.

(+58%), transferrina (-25%), haptoglobina (+33%), proteína total (-17%), globulinas (-25%), imunoglobulina A (-43%), imunoglobulina G (-24%), cálcio total (-12%), fósforo (-10%), cloretos (+5%), sódio (+4%), colesterol (-23%), triglicérides (-38.6%), bem como as atividades de aspartato aminotransferase (+14%) e fosfatase alcalina (+28%). A diminuição do teor sérico de cálcio total, fósforo, colesterol e triglicérides foi mais acentuada em vacas pluríparas do que em vacas primíparas. Esses resultados mostram que a interpretação do proteinograma e dos constituintes séricos deve levar em consideração o número de lactações e a ocorrência do parto como fatores relevantes em vacas leiteiras de alta produção no período de transição.

TERMOS DE INDEXAÇÃO: Proteinograma, bioquímica, vacas Holandesas, período periparto, período de transição, pluríparas, primíparas, proteínas de fase aguda, bovinos, morfologia.

INTRODUCTION

Laboratorial analysis is an important tool that collaborates with health status evaluation of cows, individually and as part of the herd (Cozzi et al. 2011). The determination of metabolic profile of dairy cows is also relevant for early diagnosis, treatment and prevention of production diseases in the peripartum period, as the negative consequences of health problems in the time around calving are well known and might influence production, especially in early lactation (Van Saun 2007, Silva 2009). Besides, data regarding biochemical analysis of cows in the peripartum period are usually compared to reference values, which are normally obtained from animals in mid to late lactation, which means they may not be appropriate for comparison with transition cows (Van Saun 2004).

The aim of this study was to evaluate the influence of parity on the proteinogram, and serum biochemistry profile of high yielding Holstein cows, 60 and 30 days prepartum and in the day of parturition.

MATERIALS AND METHODS

The experimental design was approved by the Animal Ethics Committee of the School of Veterinary Medicine of the São Paulo State University (Unesp), Jaboticabal Campus, under protocol number 015110/10. Forty-eight clinically healthy pregnant Holstein cows were allotted in two experimental groups comprising 24 animals each: group P (primiparous cows) and group M (multiparous cows). Cows from both groups belonged to the same herd, where mean milk yield was 32 liters/cow/day, and the experimental period extended from January 2010 to April 2011.

The diet of cows is shown in Tables 1 and 2, and consisted of two different formulations, according to the period before parturition. Between -60 days and -30 days prepartum the cows received the prepartum diet, and from -30 days pre-partum until parturition the anionic diet was provided to the animals. Animals belonging to groups P and M were allotted in different pens.

Cows from both groups were vaccinated 60 and 30 days prepartum with (1) a commercial vaccine containing strains G6P[1] and G10P[11] of inactivated rotavirus, dead coronavirus, *Escherichia coli* K99 colibacterin and type C *Clostridium perfringens* toxoid according to manufacturer's recommendations - pregnant cows and heifers received two doses, intramuscularly, with approximately 3 weeks interval between doses, and the second dose between 3 and 6 weeks

prepartum; (2) vaccine containing *Salmonella* Dublin suspension, *Pasteurella multocida*, and *Salmonella* Typhimurium in saline solution, phenicated at 0.5% inactivated by the addition of 40% formalin; (3) commercial vaccine containing a lyophilized preparation of chemically altered live samples of infectious bovine rhinotracheitis virus (IBR), and parainfluenza 3 virus (PI3) associated with a live modified bovine respiratory syncytial virus (BRSV) strain, a diluent that consisted in an inactivated combination of cytopathic and non-cytopathic BVD virus and inactivated cultures of five serotypes of *Leptospira* spp.

Blood samples were taken from the cows by jugular venipuncture; using Vacutainer® tubes (Vacutainer, Becton Dickinson, Franklin Lakes/NJ, USA) without anticoagulant, in three occasions: 60 days (M-60), and 30 days (M-30) prepartum and immediately after parturition (M0). Blood samples were centrifuged at 1,000 x g for 10 minutes, and serum was stored at -20°C until analysis.

Electrophoretic separation of serum proteins was carried out in polyacrylamide gels (SDS-PAGE) using the technique described by Laemmli (1970). After separation of protein fractions, the gel was colored in a 0.2% coomassie blue solution. Protein concentrations were determined by use of computer-assisted densitometry (Shimadzu CS-9301PC, Tokyo, Japan). Reference markers (Sigma, St Louis/MO, USA) with molecular weights of 24 kDa, 29 kDa, 36 kDa, 45 kDa, 55 kDa, 66 kDa, 97 kDa, 116 kDa, and 205 kDa were used to identify

Table 1. Formulation of concentrates received by the cows throughout the experimental period

Prepartum concentrate (-60 and -30 prepartum)	Anionic concentrate (-30 and 0 prepartum)
Vitamin E	Coline
Corn grain	Vitamin E
570 ca nutron	SALUS NA ^{®e}
Urea	Corn grain
Yeast	Vitamin AD3
Magnesium oxide	Yeast
	Urea
Mycofix ^{®a}	Mycofix ^{®a}
SELPLEX ^{®b}	SELPLEX ^{®b}
Probios ^{®c}	Biotin
Difly ^{®d}	Difly ^{®d}
Biotin	Probios ^{®c}

^a Biomin do Brasil LTDA, Piracicaba/SP, Brazil; ^b S-Vet Nutrição Animal, Pato Branco/PR, Brazil; ^c Ourofino Saúde Animal, Cravinhos/SP, Brazil; ^d Champion Fardoquímico LTDA, Anápolis/GO, Brazil; ^e Salus Group, Santo Antônio da Posse/SP, Brazil.

Table 2. Diet composition received by the cows throughout the experimental period

Prepartum diet (-60 and -30 prepartum)	kg/cow	Anionic diet (-30 and 0 prepartum)	kg/cow
Prepartum concentrate (Table 1)	0.8	Anionic concentrate (Table 1)	1.0
Soybean	1.2	Soybean	1.9
Wheat hay	1.88	Sugar cane bagasse	2.5
Sugar cane bagasse	1.33	Wheat hay	2.0
Tifton grass	14.81	Silage	14.33
Corn Silage	9.56		

protein fractions associated with the electrophoretic mobility of purified immunoglobulin A, immunoglobulin G, ceruloplasmin, transferrin, and haptoglobin (Sigma, St Louis/MO, USA).

Concentrations of total protein, albumin, urea, activities of gamma-glutamyl transferase (GGT), alkaline phosphatase (ALP), and aspartate aminotransferase (AST), as well as levels of total calcium, phosphorus, magnesium, chloride, cholesterol, and triglycerides were determined spectrophotometrically, using commercial reagents (Labtest Diagnóstica, Lagoa Santa, Minas Gerais, Brazil). Concentrations of ionized calcium, sodium, and potassium were determined by the selective ion method using an automatic electrolyte analyzer (9180 Electrolyte Analyzer, Roche Diagnostics, Mannheim, Germany). Globulin level was determined arithmetically based on the difference between total protein and albumin concentrations in serum.

Data obtained were analyzed by repeated measures ANOVA and the differences between groups and moments were compared by Tukey test, considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

In the moment of parturition (M0), there was a significant change in levels of acute phase proteins ceruloplasmin and haptoglobin, which increased 61.9% in group P and 54.3% in group M, and 24% in group P, and 33.7% in group M, respectively (Table 3). Silva (2009) and Bossaert et al. (2012) reported an increase in ceruloplasmin levels due to parturition in comparison to prepartum sampling moments, which is in agreement with the findings of the present study. The transition period is a time of immunosuppression, characterized by a marked decrease in the ability to respond to injuries and infectious challenge and so, as ceruloplasmin and haptoglobin are positive acute phase proteins in cows, this increase is expected due to the intensity of physiological and pathological events that precede parturition (Eckersall 2008). However, it is interesting to point out that although haptoglobin is considered a major acute phase protein in cows, the most significant increase observed in the acute phase protein profile evaluated by SDS-PAGE was in the concentrations of ceruloplasmin. Ceruloplasmin levels were higher in group P when compared to group M in M-30 and M0, which might be due to a stronger response of primiparous cows to the inflammatory stress caused by parturition. There was no significant difference between groups regarding haptoglobin levels, which agree with the report of Schneider et al. (2013).

Transferrin levels decreased 22.6% in group P and 28.4% in group M in M0 when compared to M-60, and did not differ between groups (Table 3). This result was expected since transferrin is a negative acute phase protein in cows, so its concentration decreases in the face of inflammatory events such as parturition or other common events in the peripartum period, as metritis, mastitis, placental retention and ketosis (Kaneko et al. 2008). Due to the limited number of animals in the present study and the low number of cows affected by these conditions (2 cows in group M presented placental retention), such events are not discussed in depth in the present study.

For the moments evaluated, α_1 -acid glycoprotein concentrations were not influenced by parturition or number of lactations, and varied between 8.43 ± 4.67 and 9.50 ± 6.94 mg/dL in group P and 9.20 ± 5.67 and 10.1 ± 6.93 mg/dL in group M. As a moderate acute phase protein in cows whose levels increase slower but are maintained for a longer period (Kaneko et al. 2008) the

evaluation of α_1 -acid glycoprotein levels would probably be more useful as a marker of puerperal diseases, and for this purpose, samples should have been taken in more occasions after parturition.

Throughout the experimental period, although in M-60 there was no difference between groups, the concentration of total protein (Table 3) was higher in group M when compared to group P, which can be explained by the cumulative effect the continuing exposure of animals to antigens present in the environment has on the globulin fraction of serum proteins, so the concentrations of total proteins tend to increase with age (Larson & Touchberry 1959). Between moments, the decrease in total protein levels in M0 in both groups is a result of the transference of globulins from the bloodstream to the mammary gland secretions, a process called colostragenesis (Birgel Junior et al. 2003). The difference observed between primiparous and multiparous cows regarding serum concentrations of total protein might influence colostrum quality, which is usually higher in multiparous cows (Rocha et al. 2012). Alvarenga et al. (2015) did not report differences in

Table 3. Mean and standard-error of protein and metabolite concentrations of primiparous (Group P) and multiparous (Group M) Holstein cows 60 (M-60), and 30 (M-30) days prepartum and in the day of parturition (M0)

Groups	Time		
	M-60	M-30	M0
Ceruloplasmin (mg/dL)			
P	50.1±5.83 Ab	51.0±3.05 Ab	82.6±6.56 Aa
M	37.1±3.46 Ab	34.6±2.35 Bb	53.4±6.58 Ba
Transferrin (mg/dL)			
P	341±21.4 Aa	333±22.1 Aa	264±26.8 Ab
M	317±22.7 Aa	303±20.2 Ab	227±23.8 Ab
Haptoglobin (mg/dL)			
P	18.9±2.93 Ab	20.8±2.88 Aab	25.8±3.21 Aa
M	20.6±1.92 Aab	18.7±1.55 Ab	25.0±2.44 Aa
Total protein (g/dL)			
P	7.93±0.11 Aa	7.81±0.10 Ba	6.48±0.17 Ab
M	8.21±0.12 Aa	8.39±0.11 Aa	6.90±0.13 Ab
Albumin (g/dL)			
P	2.64±0.06 Aa	2.55±0.04 Aa	2.54±0.04 Ba
M	2.62±0.06 Aab	2.55±0.07 Ab	2.74±0.06 Aa
Globulins (g/dL)			
P	5.29±0.11 Aa	5.26±0.11 Ba	3.94±0.16 Ab
M	5.58±0.15 Aa	5.83±0.11 Aa	4.16±0.13 Ab
Immunoglobulin A (mg/dL)			
P	79.7±6.67 Aa	89.2±7.36 Aa	50.0±5.05 Ab
M	90.9±7.56 Aa	93.6±7.45 Aa	46.4±7.56 Ab
Immunoglobulin G (mg/dL)			
P	2,098±71.2 Aa	2,242±72.4 Aa	1,715±87.0 Ab
M	2,314±94.0 Aa	2,312±71.8 Aa	1,731±138 Ab
Urea (mg/dL)			
P	17.5±1.45 Aa	20.0±1.22 Aa	19.8±1.52 Aa
M	22.0±1.66 Aa	20.7±1.48 Aa	24.8±2.05 Aa

Mean values followed by the same upper case letters in the same column and lower case letters on the same line do not differ significantly according to Tukey test ($P < 0.05$).

total protein concentrations in Holstein cows in the transition period, which disagrees with the findings of the present study.

In group M, concentration of albumin in M-30 was significantly lower when compared to M0 (Table 3). According to Birgel Junior et al. (2003), non-gestating heifers and animals at first and second thirds of gestation present higher levels of albumin than animals in the last third of pregnancy and the early postpartum. Bossaert et al. (2012) reported a decrease of albumin levels around calving, which was not noticed in the present experiment.

In M-30, the serum levels of globulins were higher in group M, when compared to group P which might also be due to the continuing exposure and increased ability of older cows to respond to antigens when compared to heifers, since vaccination schemes did not differ between groups. Between moments, a decrease in this protein fraction concentration in M0 when compared to M-30 was similar to the dynamics observed in serum levels of IgA (decrease of 43.9% in group P and 50.4% in group M) and IgG (decrease of 23.5% in group P and 25.1% in group M) (Table 3). This tendency corroborates the data reported previously by other authors, who state that the transference of immunoglobulins to the mammary gland secretions during colostrogenesis causes a typical decrease of globulin concentrations in blood serum and might influence the ability of cows to respond immunologically to challenges presented in the transition period (Birgel Junior et al. 2003, Silva 2009).

Urea concentrations were higher in group M when compared to group P in M-60 and M0, although in both groups, no difference was noticed between moments (Table 3). It is important to evaluate urea concentrations as a way to verify that dietary protein levels and nitrogen utilization efficiency are adequate (Lager & Jordan 2012). As multiparous cows receive a better diet in order to support higher milk yield, it is expected that these animals present higher levels of urea in blood serum when compared to primiparous cows.

Serum activity of GGT (Table 4) was higher in group M, when compared to group P at M-60. Birgel Junior et al. (2003) found no influence of gestational period or puerperium on the activity of this enzyme, contrary to the reported by Cozzi et al.

(2011). The difference found in the present study might be attributable to the higher incidence of hepatic disorders in older cows when compared to heifers, although the values reported here are only slightly higher than the reference values for cows found in literature (Kaneko et al. 2008) and individual evaluations did not show laboratorial evidence of hepatic disease in the animals studied (data not shown).

Serum activity of AST increased in both groups in M0 (Table 4) probably due to the intense muscular activity involved in the parturition process (Birgel Junior et al. 2003) and also due to the higher probability of liver damage as a result of negative energy balance resulting in hepatic lipidosis, especially in multiparous cows.

ALP activity was higher in group P when compared to group M in all the moments evaluated (Table 4), probably due to the higher osteoblastic activity of heifers, as reported by Cozzi et al. (2011), who found activities of ALP of 116 U/L in primiparous cows and of 90 U/L in multiparous cows. An increase in ALP activity was also found in M0, when compared to M-60 and M-30 in both groups, which is probably due to placental ALP released in the bloodstream by the time of parturition.

Serum concentration of total calcium was significantly lower in group M at M0, when compared to group P (Table 5). Although hypocalcemia may affect cows at any age, it is most common in multiparous dairy cows entering their third or greater lactation, which explains why calcium levels were lower in multiparous than in primiparous cows (Barrington 2013). Both groups presented lower total calcium concentrations at M0 when compared to M-60 and M-30. Contrary to these findings, Silva (2009) reported lower calcium levels in primiparous cows at parturition, when compared to two or more gestation cows. According to Kaneko et al. (2008), the reference range of total calcium is from 9.70 to 12.4 mg/dL in cows, which puts the present results below reference range for this parameter in all moments evaluated and in both groups. The evaluation of calcium levels around calving is an indicator of potential development of clinical or subclinical hypocalcemia (Van Saun 2007). One cow in group M presented an episode of puerperal hypocalcemia immediately after parturition, and responded well to treatment with intravenous calcium solution, although the same animal presented placental retention afterwards.

Ionized calcium levels (Table 5) did not differ between groups or moments, and varied from 0.69 ± 0.25 to 0.71 ± 0.28 mMol/L in group P and from 0.64 ± 0.23 to 0.69 ± 0.22 mMol/L in group M. Normal values for cows vary from 1.2 to 1.6 mMol/L (Kaneko et al. 2008), which indicates that in the present study, as for total calcium, levels of ionized calcium were below reference range for this species, which shows that in the period evaluated, there was an increased risk for the development of puerperal hypocalcemia and associated diseases.

There was no difference between groups regarding inorganic phosphorus concentrations (Table 5). Between moments, both groups presented lower levels of this element at M0, although values found in both groups at all moments evaluated were still within the reference range according to Kaneko et al. (2008), who reported that normal values of inorganic phosphorus concentration in cows range from 5.60 to 6.50 mg/dL. The correspondence between total calcium and inorganic phosphorus concentrations dynamics relies on a balanced mechanism which controls the homeostasis of both elements (Kaneko et al. 2008).

Table 4. Mean and standard-error of serum activities of enzymes of primiparous (Group P) and multiparous (Group M) Holstein cows 60 (M-60), and 30 (M-30) days prepartum and in the day of parturition (M0)

Groups	Time		
	M-60	M-30	M0
Gamma glutamyl-transferase (U/L)			
P	24.4±1.43 Aab	23.0±1.60 Ab	26.2±2.16 Aa
M	38.9±9.46 Aa	34.1±4.08 Aa	31.9±1.77 Aa
Alkaline phosphatase (U/L)			
P	67.6±4.91 Ab	67.5±6.14 Ab	88.4±8.96 Aa
M	44.6±4.784 Bb	49.0±4.63 Ab	61.5±5.98 Ba
Aspartate aminotransferase (U/L)			
P	53.7±1.77 Ab	53.6±1.41 Ab	59.9±1.57 Aa
M	54.6±2.98 Ab	54.6±3.01 Ab	63.5±3.79 Aa

Mean values followed by the same upper case letters in the same column and lower case letters on the same line do not differ significantly according to Tukey test ($P < 0.05$).

Table 5. Mean and standard-error of serum concentrations of minerals and lipids of primiparous (Group P) and multiparous (Group M) Holstein cows 60 (M-60), and 30 (M-30) days prepartum and in the day of parturition (M0)

Grupos	Time		
	M-60	M-30	M0
Total calcium (mg/dL)			
P	8.88±0.14 Aa	8.67±0.15 Aa	8.24±0.13 Ab
M	8.82±0.14 Aa	8.64±0.14 Aa	7.60±0.21 Bb
Ionized calcium (mMol/L)			
P	0.69±0.05 Aa	0.70±0.06 Aa	0.71±0.06 Aa
M	0.69±0.04 Aa	0.64±0.05 Aa	0.68±0.05 Aa
Phosphorus (mg/dL)			
P	6.17±0.14 Aab	6.25±0.15 Aa	5.56±0.22Ab
M	6.14±0.20 Aa	6.07±0.13 Aab	5.47±0.31 Ab
Magnesium (mg/dL)			
P	2.17±0.06 Aa	2.17±0.05 Aa	2.07±0.06 Ba
M	2.09±0.05 Ab	2.09±0.05 Ab	2.30±0.08 Aa
Chloride (mMol/L)			
P	99.8±1.28 Aab	98.6±1.37 Ab	102±1.50 Aa
M	94.9±1.33 Bb	95.4±1.59 Ab	102±1.33 Aa
Sodium (mMol/L)			
P	144±0.94 Ab	143±0.91 Ab	147±1.12 Aa
M	141±0.67 Ab	144±1.08 Ab	150±1.03 Aa
Potassium (mMol/L)			
P	4.38±0.07 Ab	4.65±0.08 Aa	4.55±0.06 Aab
M	4.45±0.05 Aa	4.53±0.06 Aa	4.51±0.06 Aa
Cholesterol (mg/dL)			
P	70.1±2.35 Ba	67.4±2.03 Aa	57.2±2.13 Ab
M	89.4±5.72 Aa	75.8±3.34 Ab	58.3±2.17 Ac
Triglycerides (mg/dL)			
P	20.6±1.05 Aa	21.6±1.08 Aa	14.3±1.19 Ab
M	21.5±1.58 Aa	21.2±1.20 Aa	13.0±0.84 Ab

Mean values followed by the same upper case letters in the same column and lower case letters on the same line do not differ significantly according to Tukey test ($P < 0.05$).

Magnesium levels were lower in group P when compared to group M at M0 (Table 5). Between moments, an increase of this elements concentration was found in group M at M0 when compared to the remaining moments evaluated. Despite of these findings, magnesium concentration remained in the normal range for cows, which varies from 1.80 to 2.30mg/dL according to Kaneko et al. (2008), which means that, associated with the fact that serum levels of magnesium reflect dietary intake, and although statistically different, the differences found in the present study do not present biological significance. Cozzi et al. (2011) reported that there was no influence of number of lactations on magnesium levels in the peripartum period, which disagrees with the findings of the present report.

Chloride concentrations varied between groups and moments and increased in M0 when compared to prepartum sampling moments in both P and M groups (Table 5). Although this difference was statistically significant, values reported here are within physiological limits established for cows that vary between 95 to 110mMol/L (Kaneko et al. 2008), which implies that these findings have no biological significance in the animals evaluated in the present study.

Although there was a variation between groups and moments in serum concentrations of sodium and potassium (Table 5), values found for both electrolytes were within reference range for cows described by Kaneko et al. (2008). According to Van Saun (2007), pre and postpartum sodium levels below 139mMol/L and prepartum potassium levels higher than 4.7mMol/L are associated with increased postpartum disease risk. In the present study, values found for these variables were not within this risk range, and clinically, there was a low incidence of diseases related to the transition period in the animals studied (data not shown).

Serum levels of cholesterol at M-60 were significantly higher in group M, when compared to group P (Table 5). Between moments, there was a gradual decrease in cholesterol levels from M-60 to M0 in groups P (-18.4%) and M (-34.8%). Alvarenga et al. (2015) also reported a decrease in cholesterol levels around parturition, and attributed this finding to a moderate decrease in food intake as well as an increase in cortisol levels around parturition, which leads to a decrease in cholesterol synthesis. Pogliani et al. (2010) reported that as multiparous cows are more subject to liver steatosis, they present a marked decrease of cholesterol concentration in the peripartum period, which agrees with the findings of the present study.

Serum levels of triglycerides did not differ between groups although, between moments, there was a marked decrease in M0 when compared to the other moments in groups P (-30.6%) and M (-39.5%) (Table 5). This decrease in triglycerides concentrations is explained by an increased hepatic uptake that occurs as a result of negative energy balance, in order to use this compound as a gluconeogenic precursor (Silva 2009, Pogliani et al. 2010), and in the present study, this was evident in both multiparous and primiparous cows as a result of onset of lactation.

CONCLUSIONS

Based on these findings, it can be concluded that parity number and the time around calving influence proteinogram and serum constituents of high yielding transition dairy cows.

The biochemical evaluation of cows in the transition period may represent an important tool for evaluation of metabolic balance in the time around calving, should parity number be taken into consideration when interpreting these parameters.

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Pelvimetry of multiparous Nellore cows in the cycling and early puerperal stages¹

Regina Lucia S. Silva², Wagner D.C. Oliveira², Daniel Biagiotti³
and Guilherme José B.C. Ferreira^{2*} 

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Pelvis is an osteoligamentous complex, which is classified as dolichopellic in ruminants. Impairments linked to incompatibility of pelvic canal and fetal size is directly related to pelvic anatomy and disposition. Heritability of pelvic area characteristics varies from moderate to high, demonstrating its importance for animal selection. Pelvimetry can be performed through direct and indirect methods that were accessed in this study aiming to establish a correlation between internal and external pelvic dimensions in multiparous Nellore cows (26 animals) at cycling and early puerperal (up to 30 days postpartum) reproductive stages. Pelvic dimensions measured by descriptive analysis were higher at early puerperal stage. Strong Pearson's correlation was determined between internal and external pelvic dimensions, between reproductive stages, and between pelvic girdle area and internal pelvic area. Significant Tukey's test differences between animals in internal and external dimensions were also found. According to the results, the pelvic anatomy of Nellore cows varies according to their reproductive stage. We detach that and provide data regarding the use of internal pelvic area for animal selection studies on this breed is determined and should be measured using the proposed formula, since it can confirm statistical differences in the areas of pelvic components between animals in the cycling and early puerperal stages.

INDEX TERMS: Pelvimetry, pluriparous cows, Nellore cows, cyclic stage, puerperium stage, cattle, pelvis, tocology.

RESUMO.- [Pelvimetria de vacas pluríparas da raça Nellore em estágio cíclico e início do puerpério.]

A pelve é considerada um complexo osteoligamentoso, classificada como dolicopélvica em ruminantes. Prejuízos ligados à incompatibilidade canal pélvico/tamanho fetal relacionam-se diretamente à anatomia e disposição pélvica. A característica de área pélvica apresenta herdabilidade variando de moderada a alta, indicando sua importância na seleção animal. A pelvimetria inclui formas direta e indireta. Neste estudo utilizaram-se vinte e seis vacas

múltiplas, da raça Nelore, agrupadas de acordo com o estágio reprodutivo, cíclico e em início de estágio puerperal (até 30 dias pós-parto). Através deste, objetivou-se evidenciar a relação das medidas pélvicas internas e externas nos diferentes períodos reprodutivos e coletar obter dados referentes a utilização da área interna do hexágono no estudo pelvimétrico dessa espécie em estágio cíclico ou puerperal. Para tanto, realizou-se, a pelvimetria direta e indireta. Os resultados obtidos, pela análise descritiva, foram maiores para animais em início de estágio puerperal. Por meio da correlação de Pearson podem-se observar fortes correlações entre mensurações pélvicas internas e externas, também entre os grupos propostos, e forte correlação entre elipse pélvica e área interna do hexágono. Pelo teste Tukey diferenças significativas tanto em medidas internas e externas puderam ser demonstradas. Conclui-se que as condições anatômicas da pelve variam de acordo com o estágio reprodutivo do animal, que aferição da área

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² Departamento de Medicina Veterinária, Universidade Federal do Piauí (UFPI), BR-135 Km 3, Bairro Planalto Horizonte, Bom Jesus, PI 64900-000, Brazil. *Corresponding author: guilherme.ferreira@ufpi.edu.br

³ Universidade Federal do Piauí, Colégio Técnico de Bom Jesus, BR-135 Km 3, Bairro Planalto Horizonte, Bom Jesus, PI 64900-000.

pélvica utilizando a formula proposta para área do hexágono interno é relevante pois foi possível comprovar a diferenças estatísticas de áreas nas fases cíclica e puerperal.

TERMOS DE INDEXAÇÃO: Pelvimetria, vacas pluríparas, raça Nellore, estágio cíclico, estágio puerpério, bovinos, pelve, tocologia.

INTRODUCTION

A commonly problem in bovine breeding is the occurrence of dystocia; it is the main cause of perinatal mortality in calves (Dematawena & Berger 1997). Racial characteristics, cow and bull conformations, calf size, and crosses with European breeds are the main causes of dystocia in bovines (Schafhauser et al. 2004). Losses due to dystocia occur because of the death of calves, and its effects on subsequent pregnancies and other reproductive events (Bellows et al. 1971).

A narrow pelvis can affect the delivery, making it difficult to expel the fetus (Derivaux & Ectors 1984). The occurrence of dystocia can be reduced by evaluating mainly the true conjugate diameter (sacropubic diameter), and the median bi-iliac breadth, since the fetus must be smaller than or equal to the female pelvis size for a normal delivery (Oliveira et al. 2003). Incompatibility between pelvic canal and fetal size involves expenses with surgical interventions, medications, longer postpartum recovery period, longer period between deliveries, and the risk of fetal and mother deaths during delivery (Hafez & Hafez 2000).

Therefore, considering the importance of the Brazilian livestock production, studies on bovine reproduction, especially females, are important to provide information on fetus and cow health to prevent expenses with reproductive problems (Hafez & Hafez 2000).

Calving occurs, on average, at 292 days in *Bos indicus*, and at 282 days in *Bos Taurus* (Paschal et al. 1991). It results from interactions between the mother and the fetus, which is a sequential maturation of communication between endocrine (Knobil & Neill 1994, Hafez & Hafez 2000), neural, and mechanical (Hafez & Hafez 2000) organs. Dilation of the pelvic region occurs during delivery; the release of relaxin - a peptidic hormone that is synthesized in the corpus luteum - is responsible for increasing malleability of the pubic symphysis and sacroiliac joints (Cunningham 1999).

A significant hormonal interference occurs on the constitution of the ligamentous fibers of the pelvic region during the delivery. This change allows a wider dilation of sacrococcygeal and intercoccygeal articulations, and ischiopubic amphiarthrosis, which only becomes completely ossified from a certain age of the animal (Derivaux & Ectors 1984).

The pelvis is a multiple-function osteoligamentous complex (Oliveira et al. 2003), its denomination derives from the Latin *pelvis* that means basin. It is classified as dolichopellic in ruminant animals because of the sacropubic diameter in females is larger than the median bi-iliac breadth (Derivaux & Ectors 1984).

The bony structure of the pelvis consists of the sacrum, first three coccygeal vertebrae, and the coxal bones (left and right) formed by the fusion of ilium, ischium, and pubis (Roberts 1986). The dorsal wall of the pelvis is formed by the sacrum and caudal vertebrae, and the ventral wall is formed by the pubic and ischial bones, including ischial tuberosity (Getty 1986); and its lateral walls are formed by the ilium and the acetabular part of the ischium bones. The articulation between coxal bones (right and left) is an amphiarthrosis called pelvic symphysis (Derivaux & Ectors 1984).

Visceral palpation via rectal and vaginal can be performed to access the pelvic area to diagnose gestations and dystocia; to explore pelvic and part of abdominal viscera; and to apply reproduction biotechniques for large animals. Thus, pelvic dimensions and delivery are closely related, mostly in cases of fetal-pelvic incompatibility (large fetus compared to mother pelvic area) that commonly results in dystocia (Deutscher 1978, Johnson et al. 1988, Nix et al. 1998). According to Koury Filho et al. (2010) every adopted criteria is the basis for genetic evaluation programs meanwhile morphological characteristics has been proved to present favorable responses to selection. For example, considering a pelvic heritability of 0.36 to 0.67 (moderate to high), and a calf weight at birth of 0.45 ($h^2 = 0.3$ to 0.45) (Deutscher 1985), the preferable characteristic for selection is the one of greater heritability (Rice 1994). This can generate calves with higher weights at birth, considering the greater bone structure of the resulting animal; however, gains will depend on the parent been selected for size of the pelvic area to allow their birth.

Pelvimetry has great applicability to the reduction of dystocia index, especially for nulliparous animals, minimizing economic losses (Meijering 1984, Ko & Ruble 1990, Oliveira et al. 2003). Two measures are fundamental for pelvimetry for pelvic classification: true conjugate diameter (sacropubic diameter), and median bi-iliac breadth. However, other measures can be considered: vertical diameters of the cranial and caudal faces of the pelvis, transversal diameter of the pelvic cavity, transversal diameter of the caudal face of the pelvis, and right and left sacroiliac oblique diameters. Pelvimetry may be direct or indirect. Four types of pelvimeter can be used in the direct method: the manual pelvimeters of Menissier-Vissac (Derivaux & Ectors 1984) and Rice, and the pneumatic/hydraulic pelvimeters of Krautmann-Litton and Equibov-Litton (Deutscher 1985, Ko & Ruble 1990). Indirect pelvimetry is the correlation between pelvic and body dimensions (Araújo et al. 2014). The present work aimed to highlight the importance and practicality of direct and indirect pelvimetry by describing the correlation between internal and external pelvic dimensions of multiparous Nellore cows at different reproductive stages (cycling and early puerperal) and provide data regarding the use of measures of internal pelvic area for studies on this breed.

MATERIALS AND METHODS

This study was carried out at the Experimental Farm of the Federal University of Piauí, in Alvorada do Gurguéia, southern State of Piauí, Brazil. Twenty-six multiparous cows of the Nellore breed were selected for the experiment and divided into two groups. The first group was consisted of 13 cows in the normal reproductive cycling stage and the second group of 13 animals in the early puerperal stage (up to 30 days postpartum).

All animals were adequate physically restrained using a cattle crush for rectal emptying and hygiene of the external anal region and identified to organize the corresponding data before the pelvimetry.

The arms of the Rice pelvimeter were then introduced, closed, and juxtaposed, keeping their internal ends close to the manipulator's hand. The bone formations of interest were located manually and the ends of the device (fixed and movable arm of the pelvimeter) were supported in the bone structures for proper measurement. The internal median bi-iliac breadth (greater internal breadth between the arms of the ilium), dorsal bi-iliac breadth (between the arms of the ilium ventrally to the sacral bone), ventral bi-iliac breadth (between the arms of the ilium at the level of the ilium pubic

eminences), sacropubic diameter (between the ventral relief of the body of the last sacral vertebrae and the projection located in the pubic symphysis), and internal bi-ischial breadth (greater breadth between ischial tuberosities) were measured (Fig.1A).

Indirect pelvimetry consisted of measuring the external median bi-iliac breadth (between lateral ends of the tuberosities of the right and left coxal bones), external bi-ischial breadth (between lateral ends of the right and left ischial tuberosities), external ilio-ischial breadth (between lateral ends of the right and left coxal and ischial tuberosities), using a tape measure (Fig.1B).

The body dimensions evaluated were animal height (from the ground to the dorsal end of the spinal processes of the first thoracic vertebrae), thoracic width (caudal tangent from the left to the right olecranon end), and thoracic depth (from the caudal wither region to the ventral portion of the sternum), using a hipometer, and body length (from the cranial end of the scapulohumeral joint to the ischial tuberosity), using a tape measure.

The pelvic girdle area (Fig.2A) was obtained by applying Equation 1; and the internal pelvic area (Fig.2B) was obtained using Equation 2.

$$PGA = \frac{MBI}{2} \times \frac{SP}{2} \times \pi \quad (1)$$

$$IPA = \left[\left(\frac{MBI + DBI}{2} \times \frac{SP}{2} \right) + \left(\frac{MBI + DBI}{2} \times \frac{SP}{2} \right) \right] \quad (2)$$

Where: *PGA* is the pelvic girdle area, *MBI* is the median bi-iliac breadth, *SP* is the sacropubic diameter, *IPA* is the internal pelvic area and *DBI* is the dorsal bi-iliac breadth.

The procedure described was approved (Issue nº 059/09) by the Animal Ethics Committee of the Universidade Federal do Piauí.

The direct and indirect pelvimetry was analyzed by descriptive statistics and Pearson correlation, using the Bioestat 5.3 program. The significance between the dimensions of animals of the different groups (cycling and early puerperal) was evaluated by the Tukey's test, using the SASM program (System for Analysis and Separation of Means).

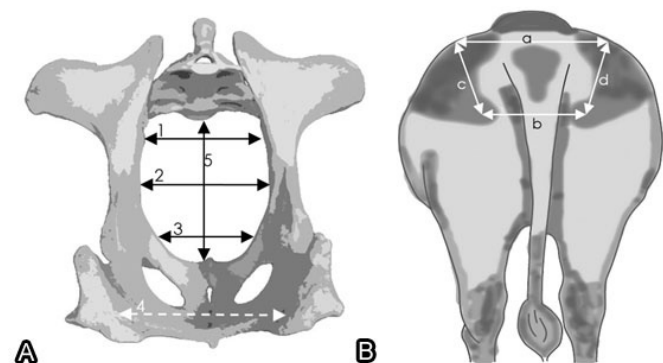


Fig.1. (A) Schematization of the pelvic bones of a bovine, where the arrows show the places used for the internal measurement, respectively: dorsal bi-iliac breadth (1), transverse diameter (2), internal ventral bi-iliac breadth (3), internal bi-ischial breadth (4) and sacropubic diameter (5). (B) Schematization of the hip of a bovine, where the arrows show the places used for the external measurement: External bi-iliac breadth (a), external bi-ischial breadth (b), external ilio-ischial breadth right (c), external ilio-ischial breadth left (d), with the convergence of arrows ac and ad the coxal tuberosity and the convergences bc and bd to the ischial tuberosities. Schemas elaborated by the authors.

RESULTS AND DISCUSSION

The descriptive analysis of the direct pelvimetry (Table 1 and 2) showed that in the pelvic girdle area presented a higher standard deviation than the other dimensions in both stages (cycling and early puerperal). It was lower than that found for the internal pelvic area, probably due to the number of variables used to obtain these areas - two for the pelvic girdle area, and four to the internal pelvic area. However, the coefficient of variation of the pelvic girdle area was lower than those found for external bi-ischial breadth, and internal pelvic area of multiparous cows in the cycling stage. Animals in the early puerperal stage presented lower coefficient of variation for pelvic girdle area than those in the cycling stage, and higher minimum and maximum areas. The other values of the direct pelvimetry of animals in the early puerperal stage were similar to the maximum values found for those in the cycling stage (Table 1 and 2).

The maximum and minimum internal dorsal bi-iliac breadth, internal median bi-iliac breadth, internal ventral bi-iliac breadth, and internal bi-ischial breadth of animals in early puerperal stage was lower than the sacropubic diameter (Table 2). This confirms the results of Oliveira et al. (2003) and classifies the pelvis of these animals as dolichopellic (Derivaux & Ectors 1984). Rezende et al. (2017) found similar results in Jafarabadi, Murrah, and Mediterranean buffaloes; these animals presented a sacropubic diameter larger than the median bi-iliac breadth, presenting pelvis with similar anatomical form to an ellipse with a vertical main axis. However, according to Tsousis et al. (2010), differences between the height of the pelvic inlet and the diagonal diameter of the pelvic inlet are not significant. These different results may be due to the methods used; pelvimetric dimensions performed in vivo and in carcasses may lead to significant differences. However, even though these differences are significant, they are too small to diversify the obstetric conduction during a possible dystocia (Kolkman et al. 2009). This denotes the consistency of the results obtained in animals when using the Rice pelvimeter.

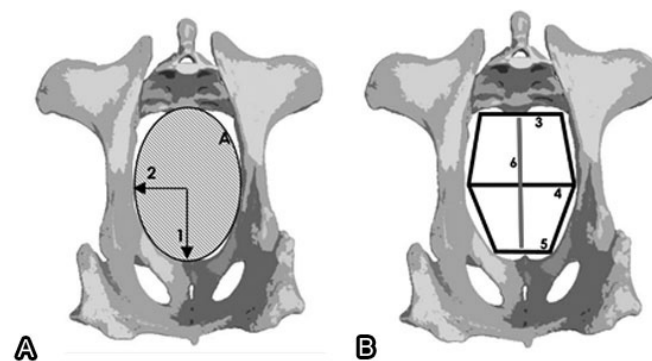


Fig.2 (A) Schematization of the pelvic bones of a bovine. The arrows represent the measurement of the rays of the pelvic ellipse, being: (1) the major radius (vertical diameter/2) and (2) the minor radius (transverse diameter/2) and the region of the pelvic ellipse area, which is obtained from formula $A = 1 \times 2 \times \pi$. (B) Schematization of the pelvic bones of a bovine. Internal median bi-iliac breadth (4), sacropubic diameter (6), internal dorsal bi-iliac breadth (3), internal ventral bi-iliac breadth (5). Schemas elaborated by the authors.

Table 1. Descriptive statistics of direct and indirect pelvimetric measurements of 13 multiparous, cyclic Nellore cows

	N	Minimum	Maximum	T.A.	A.M.	V.A.	S.D.	C.V.
AH	13	128.00	146.00	18.00	135.27	28.03	5.29	3.91%
BL	13	140.50	161.00	20.50	151.27	39.11	6.25	4.13%
TW	13	28.00	38.50	10.50	34.88	9.51	3.08	8.84%
EBIL	13	43.00	53.00	10.00	48.04	8.02	2.83	5.89%
EBIS	13	16.00	27.00	11.00	22.04	10.81	3.29	14.92%
EIIR	13	42.50	49.00	6.50	46.27	4.48	2.12	4.58%
EIIL	13	44.00	51.00	7.00	46.96	4.52	2.13	4.53%
IDBIL	13	11.00	16.50	5.50	14.54	1.89	1.38	9.47%
IMBIL	13	13.50	17.50	4.00	15.69	1.19	1.09	6.95%
IVBIL	13	12.00	16.50	4.50	14.77	1.61	1.27	8.59%
SP	13	15.00	20.00	5.00	17.65	1.68	1.30	7.35%
PGA	13	159.04	274.89	115.85	218.39	845.78	29.08	13.32%
IBIS	13	11.00	17.00	6.00	14.15	2.93	1.71	12.10%
TD	13	59.00	68.50	9.50	63.50	6.54	2.56	4.03%
IPA	13	2,087.44	4,776.20	2,688.76	3,457.00	421,048.72	648.88	18.77%

IDBIL = Internal Dorsal Bi-iliac, IMBIL = internal median bi-iliac, IVBIL = internal ventral bi-iliac, IBIS = internal bi-ischiol, SP = sacropubic, PGA = pelvic girdle area, AH = animal height, BL = body length, TW = thoracic width, TD = thoracic depth, EBIL = external bi-iliac, EBIS = external bi-ischiol, EIIR = external ilio-ischiol right, EIIL = external ilio-ischiol left, IPA = internal pelvic area, N = number of observations, A.M. = arithmetic means, S.D. = standard deviations, T.A. = total amplitude, V.A. = variance, C.V. = coefficient of variation.

Table 2. Descriptive statistics of direct and indirect pelvimetric measurements of 13 multiparous Nelore cows in puerperal stage

	N	Minimum	Maximum	A.T.	A.M.	V.A.	S.D.	C.V.
AH	13	131.00	152.00	21.00	138.81	36.56	6.05	4.36%
BL	13	145.00	162.00	17.00	153.46	38.44	6.20	4.04%
TW	13	32.00	42.00	10.00	37.42	9.58	3.09	8.27%
EBIL	13	43.00	53.00	10.00	49.00	8.67	2.94	6.01%
EBIS	13	21.00	28.00	7.00	24.54	5.39	2.32	9.46%
EIIR	13	42.00	53.00	11.00	46.77	6.36	2.52	5.39%
EIIL	13	43.00	54.00	11.00	47.27	7.03	2.65	5.61%
IDBIL	13	14.00	17.00	3.00	15.50	0.75	0.87	5.59%
IMBIL	13	14.00	17.50	3.50	15.69	1.19	1.09	6.95%
IVBIL	13	13.00	16.50	3.50	15.23	1.19	1.09	7.17%
SP	13	18.00	19.50	1.50	19.08	0.20	0.45	2.36%
PGA	13	208.92	260.36	51.44	235.06	270.87	16.46	7.00%
IBIS	13	12.00	16.50	4.50	14.04	1.85	1.36	9.70%
TD	13	63.00	72.00	9.00	66.46	5.64	2.38	3.57%
IPA	13	3,185.97	4,458.66	1,272.69	3,841.44	167,855.79	409.70	10.67%

IDBIL = Internal dorsal bi-iliac, IMBIL = internal median bi-iliac, IVBIL = internal ventral bi-iliac, IBIS = internal bi-ischiol, SP = sacropubic, PGA = pelvic girdle area, AH = animal height, BL = body length, TW = thoracic width, TD = thoracic depth, EBIL = external bi-iliac, EBIS = external bi-ischiol, EIIR = external ilio-ischiol right, EIIL = external ilio-ischiol left, IPA = internal pelvic area, N = number of observations, A.M. = arithmetic means, S.D. = standard deviations, A.T. = total amplitude, V.A. = variance, C.V. = coefficient of variation.

The arithmetic means and standard deviations found for both groups (cycling and early puerperal) regarding internal dorsal bi-iliac breadth showed differences; with lower breadths than those found in crossbred female buffaloes (Oliveira et al. 2001), and Girolando cows (Barreto et al. 2004), but higher than that found for Nellore cows (Oliveira et al. 2003) (Table 3). These results were due to conformational differences in the bone structure of crossbred buffaloes, such as their larger body diameter when compared to Nellore cows; and the genetic composition of Girolando animals, since the numerous selections for delivery ability of taurine cows possibly make, indirectly, animals from these crosses to inherit characteristics that favor delivery, such as greater

pelvic dimensions. The pelvic girdle area of animals in the early puerperal stage was larger; this may be related to the incomplete reestablishment of bone and ligamentous structures that suffered relaxation during delivery to their original conformation. However, the sacropubic diameter of animals in the cycling stage was higher than those of Nellore cows found by Oliveira et al. (2003) (Table 3). Animals in the early puerperal stage presented larger sacropubic diameter; similar results were found in Guzerá cows by Okuda et al. (1994) (Table 3). The main advantage of knowing these dimensions, measured through pelvimetry, is to avoid fetal-pelvic incompatibility (Araújo et al. 2014), i.e., including in the reproduction system animals with incompatible dimensions.

According to the Pearson's correlation, the correlations between internal and external pelvimetric dimensions of multiparous cows in the cycling and early puerperal stages were low for most animals, or moderate. The correlation between animal height and sacropubic diameter in animals in the cycling stage was $r=0.34$ (Table 4). This is a similar result to that found for crossbred buffaloes (Oliveira et al. 2001) ($r=0.33$), and Nellore cattle (Oliveira et al. 2003) ($r=0.20$). However, this correlation for animals in the early puerperal stage was moderate and negative ($r=-0.56$) (Table 5), denoting that these dimensions assume a moderate and inverse pattern of development in these animals. The correlation between sacropubic diameter and pelvic girdle area was high ($r=0.95$) in animals in the cycling stage (Table 4), and low in animals in the early puerperal stage ($r=0.18$) (Table 5). Similar results were found for the correlation between sacropubic diameter and internal pelvic area, which was $r=0.72$ for animals in the cycling stage, and $r=0.12$ for those in the early puerperal stage; and for the correlation between sacropubic diameter and internal dorsal bi-iliac breadth, internal median bi-iliac breadth, and internal ventral bi-iliac breadth, which were high in animals

in the cycling stage ($r=0.62$, $r=0.74$, $r=0.58$, respectively), and negative and low ($r=-0.32$, $r=-0.16$, $r=-0.34$ respectively) in animals in the early puerperal stage (Table 4 and 5).

These differences were probably due to instabilities of the anatomical position of the bony structures that compose the pelvis during the early puerperal stage, including changes because of the delivery reported by Cunningham (1999) and Derivaux & Ectors (1984), and incomplete reestablishment of these displaced bones. Thus, these results denote the possible equivalence between using the pelvic girdle area and internal pelvic area in pelvimetric studies.

The animal height and internal dorsal bi-iliac breadth showed correlation of 0.51 in crossbred buffaloes (Oliveira et al. 2001). This is a similar result to those found for the different animal groups in the present work, $r=0.60$ for animals in the cycling and early puerperal stages, showing a significant correlation between these characteristics. The correlation between external and internal pelvic dimensions of Nellore females is low; the correlation between external median bi-iliac and internal median bi-iliac breadths found by Oliveira et al. (2003) was 0.19, which was a similar result to that found in

Table 3. Data related to arithmetic mean and standard deviation of internal and external pelvimetric measurements observed and reported regularly in the literature by different authors and with different groups of animals

A.M. $\pm$ S.D.	IDBIL (cm)	IMBIL (cm)	IVBIL (cm)	SP (cm)	PGA (cm ²)	AH (cm)	BL (cm)	EBIL (cm)
Multiparous and cycling stage	14.54 $\pm$ 1.38	15.69 $\pm$ 1.08	14.77 $\pm$ 1.27	17.65 $\pm$ 1.29	218.38 $\pm$ 29.07	135.27 $\pm$ 5.28	151.27 $\pm$ 6.24	48.04 $\pm$ 2.82
Multiparous and early puerperal stage	15.5 $\pm$ 0.87	15.68 $\pm$ 1.09	15.23 $\pm$ 1.09	19.08 $\pm$ 0.45	235.06 $\pm$ 16.46	138.81 $\pm$ 6.05	153.45 $\pm$ 6.20	49.00 $\pm$ 2.94
Okuda et al. (1994)*	15.14 $\pm$ 0.89		13.99 $\pm$ 0.83	19.26 $\pm$ 1.33				
Oliveira et al. (2001)*	18.5 $\pm$ 1.58		17.1 $\pm$ 1.57	24.1 $\pm$ 1.78		137.7 $\pm$ 5.31	143.2 $\pm$ 8.19	63.5 $\pm$ 3.26
Oliveira et al. (2003)*	12.69 $\pm$ 1.08	14.32 $\pm$ 1.07	12.2 $\pm$ 0.86	17.68 $\pm$ 1.4	199.42 $\pm$ 26.20	129.75 $\pm$ 4.41	144.21 $\pm$ 7.10	46.19 $\pm$ 2.72
Barreto et al. (2004)*	17.12 $\pm$ 1.47		15.13 $\pm$ 1.54	19.29 $\pm$ 2.02		134.58 $\pm$ 6.27	137.17 $\pm$ 9.43	47.96 $\pm$ 3.18

* Guzerá cows (Okuda et al. 1994), multiparous crossbred buffaloes (Oliveira et al. 2001), cows of the Nelore breed multiparous (Oliveira et al. 2003), cows of the Girolanda breed multiparous (Barreto et al. 2004); A.M. = Arithmetic means, S.D. = standard deviations, IDBIL = internal dorsal bi-iliac, IMBIL = internal median bi-iliac, IVBIL = internal ventral bi-iliac, SP = sacropubic, PGA = pelvic girdle area, AH = animal height, BL = body length, EBIL = external bi-iliac.

Table 4. Pearson's correlation analysis of the direct and indirect pelvimetric characteristics of cyclic Nellore pluriparous cows

	AH	BL	TW	BILE	EBIS	EIIL	EIIR	BILID	IMBIL	IVBIL	SP	PGA	IPA	IBIS	TD
AH	1.00	---	---	---	---	---	---	---	---	---	---	---	---	---	---
BL	0.53	1.00	---	---	---	---	---	---	---	---	---	---	---	---	---
TW	-0.55	-0.20	1.00	---	---	---	---	---	---	---	---	---	---	---	---
EBIL	0.38	0.52	-0.23	1.00	---	---	---	---	---	---	---	---	---	---	---
EBIS	0.33	-0.11	-0.25	-0.18	1.00	---	---	---	---	---	---	---	---	---	---
EIIL	0.54	0.40	-0.19	0.66*	-0.35	1.00	---	---	---	---	---	---	---	---	---
EIIR	0.61*	0.43	0.07	0.46	0.04	0.75**	1.00	---	---	---	---	---	---	---	---
IDBIL	0.58*	-0.21	-0.43	-0.04	0.54	0.14	0.23	1.00	---	---	---	---	---	---	---
IMBIL	0.06	-0.37	-0.23	0.10	0.27	0.02	-0.01	0.66*	1.00	---	---	---	---	---	---
IVBIL	-0.20	-0.42	-0.01	-0.29	0.48	-0.47	-0.24	0.54	0.58*	1.00	---	---	---	---	---
SP	0.34	-0.24	-0.26	-0.05	0.27	0.23	0.30	0.73**	0.79**	0.40	1.00	---	---	---	---
PGA	0.21	-0.31	-0.25	0.05	0.27	0.17	0.19	0.73**	0.94***	0.50	0.95***	1.00	---	---	---
IPA	0.27	-0.12	-0.34	0.00	0.71**	-0.09	0.20	0.62*	0.74**	0.58*	0.72**	0.77**	1.00	---	---
IBIS	0.50	0.46	-0.59*	-0.15	0.19	0.04	-0.10	0.07	-0.43	-0.29	-0.13	-0.31	-0.14	1.00	---
TD	0.15	-0.34	-0.20	0.02	0.32	0.09	0.16	0.73**	0.94***	0.63*	0.92***	0.99***	0.80***	-0.36	1.00

IDBIL = internal dorsal bi-iliac, IMBIL = internal median bi-iliac, IVBIL = internal ventral bi-iliac, IBIS = internal bi-ischial, SP = sacropubic, PGA = pelvic girdle area, AH = animal height, BL = body length, TW = thoracic width, TD = thoracic depth, EBIL = external bi-iliac, EBIS = external bi-ischial, EIIR = external ilio-ischial right, EIIL = external ilio-ischial left, IPA = internal pelvic area; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Table 5. Pearson's correlation analysis of the direct and indirect pelvimetric characteristics of multiparous cows of the Nelore breed in the puerperal phase

	AH	BL	TW	EBIL	EBIS	EIIL	EIIR	IDBIL	IMBIL	IVBIL	SP	PGA	IPA	IBIS	TD
AH	1.00	---	---	---	---	---	---	---	---	---	---	---	---	---	---
BL	0.09	1.00	---	---	---	---	---	---	---	---	---	---	---	---	---
TW	0.35	0.13	1.00	---	---	---	---	---	---	---	---	---	---	---	---
EBIL	0.24	0.27	0.43	1.00	---	---	---	---	---	---	---	---	---	---	---
EBIS	0.63*	0.60*	0.32	0.49	1.00	---	---	---	---	---	---	---	---	---	---
EIIL	0.34	0.07	0.41	0.34	0.36	1.00	---	---	---	---	---	---	---	---	---
EIIR	0.43	0.03	0.54	0.29	0.44	0.93***	1.00	---	---	---	---	---	---	---	---
IDBIL	0.56*	-0.13	0.39	0.16	0.12	0.62*	0.54	1.00	---	---	---	---	---	---	---
IMBIL	0.19	0.00	0.43	0.14	0.19	0.32	0.38	0.62*	1.00	---	---	---	---	---	---
IVBIL	0.62*	0.11	0.22	-0.05	0.42	0.27	0.29	0.68**	0.59*	1.00	---	---	---	---	---
SP	-0.56*	0.15	0.09	0.03	-0.12	0.24	0.24	-0.32	-0.16	-0.34	1.00	---	---	---	---
PGA	0.01	0.04	0.47	0.15	0.14	0.41	0.48	0.52	0.94***	0.47	0.18	1.00	---	---	---
IPA	0.66*	0.10	0.39	0.18	0.42	0.45	0.60*	0.49	0.15	0.27	-0.18	0.10	1.00	---	---
IBIS	0.46	0.28	0.50	0.18	0.23	0.27	0.33	0.33	-0.05	0.35	-0.06	-0.06	0.51	1.00	---
TD	0.19	0.07	0.44	0.10	0.24	0.43	0.49	0.63	0.95***	0.69**	0.06	0.96***	0.16	0.05	1.00

IDBIL = Internal dorsal bi-iliac, IMBIL = internal median bi-iliac, IVBIL = internal ventral bi-iliac, IBIS = internal bi-ischiol, SP = sacropubic, PGA = pelvic girdle area, AH = animal height, BL = body length, TW = thoracic width, TD = thoracic depth, EBIL = external bi-iliac, EBIS = external bi-ischiol, EIIR = external ilio-ischiol right, EIIL = external ilio-ischiol left, IPA = internal pelvic area; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Table 6. Evaluation of significance between pelvimetric measurements of 26 cows, 13 of these multiparous in the cyclic stage and 13 in the beginning of the puerperal stage of the Nelore breed, by the Tukey test

Variable	Treatment	Mean (N = 13)	Tukey (P<5%)
Thoracic width	Cows in the early puerperal stage	37.4231	A
	Cows in the cycling stage	34.8845	B
Internal dorsal Bi-iliac	Cows in the early puerperal stage	15.5000	A
	Cows in the cycling stage	14.5385	B
Internal pelvic area	Cows in the early puerperal stage	296.1394	A
	Cows in the cycling stage	268.8317	B
		Mean (N = 13)	Tukey (P<1%)
Sacropubic	Cows in the early puerperal stage	19.0768	A
	Cows in the cycling stage	17.6538	B
Thoracic depth	Cows in the early puerperal stage	66.4614	A
	Cows in the cycling stage	63.5000	B
Thoracic area	Cows in the early puerperal stage	1956.1290	A
	Cows in the cycling stage	1736.4440	B

the present work for animals in the cycling stage (0.10) and early puerperal stages (0.14) (Table 4 and 5). Most negative correlations found between characteristic pairs in the present work were low, thus, the development of these characteristics assume opposite directions. Some correlations were classified as moderate, for example the correlation between thoracic width and internal bi-ischiol breadth in animals in the cycling stage ($r = -0.59$) (Table 4), which indicates that the development of these characteristics is inversely proportional.

The internal pelvic area encompasses better the pelvic girdle area than the internal dorsal bi-iliac breadth, which is not fully understood in the calculation of the pelvic girdle area and is fully included in the internal pelvic area. The correlation between internal pelvic area and pelvic girdle area was significant for animals in the cycling stage ($r = 0.77$), and in the early puerperal stage ($r = 0.10$) (Table 4 and 5).

Significant correlation between thoracic perimeter and internal dorsal bi-iliac breadth was also found for both

groups of animals (Table 4 and 5), indicating that this can be a parameter for selection of animals for characteristics related to pelvic area.

The comparison of means by the Tukey's test at significance level of 5% showed significant differences between external and internal dimensions of the animals in the cycling and early puerperal stages. Thoracic width, internal dorsal bi-iliac breadth, and internal pelvic area of animals in the early puerperal stage were higher than those found in animals in the cycling stage. The Tukey's test at significance level of 1% showed significantly different sacropubic diameter and thoracic perimeter between animals, with higher means in the puerperal stage when compared to animals in the cycling stage (Table 6).

CONCLUSIONS

The pelvic anatomy of Nelore cows varies according to their reproductive stage.

Assessment of the pelvic area using the formula proposed for the internal pelvic area is important, since it can confirm statistical differences in the areas of pelvic components between animals in the cycling and early puerperal stages.

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

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Ultrasonographic, macroscopic and histological characterization of the proximal insertion of the suspensory ligament in Crioulo horses¹

Grasiela De Bastiani^{2*} , Flávio D. De La Côte², Karin Erica Brass²,
Camila Cantarelli², Ligia Maria M. Malfestio³, Daniela Schwingel³,
Taiara M. Silva⁴ and Glaucia Denise Kommers⁴ 

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Although ultrasound (US) is a routine diagnostic modality, it still presents limitations for the diagnosis of lesions such as those in the proximal insertion of the suspensory ligament (PISL) because of its composition, which includes muscle fibers and adipose tissue interspersed with the ligament fibers. The objective of the present study was to describe the ultrasonographic, macroscopic and histological aspects of the PISL of thoracic limbs (TL) and pelvic limbs (PL) in Crioulo horses (CH). We selected 34 specimens of TL (right and left) and 10 specimens of PL of horses with a mean age of 5.7 years, from a private clinic or sent to the Department of Veterinary Pathology of UFSM, which died from different causes. The animals had no previous history of lameness in selected limbs associated with PISL injuries. The 34 specimens of PISL of TL were divided into CH (n=25) and Thoroughbred horses (TBH) (n=9), which composed the control group, and 11 specimens of PISL of PL were divided into CH (n=8) and TBH (n=3), which also served as control. The US examination was performed in the PISL using a *Sonosite Edge* device, 5-10 MHz linear transducer, with cross-sectional and longitudinal palmaromedial and palmarolateral images of the proximal surface of metacarpus III, II and IV (MCIII/MCII/MCIV). In PL, the evaluation was performed four centimeters below the chestnut in the plantaromedial aspect of metatarsus III and II (MTIII/MTII). PISL lobulated shape and size were compared with those of the contralateral limb, as well as the regularity of the palmar bone surface of MC III, II and IV. Subsequently, dissection of the PISL lobes was performed, as well as its macroscopic evaluation, which preceded the histological processing of the samples. In specimens of the CH breed, PISL showed echogenicity varying from peripheral dorsal hyperechogenic zones that merge into echogenic and hypoechogenic zones, where lobulation occurs. In the samples from the TBH group, PISL was also lobulated, but with differences in the echogenicity pattern such as diffuse hypoechogenicity and echogenicity. Macroscopically, CH samples presented a large amount of adipose tissue that corresponds to the dorsal peripheral zone of PISL, which ends in the connective tissue that delimits the ligamentous lobes. On a macroscopic cross-section of PISL, muscle fibers in red are mixed with white ligament fibers in the center of the ligament. This macroscopic finding was not observed in TBH samples, in which muscle fibers overlap ligament fibers throughout the ligament extension and a small amount of fat is present in the dorsal periphery of the ligament. PISL of PL had a triangular

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² Departamento de Clínica de Grandes Animais, Hospital Veterinário Universitário (HVV), Universidade Federal de Santa Maria (UFSM), Av. Roraima 1000, Campus Universitário, Santa Maria, RS 97105-900, Brazil.

*Corresponding author: grasiela.bastiani@ufsc.br

³ Departamento de Histologia, IMED, Rua Senador Pinheiro, Campus Passo Fundo, Passo Fundo, RS 99070-220, Brazil.

⁴ Departamento de Patologia, Centro de Ciências da Saúde (CCS), UFSM, Av. Roraima 1000, Campus Universitário, Santa Maria, RS 97105-900, Brasil.

shape with echogenicity characteristics very similar to those observed in TL. In ultrasonographic, macroscopic and histological evaluation, PISL samples of TL and PL in CH showed a larger amount of peripheral dorsal adipose tissue, as well as a larger number of merged ligament and muscle fibers compared with those in TBH.

INDEX TERMS: Ultrasonography, macroscopic assessment, histology, proximal insertion, suspensory ligament, Crioulo horses, ultrasound, equine, horses.

RESUMO.- [Caracterização ultrassonográfica, macroscópica e histológica da inserção proximal do ligamento suspensório em cavalos Crioulos.]

Embora uma modalidade diagnóstica rotineira, a ultrassonografia ainda possui algumas limitações para o diagnóstico de lesões como as que afetam a inserção proximal do ligamento suspensório (IPLS). Uma dessas limitações é relacionada à composição desse ligamento que inclui fibras musculares e tecido adiposo intercalados entre as fibras ligamentares. O objetivo do presente trabalho foi descrever os aspectos ultrassonográficos (US), macroscópicos e histológicos da IPLS de membros torácicos (MT) e membros pélvicos (MP) de equinos da raça crioulo (CC). Foram selecionados 34 espécimes de MT (direito e esquerdo) e 10 espécimes de MP de equinos com idade média de 5,7 anos, que vieram a óbito por diferentes causas, oriundos de uma clínica privada ou destinados ao Laboratório de Patologia Veterinária da UFSM. Não havia histórico prévio de claudicações nos membros selecionados que pudessem estar relacionadas a lesões da IPLS. Os 34 espécimes da IPLS MT foram divididos pela raça CC (n=25) e Puro Sangue Inglês (PSI) (n=9), o qual serviu como grupo controle e, 11 espécimes da IPLS do MP divididos em raça CC (n=8) e PSI (n=3) também como grupo controle. O exame US foi realizado na IPLS com um aparelho *Sonosite Edge*, transdutor linear de 5-10 MHz, com imagens transversais e longitudinais palmaromedial e palmarolateral da face proximal do metacarpiano (MC) III, II e IV. No MP a avaliação foi realizada quatro centímetros abaixo da castanha no aspecto plantaromedial do metatarsiano III e II (MTIII / MTII). Foram também observadas a forma lobulada da IPLS e o tamanho em comparação ao membro contralateral, bem como a regularidade da superfície óssea palmar do MC III, II, IV. Posteriormente foi realizada a dissecação dos lobos IPLS, bem como a avaliação macroscópica dos mesmos que antecedeu o processamento das amostras para histologia. Em espécimes CC, a IPLS possui uma ecogenicidade que varia de zonas periféricas dorsais hiperecogênicas que se mesclam a zonas ecogênicas e hipoeecogênicas onde ocorre a sua lobulação. Nas amostras do grupo PSI, a IPLS também é lobulada, mas com diferenças no padrão de ecogenicidade como, hipoeecogenicidade e ecogenicidade difusas. Macroscopicamente, as amostras CC apresentaram uma grande quantidade de tecido adiposo que corresponde à zona periférica dorsal da IPLS, a qual termina no tecido conjuntivo que delimita os lobos ligamentares. Em uma secção transversal macroscópica da IPLS as fibras musculares em vermelho se mesclam as fibras ligamentares brancas no centro do ligamento. Este achado macroscópico não foi observado na raça PSI, onde as fibras musculares intercalam as fibras ligamentares em toda a extensão do ligamento e pequenas quantidades de gordura estão presentes na periferia dorsal do mesmo. A IPLS no MP possui um formato triangular com

características de ecogenicidade muito similares as citadas no MT. Na avaliação US, macroscópica e histológica as amostras da IPLS em MT e MP de equinos na raça CC demonstraram uma maior quantidade de tecido adiposo dorsal periférico bem como, uma maior quantidade de fibras musculares e ligamentares mescladas em comparação às amostras PSI.

TERMOS DE INDEXAÇÃO: Ultrassonografia, macroscopia, histologia, inserção proximal, ligamento suspensório, cavalos Crioulos, ultrassom, histologia, equinos.

INTRODUCTION

Lameness originating from the suspensory ligament (SL) is a common finding in performance horses (Dyson 1991). There are many studies in the literature addressing the diagnosis, occurrence, and treatment alternatives for proximal desmitis of the SL. However, when considering the main modality of their diagnosis - ultrasound (US) examination, changes in the SL associated with its structure and shape cannot always be easily differentiated from normal findings (Zauscher et al. 2013).

In the thoracic limb (TL), the SL originates in the proximal palmar aspect of metacarpus III (MCIII) (Gibson & Steel 2002) and in the third carpal bone (Werpy et al. 2013), whereas in the pelvic limb (PL), the SL starts in the distal row of the tarsal bones and in the proximal plantar aspect of metatarsus III (MTIII) (Gibson & Steel 2002), and it has the shape of a triangle (Werpy et al. 2013). An additional portion coming from the calcaneus is also described (Schulze 2007, Schulze & Budras 2008).

US imaging is the most frequently used technique to assess of the suspensory apparatus (Carnicer et al. 2012). Although it was the imaging modality indicated for the evaluation of tendons and ligaments according to Denoix (1994), the interpretation of US images of the proximal insertion of the suspensory ligament (PISL) may become a challenge because of the diverse echogenicity found in this specific region. This diversity is due to the emergence of artifacts as a result of the unique histological composition of the SL, which has muscle fibers, that is why it is also called interosseous muscle III (Budras et al. 2003). This observation also stems from the different acoustic properties of the different tissues that compose the SL (O'Neil 2008). Muscle fibers are less echogenic than ligament fibers, creating a variation of echoes in the regular proximal insertion of the suspensory ligament (PISL) (Agut et al. 2009). In addition to muscle fibers, there is also fat, which is usually hyperechogenic (Bischofberger et al. 2006). However, the adipose tissue is often interspersed with muscle fibers within the ligament fascicles, contributing to variation in SL echogenicity (Schramme et al. 2012). The PISL consists of a strong tendinous band containing a variable amount of muscle tissue and fat (Dyson 1998).

It is also composed of muscle fascicles surrounded by dense connective tissue (DCT) or, specifically, collagen bundles in a parallel arrangement of fibers. The surrounding loose connective tissue (LCT) could hardly be described as the classical perimysium, because it presents characteristics similar to those of the tissue surrounding the tendon tissue (Soffler & Hermanson, 2006).

Interpretation of routine US examination of the PISL in Crioulo horses (CH) can be difficult due to lack of information on its tissue composition, which directly influences its echogenicity pattern. To this end, this study aimed to describe the association of ultrasonographic, macroscopic and histological findings, thus providing a more accurate characterization of the PISL in the CH breed.

MATERIALS AND METHODS

We selected 34 specimens of TL and 10 specimens of PL of Crioulo (study group - CH) and Thoroughbred (control group - TBH) horses that died from different causes at a mean age of 5.7 years, from a private equine clinic in southern Brazil, or that were sent to the Department of Veterinary Pathology of the Federal University of Santa Maria (UFSM). The TL specimens were disarticulated in the intercarpal region, whereas the PL specimens were disarticulated in the proximal intertarsal joint. The specimens were frozen at the origin, sent to the UFSM, and conditioned at 4°C. US imaging was used to characterize the echogenicity, size, shape, and architecture of the PISL in CH, and the images were compared with those obtained under the same conditions from the PISL in TBH. The specimens were trichotomized on the proximal palmar surface in the TL and proximal plantaromedial aspect below the chestnut in the PL. In order to facilitate passage of the US waves, the specimens were immersed in warm water and gel was used for better coaptation of the transducer to the skin. A stand-off pad was attached to the transducer to enlarge the contact surface between the transducer and the structure to be assessed. The US images were produced using a Sonosite device equipped with a 5-10 MHz linear transducer. Examination of the PISL of TL was performed through three approaches: a proximal, palmar cross-section, aiming to observe the two lobes together and the bone surface of MCIII in the same image and, in a second moment, observe each lobe separately in a palmaromedial and palmarolateral cross section, and a longitudinal and transverse section, examining the entheses of MCIII between MCII and MCIV. Assessment of the PISL of PL was conducted using a cross-sectional and longitudinal palmaromedial image taken approximately 4cm below the chestnut. Aiming to facilitate the US approach and maintain limb stance similar to the normal standing biomechanics, the specimens were placed in a hydraulic press under a force of 400-500kg. Criteria such as echogenicity, size, shape, architecture and entheses of the PISL were evaluated for the CH and TBH breeds in the TL and PL. Specimens of both breeds that did not present changes in the US examination of the PISL were selected for the macroscopic and histological assessments. Subsequently, the medial and lateral lobes of the PISL were dissected in the proximal palmar region of the TL, approximately 2cm below the carpo-metacarpal joint, and in the proximal palmaromedial region of the PL, approximately 4cm below the chestnut. The medial and lateral lobes of the PISL of the TL were cross-sectioned, as well as the rounded lobe of the PL, and photographed for documentation. After that, the samples were fixed in 10% buffered formalin for 14 days. Soft tissue samples were routinely processed for histology in 3µm-thick sections and stained with hematoxylin-eosin (HE).

Following, morphometric evaluation of the variables DCT, LCT, fat, and muscle fibers of the PISL was performed, and the results of the study (CH) and control (TBH) groups were compared. The 34 PISL specimens of the TL were divided into CH (73.52%; X/n total n=25) and TBH (26.37%; X/n total n=9), whereas the 11 PISL specimens of the PL composed CH (72.77%; X/n total n=8) and TBH (27.27%; X/n total n=3).

Statistical analysis. In order to linearize the data, the variables DCT, LCT, fat, and muscle fibers were transformed into numerical variables and then into linear score ($LS = [\log_2(\text{variable})] + 3$). After transformation into LS, the dependent variables (DCT, LCT, fat, and muscle fibers) were tested for residual normality by the Kolmogorov-Smirnov test and for homogeneity of variance using the Levene's test. Subsequently, they were submitted to univariate ANOVA using the framework for general linear models (PROC GLM) in a completely randomized design. Finally, the means were adjusted by the ordinary least squares method using the command LSMEANS (Least Squares Means) and compared with application of the Tukey's test at 5% significance level. Statistical data were processed in the SAS® System for Windows™ 9.0 software (Table 1).

RESULTS

Ultrasound (US) examination

The surfaces of MCIII, MCII, and MCIV of some specimens showed slight irregularities; however, they were included in this study because they did not present bone fragments and/or changes regarding echogenicity, shape, size and architecture of PISL.

PISL of CH had a lobular shape similar to that of TBH. As for size, it was observed that 73.52% of the PISL of CH showed no difference between lobes; however, in 26.47% of them, the medial lobe was larger comparison with the lateral lobe, and the same was observed for TBH. With respect to echogenicity, PISL of CH (Fig. 1A) was characterized by hypoechogenicity throughout the dorsal peripheral zone, which was also verified in the lobulation. The lobes were echogenic, interspersed with some hypoechogenic spots. In the TBH group (Fig.2A), the lobulated dorsal peripheral zone in direct contact with MCIII, MCII, and MCIV was echogenic compared with that in the CH group. The PISL lobes in the TBH group were echogenic, more intensely interspersed with hypoechogenic area compared with those in the CH group. In the negative incidence on the PISL of CH (Fig.1B,C), some hyperechogenic lines and spots were evidenced in all its peripheral region; in some specimens,

Table 1. Comparison of the constitution of the proximal insertion of the suspensory ligament (PISL) between the study (Crioulo horses - CH) and control (Thoroughbred horses - TBH) groups with respect to dense connective tissue (DCT), loose connective tissue (LCT), fat, and muscle fibers

Variable	Treatment		p value*
	Control (TBH)	Study (CH)	
DCT	4.49 (0.05)	4.55 (0.03)	0.2953
LCT	3.08 (0.06)	3.03 (0.04)	0.4717
Fat	3.67 (0.09)	3.99 (0.05)	0.0038
Muscle fibers	3.88 (0.14)	3.67 (0.09)	0.2187

* p value >0.05 do not differ by the Tukey's test at 5% probability level; values in parentheses indicate standard error of the mean.

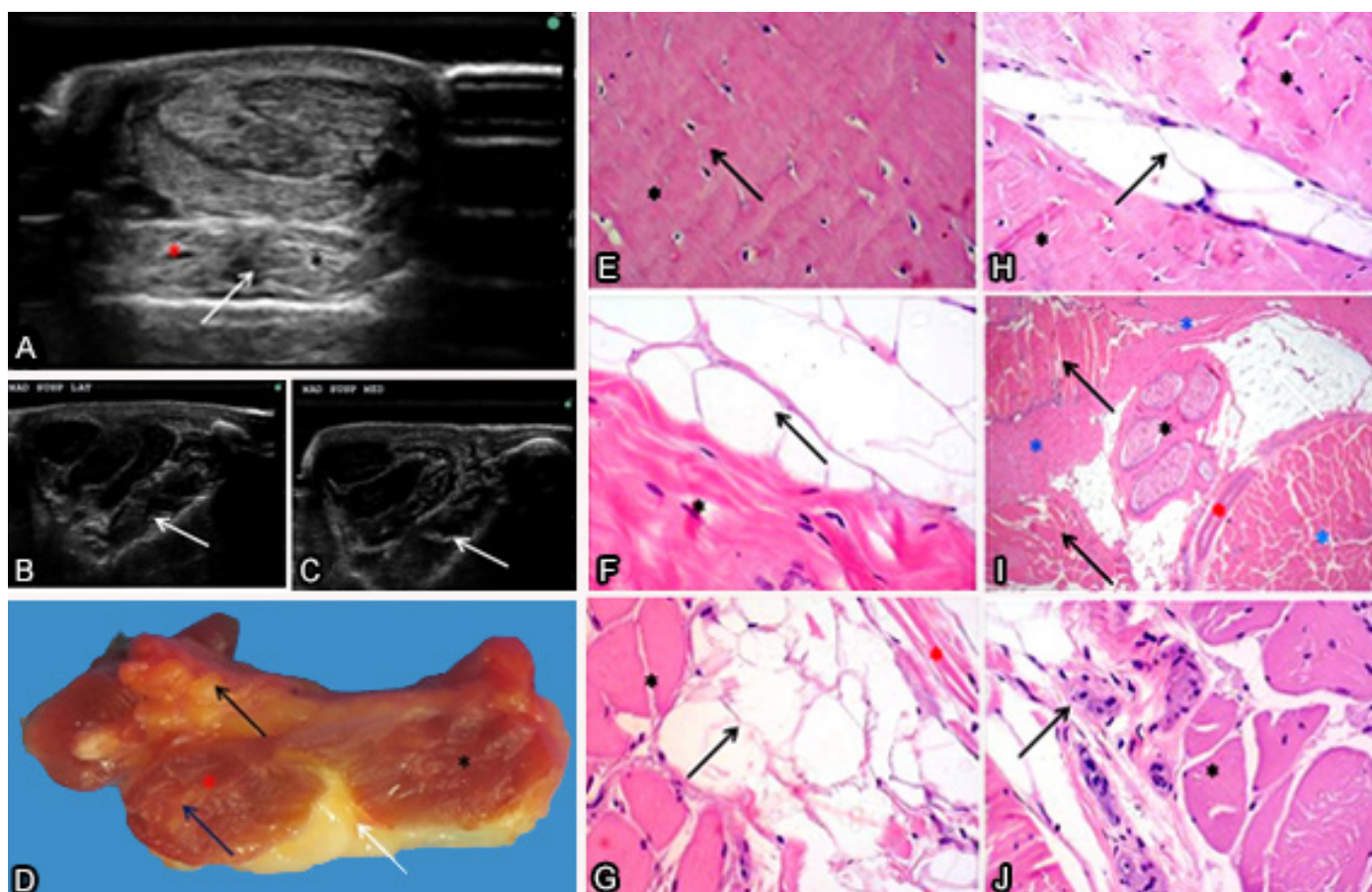


Fig.1. Proximal insertion of the suspensory ligament (PISL) of thoracic limb (TL) in the study group (Crioulo horse - CH). (A) Cross-sectional, proximal, palmaromedial (left) and palmarolateral (right) US images; hypoechogenicity of the peripheral region in the point where lobulation occurs (arrow). The lobes are echogenic, interspersed by some hypoechogenic spots (red asterisk, medial lobe; black asterisk, lateral lobe). (B) Cross-sectional, proximal lateral and (C) proximal medial, medial (left) and lateral (right) ultrasound (US) images; some hyperechogenic lines and spots are observed throughout the peripheral region reaching the interior of the lobe (arrows). They are not angle-negative dependent. (D) Gross cross-section; medial lobe (red asterisk), lateral lobe (black asterisk). Fat on the dorsal peripheral region (white arrow), inside the ligament (blue arrow), and in contact with the carpal joint (black arrow). (E) PISL composed of dense connective tissue - DCT (asterisk) and collagen fibers (arrow). HE, obj.20x. (F) Adipocytes are observed in the dorsal peripheral region (arrow), delimiting the loose connective tissue - LCT (asterisk). HE, obj.20x. (G) Adipocytes (black arrow) interspersed with muscle fibers (black asterisk) and LCT (red asterisk). HE, obj.20x. (H) Adipocytes (arrow) interspersed with the DCT (asterisk). HE, obj.20x. (I) PISL; ganglion nerve nuclei (black asterisk) between adipocytes (white arrow), delimited by LCT (red asterisk), DCT (blue asterisk), and bundles of muscle fibers (black arrow) are observed. HE, obj.20x. (J) Blood vessels (arrow) are observed between fascicles of muscle fibers (asterisk). HE, obj.20x.

these lines start from the periphery reaching the interior of the lobe, and they were not angle-negative dependent. This situation was not verified in the TBH group (Fig.2B,C), in which only subtle hyperechogenic spots were observed in the PISL peripheral region. In some specimens from younger CH breed animals (3.0 years old on average), the hypoechogenic area throughout the dorsal peripheral zone of the PISL was echogenic. In one specimen, of approximately six months of age, this situation did not occur; on the contrary, the dorsal peripheral region was markedly hypoechogenic, as previously described.

In the PL, the PISL of CH showed a rounded shape and was arranged under the surface of MTIII and the entheses of MTII; it was echogenic, interspersed with hypoechogenic spots (Fig.3A). On its dorsal surface, there was a well-defined hypoechogenic region in close contact with the surface of

MTIII. In the TBH group (Fig.4A), the dorsal surface was echogenic and it was possible to observe a PISL in the shape of a triangle. In some specimens from CH and TBH, poorly delimited hyperechogenic zones were present within the SL, and hyperechogenic lines merged within the SL were also observed.

Macroscopic assessment

PISL of TL was observed in cross sections from the proximal dissection of the skin, superficial digital flexor tendon, deep digital flexor tendon, and the carpal joint. Macroscopically, the PISL of CH (Fig.1D) was divided into two well-defined lobes, medial and lateral, and no difference in size and shape was observed between the lobes. In specimens from the TBH group, the lateral lobe

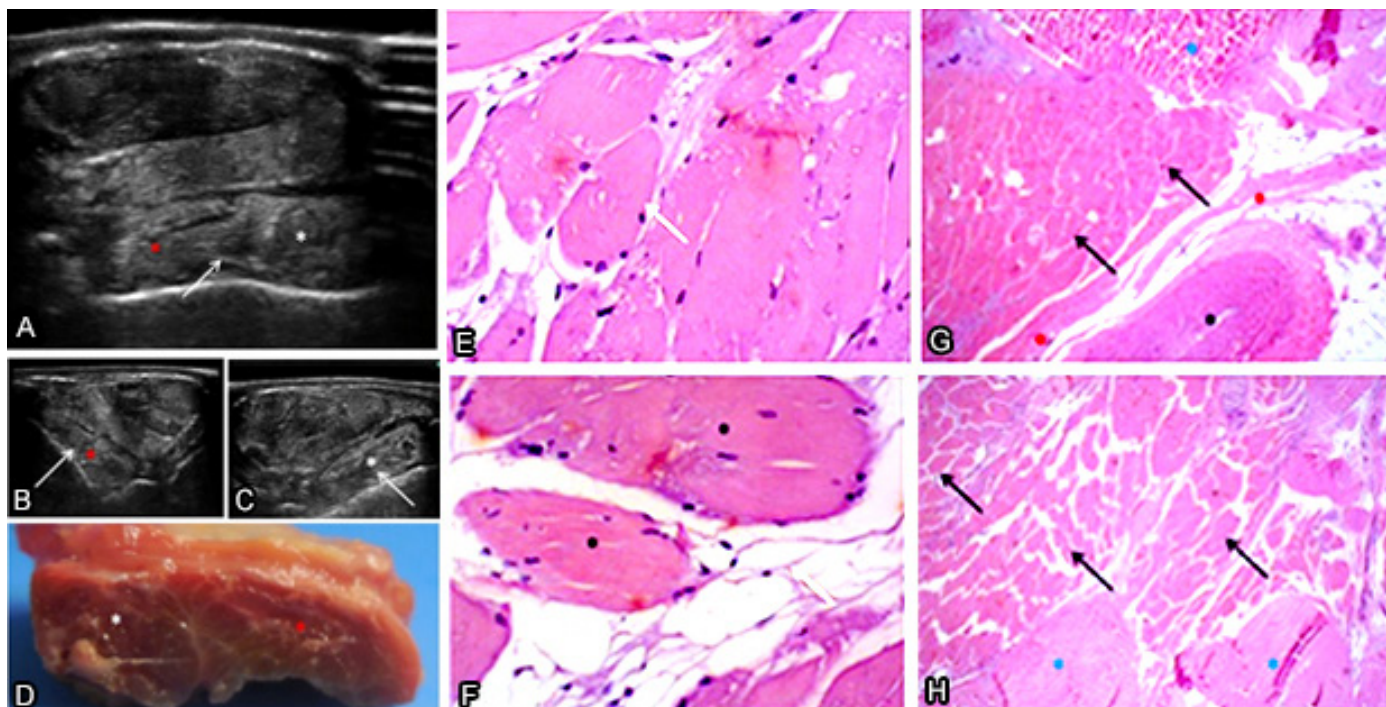


Fig.2. Proximal insertion of the suspensory ligament (PISL) of thoracic limb (TL) in the control group (Thoroughbred horse - TBH). (A) Cross-sectional, proximal, palmaromedial (left) and palmarolateral (right) ultrasound (US) images. Lobulated dorsal peripheral region in direct contact with MCIII (arrow). Medial (red asterisks) and lateral (white asterisks) lobes are echogenic, interspersed with hypoechogenic areas more intensely. Difference in size and shape is observed between them. Cross-sectional (B) proximal medial and (C) proximal lateral, medial (left) and lateral (right) US images; difference in size and shape between the medial (red asterisk) and lateral (white asterisk) lobes is observed. Subtle hyperechogenic spots located on the dorsal peripheral region in contact with metacarpus III - MCIII (arrow). (D) Gross cross-sectional; it can be observed that the lateral lobe is more rounded and prominent (white asterisk) compared with the medial lobe, which is flattened and thin (red asterisk). (E) Arrangement of fascicles of muscle fibers (arrow). HE, obj.20x. (F) Muscle fibers (asterisk) delimited by a small number of adipocytes (arrow). HE, obj.20x. (G) Fewer adipocytes (white arrow) are observed among the loose connective tissue - LCT (red asterisk), dense connective tissue - DCT (blue asterisk), muscle fiber fascicles (black arrow), and blood vessels (black asterisk). HE, obj.10x. (H) DCT (blue asterisk) delimited by a large number of muscle fiber fascicles (black arrow). HE, obj.10x.

was more rounded (Fig.2D) and prominent compared with the medial lobe, which was flatter and thinner. The PISL was in direct contact with MCIII and with its entheses between MCII and MCIV. In the dissection of specimens of the CH group, a moderate layer of fat was observed in contact with the carpal joint. Observation of the same specimens in cross section showed a thick layer of fat on the dorsal peripheral zone of the SL (Fig.1D), which was yellowish in color and also filled the space where SL lobulation occurs. Adipose tissue was also often well delimited in the lobes. In contrast, no fat was found in the dorsal peripheral region in samples of the TBH group. A thin layer of fat was observed on the ventral border of the ligament in some cases of the TBH group. In only one TBH specimen location of adipose tissue was similar to that found in CH specimens. PISL color in CH varied from a light to off-white red, with fascicles well delimited by pearly white tissue similar to that observed in the tendinous tissue. Pearly white tissue can also be observed peripherally to the ligament in some cases. The palmar artery and palmar vein were observed, and they were supported by fat and the same pearly white tissue close to the PISL. Samples of the TBH group showed

staining and organization of the fascicles similar to those verified in samples of the CH group.

Cross sections of PISL specimens were performed in the plantaromedial aspect of PL in order to observe the shape, fascicular architecture and staining of the composing tissues and the results were compared between the CH and TBH groups. PISL showed rounded shape in CH (Fig.3B) and triangular shape in TBH, with color varying from light to off-white red, in a few cases. The fascicles were clearly delimited by pearly white tissue. A thick layer of this tissue was observed on the dorsal margin of the SL in the TBH group (Fig.4B). In contrast, a thick layer of yellowish adipose tissue was identified in the PISL border in the CH group. In these CH specimens, fat was also focally located within the SL.

Histological assessment

In the samples of TL, the PISL was composed of DCT, and bundles of collagen fibers were observed (Figs.1E and 2H). This tissue was delimited by LCT, which resulted in the formation of fascicles similar to the peritendon observed in the tendinous tissue. Adipocytes were densely present in the dorsal border of the PISL filling the space formed by their lobulation (Fig.1F). Adipocytes were also present inside

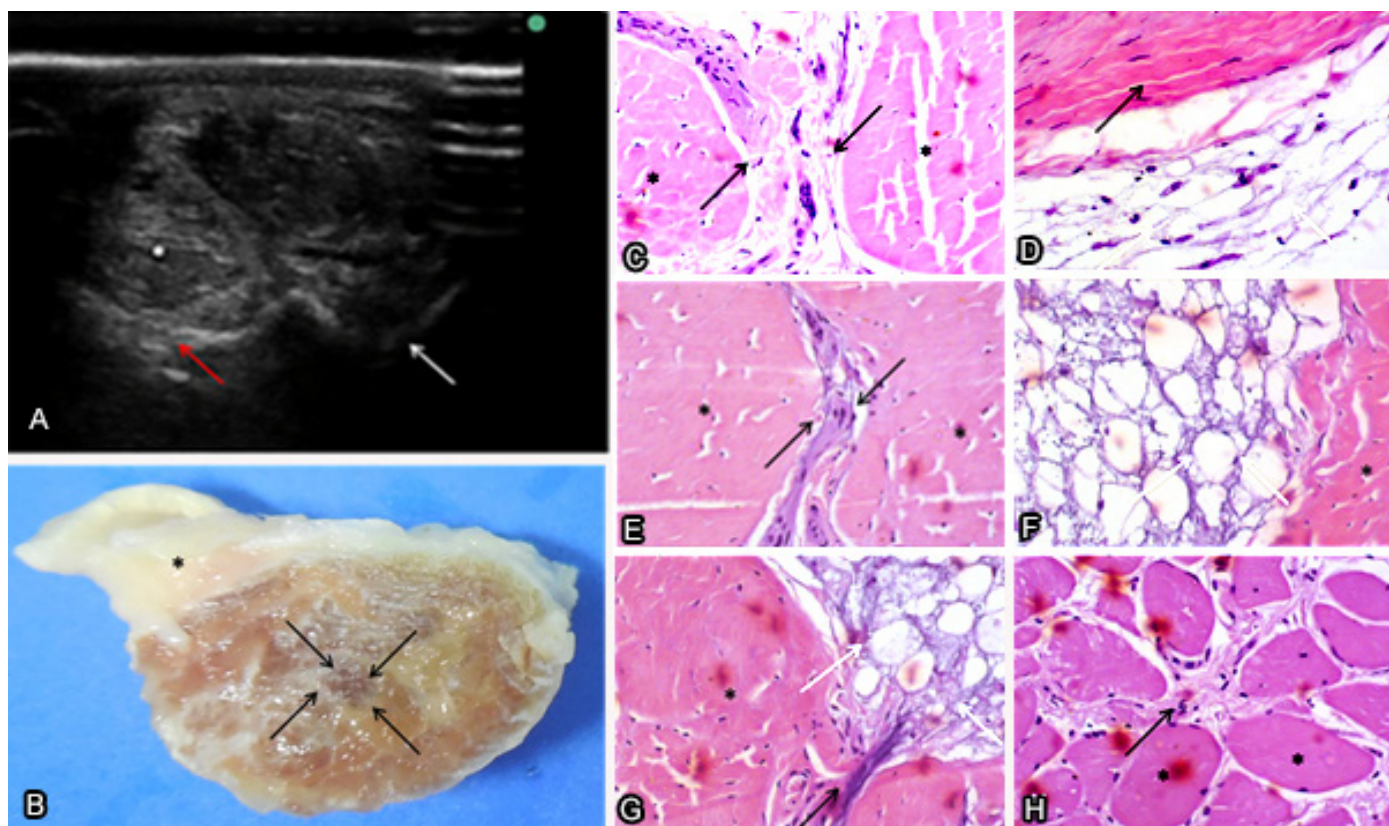


Fig.3. Proximal insertion of the suspensory ligament (PISL) of pelvic limb (PL) in the study group (Crioulo horse - CH). (A) Cross-sectional, plantaromedial (left and right) ultrasound (US) images. It is possible to observe a rounded SL (asterisk) arranged under the bone surface of metatarsus III - MTIII (white arrow) and the entheses of MTII (red arrow), echogenic interspersed with hypoechogenic spots. (B) Gross cross-section. It is possible to observe a rounded suspensory ligament (SL) organized in fascicles (black arrow) and delimited by pearly white tissue (asterisk). (C-D) dense connective tissue (DCT) fascicles (asterisk) delimited by the loose connective tissue - LCT (arrow) are observed. HE, obj.20x. (E-G) Adipocytes (white arrow) arranged between the DCT (asterisk) and the LCT (black arrow). HE, obj.20x. (H) Muscle fiber fascicles (asterisks) are observed interspersed with the LCT (arrow). HE, obj.20x.

the lobes and, in some samples, delimited by muscle fiber fascicles and by DCT (Fig.1G,H), and LCT and DCT (Fig.1I). Numerous nerve ganglia (Fig. 1I) and blood vessels (Fig. 1J) were observed among adipocytes located on the dorsal border. Muscle fiber fascicles composed both groups and were mainly located inside the lobes surrounding the DCT and LCT (Figs.1I and 2E). An important characteristic observed in the TBH group refers to the smaller number of adipocytes per field evaluated subjectively (Fig.2G) compared with the CH group both in the border and inside the lobes.

Tissue composition and organization in the PISL in the PL samples are similar to those in the TL samples (Fig.3C,D). In the CH group, there was greater concentration of adipocytes per field (Fig. 3E-G) in the dorsal peripheral region of PISL and there were blood vessels and ganglion nerve clusters among them. In these specimens, muscle fiber fascicles (Fig.3H) were interspersed with adipocytes and delimited by LCT and DCT. In the TBH group, there was smaller concentration of adipocytes inside (Fig.4C), between muscle fiber fascicles (Fig.4D), and in the border (Fig.4E) of the PISL compared with that in the CH group. Greater concentration of muscle fiber fascicles was observed in the SL delimiting the DCT and

LCT (Fig.4F,G), as well as in the peripheral regions of the SL (Fig.4H) in the TBH group.

DISCUSSION

The selection criterion used in this study was based on the US, macroscopic and histological assessments of PISL specimens of the TL and PL of CH that were compared with those of TBH. Only data regarding the age and breed of the horses were obtained. The animals selected for this study did not have previous history of PISL-related lameness of the fore and hind limbs. The selected specimens did not show US changes compatible with PISL changes when compared with the contralateral limb. The choice of the US imaging as sample selection was due to the fact that it is the most frequently used modality in veterinary clinical routine for soft tissue evaluation. According to Carnicer et al. (2012), US is the most commonly used diagnostic imaging modality in the assessment of the suspensory apparatus, but interpretation of the images obtained from the PISL may vary because of its echogenicity. This diversity may be due to artifacts produced by the unique histological composition of the SL (Budras et al. 2003). It is worth considering that there are individual

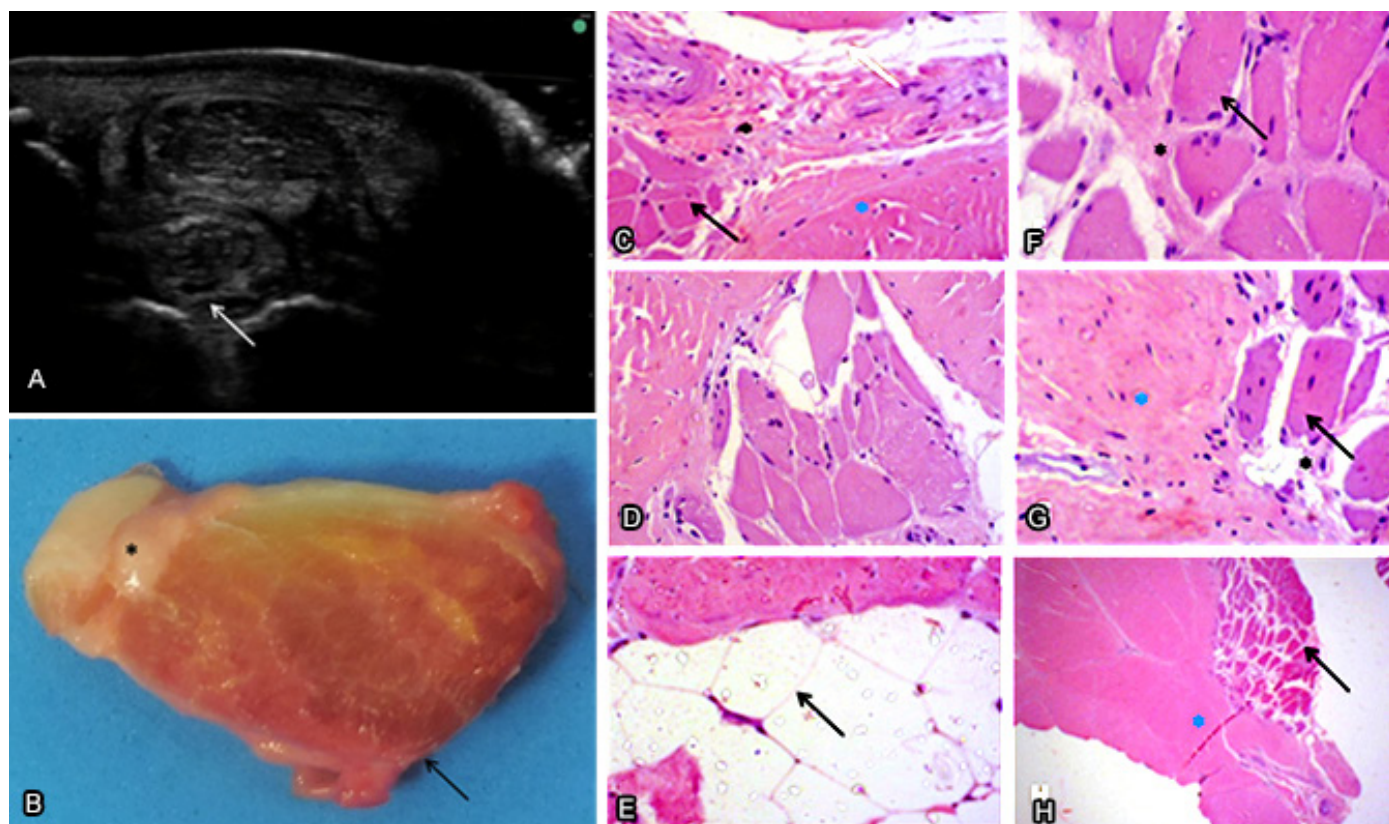


Fig.4. Proximal insertion of the suspensory ligament (PISL) of pelvic limb (PL) in the control group (Thoroughbred horse - TBH). (A) Cross-sectional, plantaromedial ultrasound (US) images. The dorsal peripheral zone of the echogenic suspensory ligament - SL (arrow) is observed. (B) Gross cross-section of the PISL. It is possible to observe the pearly white tissue that surrounds the SL (asterisk) and its triangular shape in light red (arrow). (C-D) A lower concentration of adipocytes in the SL among the loose connective tissue - LCT (black asterisk), dense connective tissue - DCT (blue asterisk), and muscle fiber fascicles (black arrow) is observed. HE, obj.20x. (E) A small number of adipocytes are observed in the SL. HE, obj.20x. (F-G) Arrangement of muscle fiber fascicles (arrow) between the LCT (black asterisk) and the DCT (blue asterisk). HE, obj.20x. (H) The dorsal peripheral region of the SL with muscular fiber fascicles (arrow) and the DCT are observed. No adipocytes are observed. HE, obj.10x.

differences regarding the number of muscle fibers in the PISL, which can cause variation in US echogenicity (Zauscher et al. 2013). No statistically significant difference regarding the number of muscle fibers was observed between the CH and TBH groups ($p=0.2187$). However, an important histological feature is that muscle fiber fascicles were not peripherally present in the SL, but rather in the center of DCT fascicles, often delimited by the SL or by LCT and fat.

US diagnosis is used to differentiate changes in echogenicity as well as irregularities in the SL border (Imboden et al. 2009). Results reported by Reeding & Schramme (2007) demonstrated that US specificity is low when compared to magnetic resonance imaging (MRI) to document lesions associated with lameness originating in the plantar surface of MTIII. Nevertheless, Denoix et al. (2008) described the US technique as the most commonly used imaging modality in the investigation of SL changes.

Muscle fibers are less echogenic than ligament fibers, creating a variation of echoes in the regular PISL (Agut et al. 2009). This variation was observed in specimens of the study and control groups in the present study, creating hypoechoic spots or lines delimited within the SL in some evaluations. In US, the muscle fibers present

lower echogenicity compared with that of the ligament fibers because of their tissue architecture, thus creating a variation in the pattern of produced echoes. Adipose tissue is hyperechogenic when the limb is in flexion, but when the limb is in bipodal stance, it is less echogenic than the ligament fibers. Corroborating Dyson (1998), the variable number of muscle fibers in the PISL could contribute to the production of hypoechoic zones in the US images, thus hindering accurate US evaluation of the ligament architecture in the PISL. Echogenicity of the muscle fibers is smaller than that of the collagen fibers of the SL. In contrast, echogenicity of adipose tissue is not angle-negative dependent. Fat is hyperechogenic and may also present with echogenicity similar to that of the collagen fibers of the SL (Bischofberger et al. 2006).

According to Micklethwaite et al. (2001), US examination should be performed with the horse supporting the limbs on the ground, because if it is conducted without the weight tension in the ligaments and tendons, echogenicity can be reduced, possibly producing artifacts that can be mistaken for lesions. In this study, it was observed that when the limb was fully extended - a situation simulated by the press where the limbs were placed - adipose tissue was hypoechoic;

however, when full extension was not reached, similarly to the biomechanics of the animal, fat was hyperechogenic. These data were verified when, with the limb in full extension, a well delimited thick hypoechogenic line was observed in close contact with the surface of MCIII and its entheses between MCII and MCIV, following the bifurcation area of the SL of TL, and became hyperechogenic when in slight flexion. In the gross assessment, it corresponded to thick yellowish adipose tissue present in the dorsal peripheral zone of PISL filling the space formed by its lobulation. Morphometric histological assessment showed ($p=0.0038$) the presence of a large number of adipocytes interspersed with blood vessels and numerous nerve ganglia in the CH group. Adipocytes were also present in smaller number among the muscle fibers, LCT and DCT of the PISL. This composition was observed in 96% of the samples from the CH group, and only one sample (4%) did not present this characteristic. In contrast, these findings were not observed in the TBH group.

In the gross assessment, the organization of adipose tissue was not presented in the same way in some samples of the CH group where there was a thin layer in the aspect that is in close contact with the carpal joint. This finding was compatible with the histological evaluation, and adipocytes were also present between muscle fiber fascicles, LCT and DCT. As reported by Werpy et al. (2013), although the regions with presence of fat and muscle fibers are frequently located inside the lobes, they can be observed in histological sections dissecting the collagen fibers of the SL and located in the border. However, fat and muscle fibers are often interspersed within the SL, thus contributing to variation in their echogenicity (Schramme et al. 2012).

PISL of TL has a bilobed appearance, with the medial lobe usually thinner and wider than the lateral lobe (Werpy et al. 2013). In the TBH group, it was observed macroscopically that the medial lobe was wider and flatter and the lateral lobe was more rounded and prominent. This gross finding was not visualized in the CH group, with no differences in shape and size between the lobes. In the PISL of PL, the triangular shape of the SL is thinner medially than laterally, and is close to the MTIV, and separates from the MTII due to a greater amount of connective tissue (Werpy et al. 2013). This finding was observed macroscopically in both groups except that, in the CH group, there was a thick layer of peripheral fat that invaded the SL in most of the samples. In horse of the Standardbred breed, it was observed a larger number of muscle fibers in the SL of TL than in that of PL, and that this ratio is inverse in the TBH breed (Soffler & Hermanson 2006). This difference was not observed between the CH and TBH groups.

PISL of TL and PL shows tissue organization in fascicles very similar to that observed in tendons. DCT is delimited by LCT and interspersed with muscle fibers and adipocytes. Histologically, as described by Schramme et al. (2012), the SL was composed of interfascicular spaces containing DCT and its collagen fibers.

The relationship between the US, macroscopic and histological findings of the PISL of TL and PL in the CH group, correlated with the findings for the TBH group, enables high accuracy in the interpretation of US images, providing information on tissue morphology and organization of the SL in CH. These results enable better understanding of US images that do not

correspond to PISL lesions, emphasizing the need for more comprehensive knowledge about the behavior of US changes and their possible misinterpretations.

CONCLUSION

The proximal insertion of the suspensory ligament (PISL) in horses of the Crioulo breed (CH) shows individual characteristics with regards to its shape, size, and tissue composition and, consequently, is subject to echogenic variations. These findings are of fundamental importance for interpretation of ultrasound (US) images.

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

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The effect of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) on mice myocardial morphology¹

Tais H.C. Sasahara^{2*}  and Marcia R.F. Machado³

ABSTRACT.- Sasahara T.H.C & Machado M.R.F. 2019. **The effect of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) on mice myocardial morphology.** *Pesquisa Veterinária Brasileira* 39(5):364-370. Departamento de Cirurgia, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Cidade Universitária, Avenida Prof. Orlando Marques de Paiva 87, São Paulo, SP 05508 270, Brazil. E-mail: tsasahara@gmail.com

Studies have demonstrated sympathetic cardiac denervation in the MPTP mouse model. MPTP toxicity causes sympathetic nerve damage and depletion of heart norepinephrine. Previous evaluations of impairments in heart innervation have been based on imaging, electrophysiological and biochemical methods. However, these studies lacked information that can be obtained from morphoquantitative analyses. Thus, this study aimed to apply a design-based stereological method for evaluating the morphoquantitative alterations of myocardium following treatment with the neurotoxin MPTP in the C57/BL mouse. Our results showed that MPTP reduced the number of cardiomyocytes in the left ventricle.

INDEX TERMS: Methyl, phenyl, tetrahydropyridine, MPTP, mice, myocardial, morphology, heart, rodent, stereology.

RESUMO.- [O efeito do 1-metil-4-fenil-1,2,3,6-tetrahidropiridina (MPTP) na morfologia do miocárdio de camundongo.]

Estudos têm demonstrado a desnervação simpática cardíaca no modelo da administração do MPTP em camundongo. A toxicidade do MPTP causa lesão ao nervo simpático e depleção da norepinefrina. As avaliações dos danos na inervação do coração são baseadas em métodos de imagem, eletrofisiológico e bioquímico. Contudo, estes estudos carecem de informações provenientes de análises morfoquantitativas. Assim, objetivou-se aplicar métodos estereológicos para avaliar as alterações morfoquantitativas do miocárdio após o tratamento com a neurotoxina MPTP no camundongo C57/BL. Nossos resultados mostraram que o MPTP causa redução no número de cardiomiócitos no ventrículo esquerdo.

TERMOS DE INDEXAÇÃO: Metil, fenil, tetrahidropiridina, MPTP, morfologia, miocárdio, camundongo, coração, roedor, estereologia.

INTRODUCTION

Several studies have demonstrated sympathetic cardiac dysfunction in MPTP-induced animal models, especially in C57/BL mice (Goldstein et al. 2000, Li et al. 2002, Ren et al. 2004, Haensch et al. 2009). Animal models of cardiac dysautonomia provide an opportunity to evaluate and understand the neurodegenerative process and develop unbiased methods of evaluation (Joers & Emborg 2014). One of the most commonly used chemicals in such animal models is 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which is considered the gold standard model cardiac denervation in neurodegenerative disease as Parkinson's disease (Jackson-Lewis et al. 2012).

The findings of cardiac denervation are based on radiolabelling imaging techniques, electrophysiological studies, pharmacological dosage or immunohistochemistry in either humans or animal models (Takatsu et al. 2000, Ren et al. 2004, Amino et al. 2005, Goldstein et al. 2005, Haensch et al. 2009, Ziemssen & Reichmann 2010, Jain & Goldstein 2012), but little attention has been given to the morphological aspects of the heart, especially in studies using efficient 3-D quantitative methods in MPTP models. Assuming that sympathetic cardiac dysfunction and cardiac dysautonomia have negative consequences on cardiac muscle, this study aimed to describe a design-based stereological method for evaluating the morphoquantitative alterations

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² Departamento de Cirurgia, Faculdade de Medicina Veterinária e Zootecnia (FMVZ), Universidade de São Paulo (USP), Cidade Universitária, Avenida Prof. Orlando Marques de Paiva 87, São Paulo, SP 05508 270, Brazil. *Corresponding author: tsasahara@gmail.com

³ Faculdade de Ciências Agrárias e Veterinárias (FCAV), Universidade Estadual Paulista "Júlio de Mesquita Filho" (Unesp), Campus de Jaboticabal, Via de Acesso Prof. Paulo Donato Castellane s/n, Jaboticabal, SP 14884-900, Brazil.

of myocardium following treatment with the neurotoxin MPTP in the C57/BL mouse model. The present results may contribute to a better understanding of the pathophysiological mechanisms underlying sympathetic cardiac denervation and the extent to which cardiac dysautonomia affects the myocardium.

MATERIALS AND METHODS

In this study were used ten two-month-old male C57/BL mice with a mean body weight of 20g. These animals were obtained from the Centre of Experimental Model Development (Cedeme) at Federal University of São Paulo (Unifesp). This study was approved by the Animal Care Commission of the College of Veterinary Medicine at the University of São Paulo (Application number 1068/2007) in accordance with the directives of the Brazilian College of Experimentation (COBEA) and with the Code of Ethics of the World Medical Association. The animals were assigned to two groups. Group I included MPTP-induced animals (5 animals), whereas Group II was the control and included sham animals (5 animals).

The neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP Sigma®) was diluted in 100ml of saline solution to obtain 1% solution. The animals received two doses of 50mg/kg with a 16h interval between doses. This dose was previously reported to induce an almost complete depletion of dopaminergic cells (Sonsalla & Heikkilä 1986, Sundström et al. 1987) and was sufficient to induce cardiac alterations (Ren et al. 2004, Amino et al. 2008). The animals in Group II received saline solution (0.9%) with the same administration schedule as Group I. The administration of the MPTP and saline solutions was intraperitoneal by using an insulin syringe.

The animals were euthanised with an overdose of sodium pentobarbital (120mg/kg/i.p) seven days after the last administration of MPTP. The animals were perfused through the ascending aorta with phosphate buffered saline (PBS; Sigma®, 0.1 M, pH 7.4) containing 2% of heparin (Roche®), followed by fixation with 4% paraformaldehyde in phosphate buffered saline (PBS; Sigma®, 0.1 M, pH 7.4). Then, the hearts were removed and immersed in the same fixative solution and stored at 4°C for 24 hours.

Cardiac morphoquantitative study

Left ventricle volume V (LV). The V(LV) was estimated according to the Cavalieri principle. The left ventricle was sectioned in 1mm thick slices. The following formulae were applied in the analysis:

$$V(W): = \Sigma p_w \times a(p) \times t \text{ and } V(L): = \Sigma p_l \times a(p) \times t$$

Σp_w is the number of points hitting the wall and Σp_l is the number of points hitting the lumen. The area associated with each test point (area per point) was 0.518mm for the wall and 0.038 for the lumen. The thickness (t) was 1mm (Fig.1). The V(LV) was calculated as the sum of V(W) + V(L). The error variance of the Cavalieri principle was estimated according to Gundersen et al. (1999).

Sampling of the left ventricle. The left ventricle (LV) sampling was based on the Smooth Fractionator (Gundersen 2002). Following the LV volume by Cavalieri principle, the LV was sectioned in slices, bars and in fragments with different sizes. These fragments were organised in ascending order and the LV fragments were systematic randomly sampled (SURS) (Gundersen et al. 1999) (Fig.2). They were then embedded in agar in rubber moulds to generate small spheres (Isectors) (Nyengaard & Gundersen 1992) (Fig.3). To create

isotropic orientation, the isectors were rolled on a surface before embedding in agar or in paraffin. The sections were stained by cresyl violet.

Heart microstructure quantification

Cardiac muscle fibre (CMF) density volume and cardiac interstitial (CI) density volume (Vv). In each section sampled, a point system, which was generated with the stereological software NewCAST™ (3.4.1.0 version) (Hoersholm, Denmark), was randomly superimposed on the image sections. The points hitting the CFM and CI were counted. The Vv (CMF) was estimated by dividing the number of CMF points counted by the total points hitting the entire ventricle wall. The same calculations were performed for CI: the total number of CI points divided by the total points hitting the entire ventricle wall. The error variance was calculated according to previous reports (Gundersen et al. 1999, Nyengaard 1999). The error variance was 0.09 for control and MPTP groups and 0.11 for CMF and CI.

Cardiac muscle fibre total volume (V(CMF) and cardiac interstitial total volume (V(CI). Total volume of CMF was estimated by multiplying the Vv (CMF) by the left ventricle wall volume (VW).

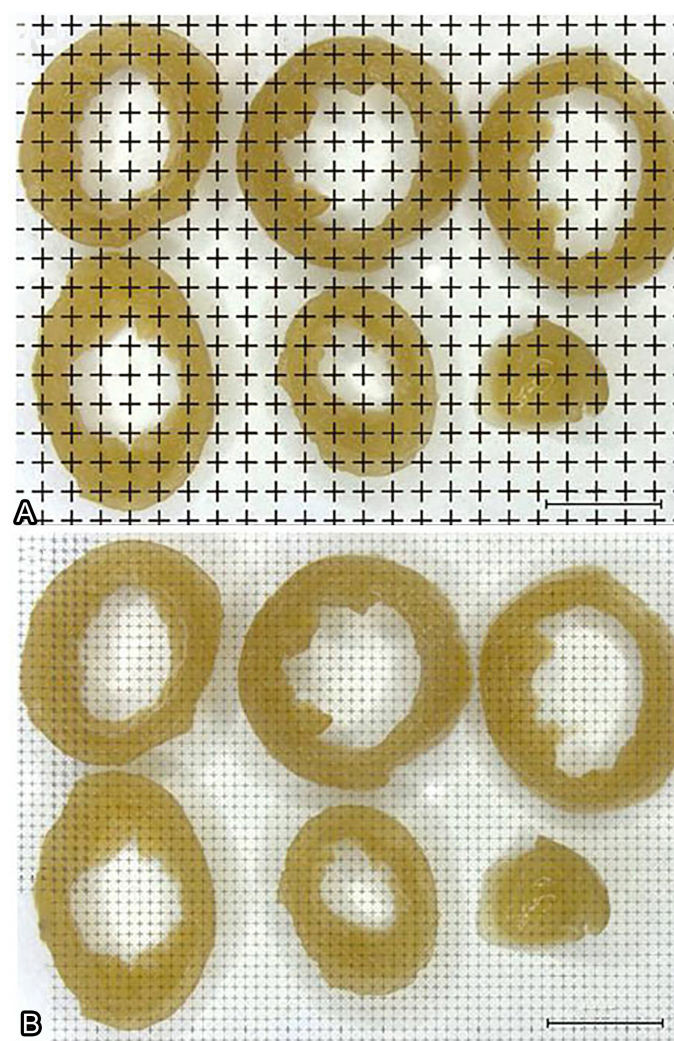


Fig.1. Estimation of the left ventricle (LV) volume by Cavalieri principle. (A) Points hitting the left ventricle wall, (B) points hitting the lumen. Bar = 2mm.

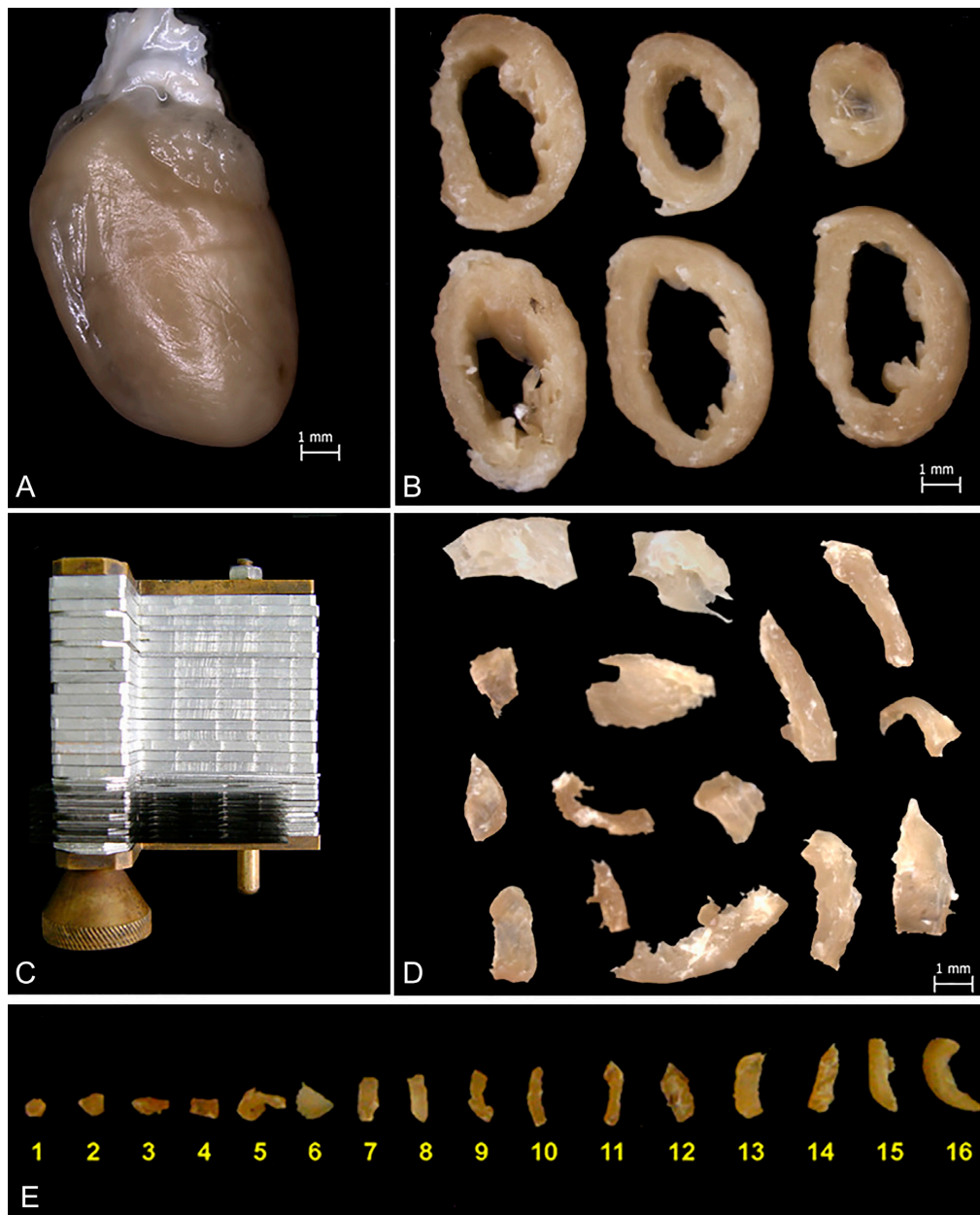


Fig.2. Left ventricle sampling. (A) Heart, (B) left ventricle slices, (C) tissue slicer, (D) left ventricle (LV) fragments with different size, (E) LV fragments arranged in ascending order.

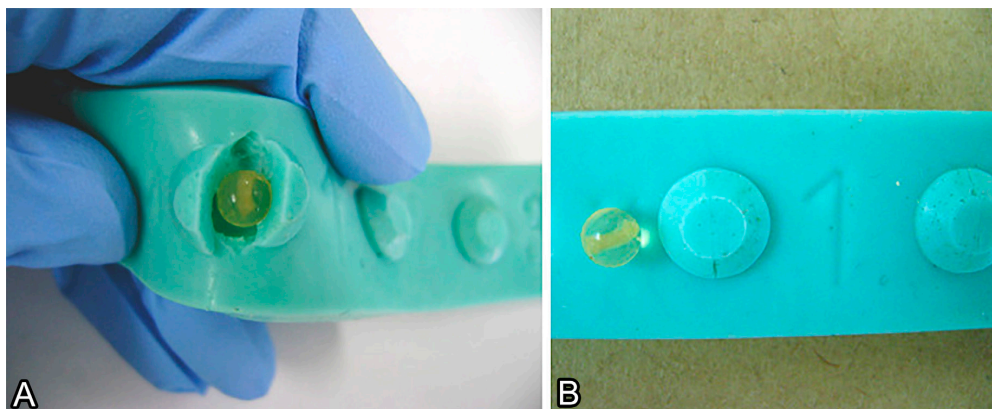


Fig.3. (A) Rubber mould to generate small spheres (Isectors). (B) Left ventricle fragment embedded in agar.

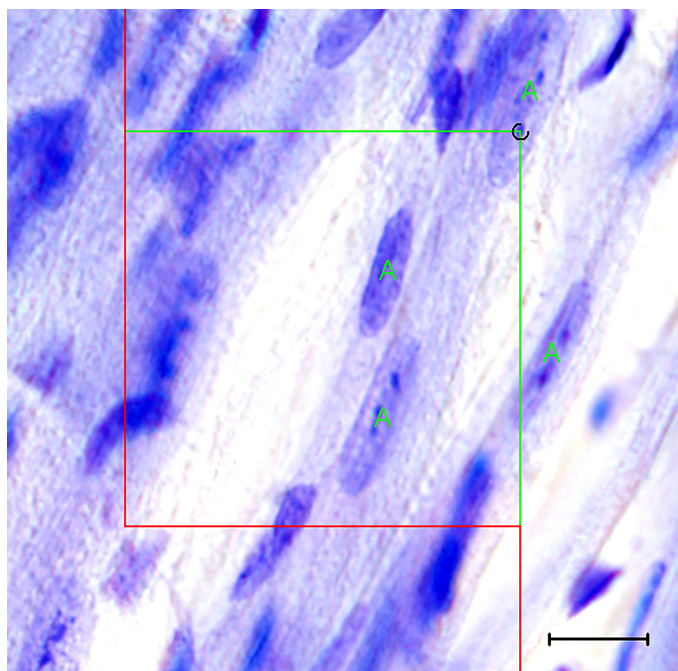


Fig.4. Left ventricle showing the counting frame. The green line is the acceptance line and the red line is the forbidden line. Counted cardiomyocyte nuclei (A). Cresyl violet stain.

Following the same principle, the total volume of CI was estimated by multiplying the V_v (CI) by the left ventricle wall volume (VW).

Total number of cardiomyocyte nuclei. The total number of cardiomyocyte nuclei was estimated using the optical fractionator principle (West et al. 1991). The nuclei were counted using the stereological software NewCAST™ and sampled following the SURS principle (Gundersen et al. 1999). The nuclei hitting the green line or inside the counting frame were counted (Fig.4). The area fraction was obtained by dividing the counting frame area ($1600\mu\text{m}^2$) by the step length ($375.16\mu\text{m} \times 375.16\mu\text{m}$). The height fraction was calculated by dividing the disector height by the overall mean section thickness. The error variance for estimation of the total number of cardiomyocyte nuclei ($CE(N)$) was estimated according to the procedures of Gundersen et al. (1999).

Mean number of cardiomyocyte nuclei per cardiomyocyte.

Immunohistochemistry was used to define the cardiomyocyte limits and to estimate the mean number of nuclei per cardiomyocyte. Two antibodies were used for this purpose. Intercalated discs were visualised using the polyclonal rabbit pan-cadherin (1:500) (H-300 Santa Cruz Biotechnology, California, USA) and cardiomyocyte membranes were visualised using the polyclonal rabbit dystrophin (1:200) (Abcam, Cambridge, UK) (Fig.5).

Total number of cardiomyocytes. The total number of cardiomyocytes was estimated by dividing the total number of cardiomyocyte nuclei by the mean number of cardiomyocyte nuclei, which was obtained by sequencing the same cell stained by immunohistochemistry (IHC).

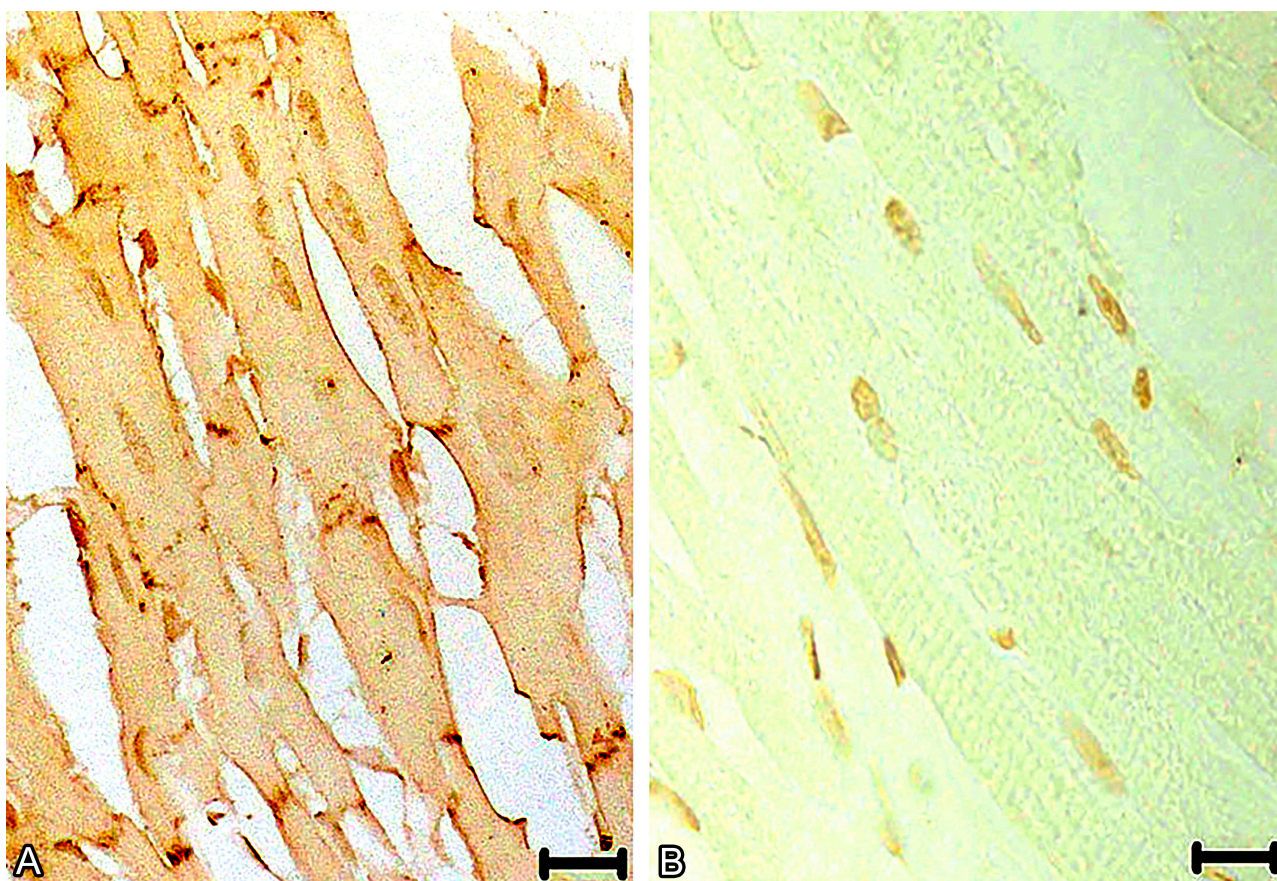


Fig.5. (A) Cardiomyocyte immunostained by Pan-cadherin, (B) negative control. Bar = $10\mu\text{m}$.

Statistical analysis

The results are presented as the mean of the observed coefficient of variation (CVobs), where $CV\ obs = SD/mean$. The data were analysed by the non-parametric Mann-Whitney test using Minitab statistical software version 17. Group differences were considered significant at $p < 0.05$.

RESULTS

Left ventricle volumes V (LV)

The wall volume $V(W)$ was 73.25mm^3 (0.05) and 73.87mm^3 (0.09) for control and MPTP groups, respectively ($p=0.67$). The lumen volume $V(L)$ was 13.58mm^3 (0.49) for the control group and 12.15mm^3 (0.22) for the MPTP group ($p=0.52$). The volume of the left ventricle $V(LV)$ was 86.83mm^3 (0.09) and 86.02mm^3 (0.10) for the control and MPTP groups, respectively ($p=0.52$) (Fig.6A).

Cardiac muscle fibre (CMF) total volume and cardiac interstitial (CI) total volume

The CMF total volume was 43.17mm^3 (0.12) and 42.20mm^3 (0.12) for control and MPTP groups, respectively ($p=0.53$). The CI total volume was 30.07mm^3 (0.14) and 31.70mm^3 (0.26) for control and MPTP groups, respectively ($p=0.67$) (Fig.6B).

Total number of cardiomyocytes

The total number of cardiomyocytes was 5.7×10^6 (0.31) for the control group and 3.1×10^6 (0.44) for the MPTP group ($p=0.02$) (Fig.6C).

DISCUSSION

We applied three-dimensional and unbiased methods to evaluate the heart in mice. Stereology has been used previously to quantify components of the heart to understand the consequences and progression of heart disease (Gruber et al. 2012, Noorafshan et al. 2013, Schipke et al. 2014, 2016).

Cardiac dysautonomia and sympathetic nervous system impairments of the heart have been reported in neurodegenerative diseases (Goldstein 2014, Joers & Emborg 2014, Piqueras-Flores et al. 2017). MPTP toxicity studies in mice have suggested a selective vulnerability of monoamine cells. These cells contain transporters that allow MPP+ to move into the cell interior. Therefore, using these transporters with MPP+ could provide access to postganglionic sympathetic nerves and subsequently damage those (Takatsu et al. 2000). According to Fuller & Hemrick-Luecke (1986), the conversion of MPTP to MPP+ is not required to deplete the heart norepinephrine.

The effects of MPTP vary according to mouse strain, sex, age, dose and intervals of application. The most susceptible strain is the C57/BL and male mice (Sonsalla & Heikkila 1986, Przedborski et al. 2001, Emborg 2004, Luchtman et al. 2009). The MPTP dose used in this study was reported in previous studies (Ren et al. 2004, Amino et al. 2008) to induce sympathetic cardiac dysfunction and contractile function impairments. Three different doses were evaluated by Takatsu et al. (2000): 5mg/kg (one single dose), 5mg/kg (two doses) and 50mg/kg (two doses). The interval between doses was 16h. The later dose was reported to reduce more the 123-I-MIBG, an analogous of norepinephrine, indicating more cardiac damage and almost complete dopaminergic cell depletion. Based on this evidence, and to investigate the possible correlation between the loss of dopaminergic innervation and its consequences in cardiac muscle, this study focused strictly on morphological study of the myocardium at the macroscopic level and no functional or clinical study was done. In addition, the left ventricle volume was examined at the microscopic level and included the cardiac muscle fibre,

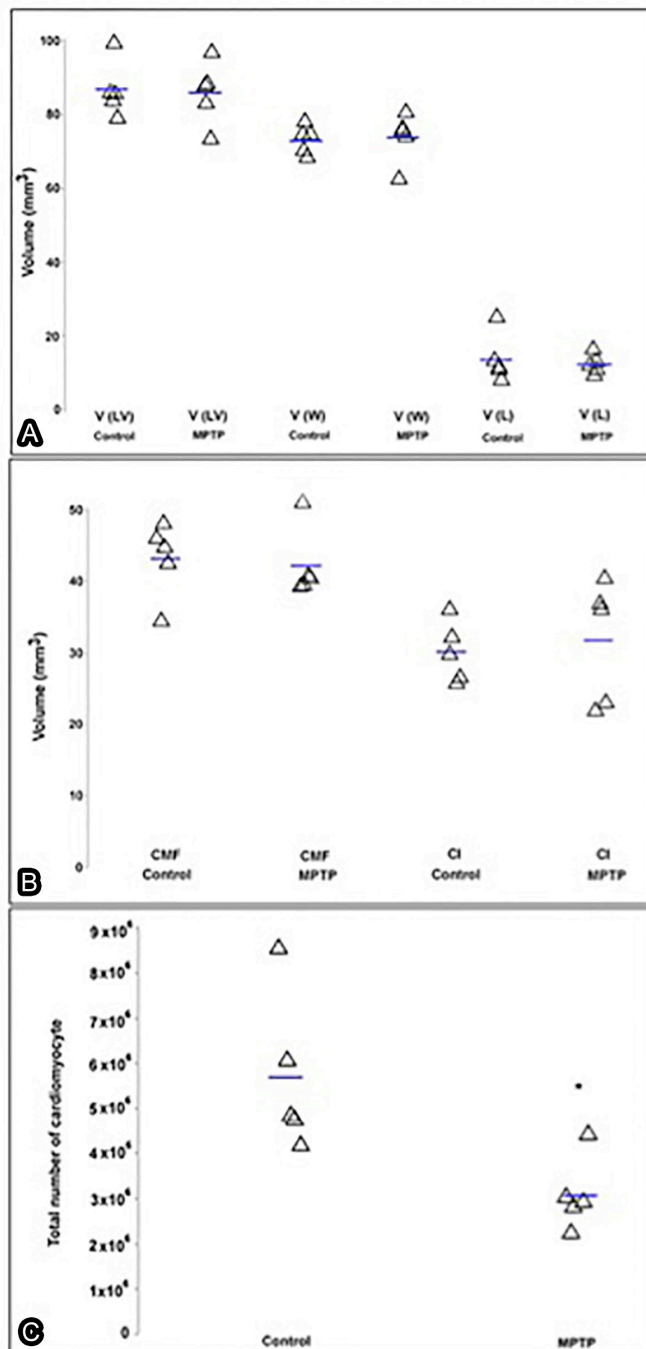


Fig.6. (A) Wall volume $V(W)$, lumen volume $V(L)$ and left ventricle volume $V(LV)$ estimated using the Cavalieri principle. (B) Total volumes of cardiac muscle fibre (CMF) and cardiac interstitium (CI). (C) Total number of cardiomyocytes. Triangles indicate individual values; horizontal bars indicate the group means.

along with cardiac interstitial volume and the number of cardiomyocytes, the functional heart unit.

The volume of the left ventricle was estimated using the Cavalieri principle (Gundersen et al. 1988, Michel & Cruz-Orive 1988). Although this is a time-consuming method, it is an efficient and accurate method (Gundersen et al. 1999, Yan et al. 2003, Van Vré et al. 2007), and it is the most appropriate method for estimating the volume of cavity organs. We estimated the left ventricle volume (LV) by summing the lumen volume (L) and the wall volume (W). These two volumes indicate the size of the left ventricle chamber and the thickness of the wall, respectively. An analysis of each of these volumes is useful because they might shed light on how the heart responds to factors such as haemodynamic load, as well as neuroendocrine and chemical stimuli that may cause cardiac tissue remodelling. In our study, the chemical effects of MPTP did not change the left ventricle volume, including the lumen and wall volumes.

At the microscopic level, the cardiac muscle fibre (CMF) and the cardiac interstitial (CI) total volumes can assess cardiac tissue remodelling. Cardiac remodelling is a complex process involving changes in cardiomyocytes and in the interstitium that results in progressive structural and functional alterations, including cardiac dilatation, interstitial fibrosis and reduction in contractility and relaxation (Remme 2003). The use of MPTP did not change the CMF or the CI total volumes, but the toxic effect of MPTP decreased the total number of cardiomyocyte by 45%. Analysing the cardiac tissue, from the remodelling point of view, this reduction in the number of cardiomyocytes should be accompanied by a reduction in the CMF total volume. In our case, however, this did not happen. Although we did not estimate the cardiomyocyte volume, we suggest that the remaining cardiomyocyte may have increased in size as a compensatory process to maintain cardiac function. Hypertrophy is one of the several mechanisms of cardiac musculature that result from factors (ischaemia, toxic substances, microorganisms etc.) that damage cardiomyocytes (Pangonytė et al. 2008).

In summary, this study showed that the effects of the MPTP reduced the number of cardiomyocytes in the mouse left ventricle without changing the ventricular volume. We believe our morphoquantitative results, obtained using a designed stereological method, can be applied in the heart of other animal models to better elucidate the pathophysiological mechanisms of cardiac sympathetic denervation.

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

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