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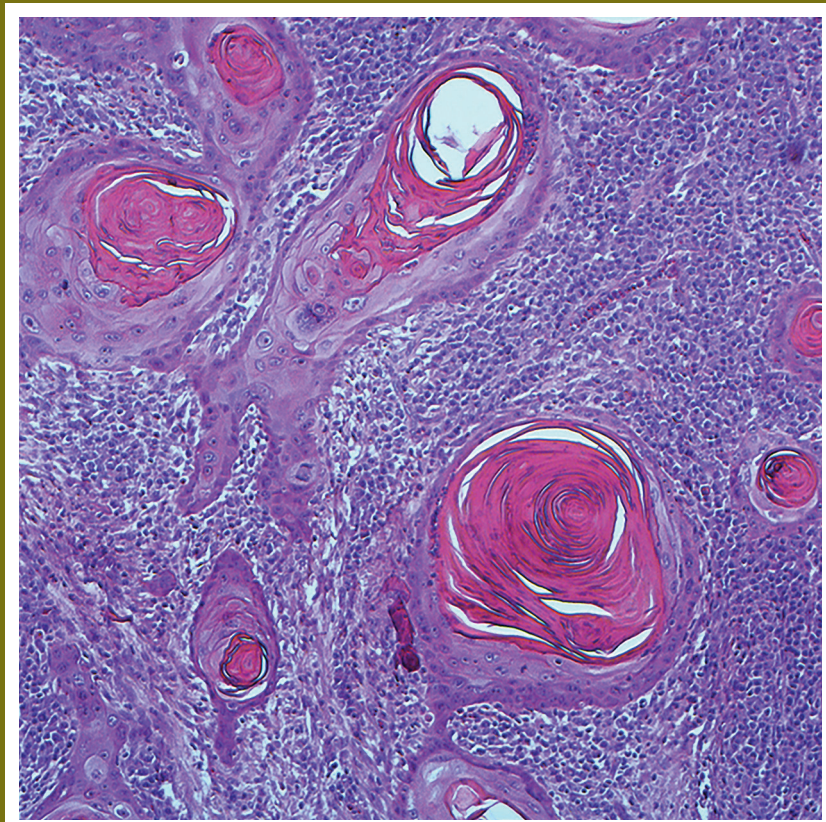
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Cover illustration: Keratin pearls, surrounded by neoplastic keratinocytes and abundant inflammatory infiltrate in sheep's skin. HE, obj.5x. (Costa et al., p. 254)

Phytotoxins eliminated by milk: a review¹

José Radmácyo G. Lopes^{2*} Franklin Riet-Correa³ and Rosane M.T. Medeiros²

ABSTRACT. Lopes J.R.G., Riet-Correa F. & Medeiros R.M.T. 2019. **Phytotoxins eliminated by milk: a review.** *Pesquisa Veterinária Brasileira* 39(4):231-237. Hospital Veterinário, Centro de Saúde e Tecnologia Rural, Universidade Federal de Campina Grande, Avenida Universitária s/n, Bairro Santa Cecília, Patos, PB 58780-110, Brazil. E-mail: macyo_mv@hotmail.com

Milk is a complex emulsion of lipids suspended in aqueous protein solution that can be a carrier of various contaminants, but generally it is not an important route of toxic excretion. The main problem is chronic repetitive exposure, as it occurs with ingestion of toxic plants and its potential danger to animals that consume the milk. Previously reported hazardous phytotoxins eliminated by milk include: indolizidine alkaloids, causing oligosaccharide storage disease; piperidine alkaloids, causing acute poisoning or malformations; pyrrolizidine alkaloids, which cause hepatic lesions; quinolizidine alkaloids, as a cause of skeletal defects; glucosinolates, which cause changes in the thyroid; tremetol (or tremetone), which causes a disease characterized by tremors in animals and milk sickness in humans; sodium monofluoroacetate, which causes the death of kids after ingestion of colostrum from goats that have ingested *Amorimia septentrionalis* during gestation; ptaquiloside, which induces carcinogenesis in animals that ingest milk or derivatives produced by animals that have ingested *Pteridium* spp. *Ipomoea asarifolia*, which contains indole diterpenes causing tremors in suckling pups. *Chrysocoma ciliata* causes alopecia in suckling pups, but its toxic compound is still unknown. Knowledge about the risk of exposure to these substances via milk and its dissemination are important for veterinary and human health.

INDEX TERMS: Toxic plants, phytotoxins, elimination by milk, goats, milk, toxicoses.

RESUMO. [Fitotoxinas eliminadas através do leite: uma revisão.] O leite é uma mistura complexa de lipídeos suspensos em solução aquosa de proteínas e pode ser veículo de diversos contaminantes, mas, geralmente, não é uma importante via de excreção de tóxicos. O principal problema é a exposição repetida, como ocorre com a ingestão de plantas tóxicas, e seu potencial perigo para os animais que consomem o leite. As fitotoxinas já descritas, que são eliminadas através do leite e podem causar danos à saúde incluem: alcalóides indolizidínicos, causam a doença do armazenamento de oligossacarídeos; alcalóides piperidínicos, que causam intoxicação aguda, pela sua capacidade de dessensibilizar os receptores nicotínicos da acetilcolina, ou malformações; alcalóides pirrolizidínicos, que provocam alterações hepáticas; alcalóides quinolizidínicos, que causam defeitos esqueléticos; glucosinolatos, que

provocam alterações na tireoide; tremetol (ou tremetone), que provoca, em animais, uma doença caracterizada por tremores, e em humanos, a doença conhecida como doença do leite; monofluoroacetato de sódio, que causa a morte de filhotes após a ingestão do colostro de fêmeas que ingeriram *Amorimia septentrionalis* durante a gestação; ptaquilosídeo, que induz a carcinogênese em animais que ingerem leite ou derivados, produzidos por animais que ingeriram *Pteridium* spp. *Ipomoea asarifolia* contém indol diterpenos que causam tremores em filhotes lactentes. *Chrysocoma ciliata* causa alopecia em filhotes lactentes, porém seu princípio ativo tóxico ainda é desconhecido. O conhecimento e a divulgação sobre o risco da exposição a essas substâncias eliminadas através do leite são importantes tanto para a saúde animal quanto para a saúde humana.

TERMOS DE INDEXAÇÃO: Plantas tóxicas, fitotoxinas, eliminação através do leite, cabras, leite, caprinos, toxicoses.

INTRODUCTION

Milk can be defined as the whole product of the total or uninterrupted milking of a healthy, well-nourished, non-fatigued dairy female. On average, it is composed of 1/8 of solid

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substances (fat, lactose, proteins, and minerals) and 7/8 of water (Valsechi 2001).

Milk is a route of excretion of several contaminants, such as microorganisms, toxins, antibiotic residues, antiparasitics, and organochlorine pesticides (Heck et al. 2007). Milk contamination can occur before milking (indirect form), either by a pathogen infecting the animal, drugs used, or substances present in feed (Fink-Gremmels 2008), or during or after milking (direct form), through contamination by the milker's hands or machinery used in milking until the final industrialization process (Pereira 2011).

Because milk is a complex emulsion of lipids suspended in aqueous protein solution, it is considered a minor route of toxicant excretion. However, depending on the toxin concentration in the blood, the ability of a xenobiotic to diffuse across the cell membrane, its affinity for certain constituents in the milk, the efficiency of major detoxification and excretion routes (liver, urine, feces, etc.) and, specifically, the lipophilicity and basicity of the toxin and the type of plant toxin and its metabolites, toxicants can be irreversibly excreted in milk (Panter et al. 1988).

Milk may contain xenobiotics ranging from polar to lipophilic compounds; however, elimination of a toxin depends on its half-life in body water. The half-lives of most polar substances and the rapidly metabolized lipophilic compounds are usually short in milk, because these toxins are rapidly eliminated by the major routes of excretion. Thus, the percentage of the total amount of such compounds that is eliminated via milk is rather small. The main problem is chronic repetitive exposure, as it occurs from ingestion of poisonous plants and its potential danger to animals consuming the milk (Panter et al. 1988).

Some poisonous principles are readily excreted in milk, including those of high fat solubility that are concentrated in the lipid portion of the milk. These lipophilic xenobiotics have long biological half-lives and are frequently detected in milk at the highest concentrations and for the longest periods of time (Mathews 1980).

Elimination of plant toxins via milk is beneficial to lactating animals that ingest toxins; however, consumption of milk from intoxicated animals may induce intoxication in neonates or suckling animals. Poisoning that may occur in suckling offspring may be more severe than in lactating animals, because of toxin concentration in the milk, and young animals lack the ability to efficiently metabolize or detoxify xenobiotics (Panter & James 1990).

Because of the importance of milk to public health and food safety, the need to determine the disposition of xenobiotics and, in particular, of natural phytotoxins in milk and other dairy products, is important. Therefore, the present study aimed to revise the main phytotoxins eliminated in milk and their potential danger to neonates and suckling animals.

LITERATURE REVIEW

Phytotoxins eliminated by milk

Indolizidine alkaloids. The main toxin in this group, called swainsonine, was first identified in *Swainsona canescens* (Colegate et al. 1979) and later in *Oxytropis serecia* and *Astragalus lentiginosus* (Molyneux & James 1982). In Brazil, it is chiefly found in *Ipomoea carnea* subsp. *fistulosa* (Oliveira et al. 2009), *Ipomoea riedelii*, *Ipomoea sericophylla* (Barbosa et al. 2006), *Ipomoea marcellia* (*Ipomoea verbascoidea*)

(Mendonça et al. 2012), *Turbina cordata* (Dantas et al. 2007), and *Sida carpinifolia* (Driemeier et al. 2000).

Swainsonine inhibits activity of the lysosomal α -mannosidase and Golgi α -mannosidase II enzymes (Dantas et al. 2007), resulting in lysosomal accumulation of incompletely processed oligosaccharides and loss of cellular function followed by cell death (Riet-Correa et al. 2009), causing lysosomal storage diseases (Mendonça et al. 2012).

Regardless of palatability, animals that begin to ingest these plants develop the habit of ingesting them compulsively and, through a social facilitation mechanism, induce other animals of the same species to ingest them. Intoxicated animals show mainly nervous clinical signs, which include ataxia, hypermetria, lateral gait, spastic paresis, intention tremor, and widened base, and may also present somnolence, apathy, anorexia, progressive weight loss, and creepy hair (Dantas et al. 2007, Riet-Correa et al. 2009). The poisoning presents a chronic course, and animals can survive for several months after appearance of the clinical signs. If the animals fail to ingest these plants right after nervous clinical signs are observed or even 15-30 days afterwards, the signs are reversible (Riet-Correa et al. 2009). Histologically, intoxication is characterized by distension and vacuolization in the pericary of neurons, mainly of Purkinje cells of the cerebellum (Oliveira et al. 2009).

In a series of experiments conducted with suckling calves and lambs following a single oral exposure to swainsonine (*Oxytropis serecia*) (0.8 and 2.0mg of swainsonine for kg of body weight, respectively), this locoweed toxin was observed in the milk of both species and confirmed by quantification (Taylor & Strickland 2002). James & Hartley (1977) fed calves, lambs and kittens milk from cows that had ingested *A. lentiginosus* and later found that they showed microscopic lesions characteristic of poisoning caused by this plant, marked by neurovisceral cytoplasmic foamy vacuolization; increased amount of serum aspartate aminotransferase was also observed.

Piperidine alkaloids. The most common toxins in this group are found in *Conium maculatum*, causing skeletal defects in calves, piglets, and lambs born to dams that had ingested these toxins at certain stages of pregnancy (Panter et al. 1988). Perhaps the first notable example of poisoning by a member of this group of toxins was the death of Socrates in 399 BC. According to Plato's famous account in "Phaedo", the philosopher ingested *C. maculatum*, a plant with high concentrations of coniine and γ -coniceine (Reynolds 2005).

In humans, nicotine contained in domestic tobacco (*Nicotiana tabacum*) has been blamed for the occurrence of acute intoxications whose intensities range from mild to severe (Schep et al. 2009). Many other plants of the genera *Nicotiana*, *Lobelia*, *Pinus*, *Duboisia*, *Sedum*, *Withania*, *Carica*, *Hydrangea*, *Dichroa*, *Cassia*, *Prosopis*, *Genista*, *Ammodendron*, *Lupinus*, *Liparia*, and *Collidium* also contain piperidine alkaloids (Keeler & Crowe 1985).

Consumption of plants with high contents of piperidine alkaloids can produce acute poisoning (Panter et al. 1999), characterized by frequent urination and defecation, tachycardia, muscle weakness and fasciculation, ataxia, collapse and, eventually, death due to respiratory failure (Panter et al. 1998).

Acute intoxications caused by piperidine alkaloids are assigned to their ability to desensitize the nicotinic

acetylcholine receptors (Green et al. 2010) and inhibition of cation conduction (Na^+ , Ca^{2+} , and K^+). Through their action at the neuromuscular junction, for instance, alkaloids cause transient fasciculation in the skeletal muscles, which are quickly followed by paralysis, progressing to respiratory failure (Arias 2000).

Many plants of these genera are consumed directly or indirectly by humans and animals, and some of them are used in human medicine. Piperidine alkaloids can be eliminated in milk, placing nursing infants at risk of toxicity and pregnant women at risk of teratogenicity. Few cases of transfer of piperidine alkaloids via milk have been reported, but maybe this is due to lack of research (Panter & James 1990).

In 1980, there was an incident in which dairy cows were fed green chopped hay containing approximately 60% *C. maculatum*. It was reported that 160 lactating cows and 66 heifers were intoxicated. Subsequently, 10 cows and 14 heifers died, and an additional 14 cows and 12 heifers were slaughtered (Kubik et al. 1980). Piperidine alkaloids were detected using thin layer chromatography, and odor typical *C. maculatum* was detected in the milk and urine of the cows. To avoid hazard to humans, the milk obtained for a period of several days was discarded (Panter & James 1990).

Pyrrolizidine alkaloids. Pyrrolizidine alkaloids are naturally occurring phytochemicals in approximately 6,000 plant species of different genera and families (Smith & Bababunmi 1980).

They are hepatotoxins that cause irreversible damage to the liver, and many of them may present carcinogenic action. Most plants containing pyrrolizidine alkaloids occur in three families, namely, Boraginaceae, Compositae, and Leguminosae. *Senecio brasiliensis* (Compositae) and *Crotalaria* spp. (Leguminosae) are the main plants associated with pyrrolizidine alkaloid intoxication in Brazil (Cheeke 1998).

In domestic animals, poisoning by plants containing pyrrolizidine alkaloids is mainly due to ingestion when there is shortage of pasture or through prolonged consumption of hay or grain contaminated with these plants, because these compounds present cumulative action (McLean 1970) and can cause a variety of symptoms, including hepatotoxicity, carcinogenicity, genotoxicity and pneumotoxicity (Boppré 2011).

In isolation, these alkaloids are nontoxic, but they become toxic when bio-transformed by the hepatic monooxygenase enzyme into a highly reactive form known as pyrrole (Prakash et al. 1999). The toxicity of pyrroles is associated with their high reactivity, acting as alkylating agents and easily binding to DNA molecules. In addition, pyrroles act by inhibiting mitosis in hepatocytes, leading to megalocytosis, necrosis and, consequently, reduction in the number of hepatocytes, which are replaced with fibrous connective tissue, giving rise to clinical signs and death owing to hepatic dysfunction (Santos et al. 2008). Part of the pyrroles escape into the general circulation, causing lesions in other tissues such as the kidneys and lungs (Radostits et al. 2002).

Natural poisoning can occur acutely or chronically, and is primarily characterized by apathy, anorexia, ascites, jaundice, photosensitization, incoordination, aggression, and head pressure against objects (Lucena et al. 2010).

Aiming to determine the transfer of pyrrolizidine alkaloids by milk, Medeiros et al. (1999) fed a goat with *Crotalaria spectabilis*

seeds, which contain the pyrrolizidine alkaloid monocrotaline, and incorporated its milk into the diet of young rats for 60 days; at the end of the experiment, the pups showed lower weight gain and pulmonary and hepatic lesions compared with those in the control group. Goeger et al. (1982) fed lactating goats *Senecio jacobaea*, used their milk in the diet of rats for a prolonged period, and observed mild hepatic pathologies at the end of the experiment.

Quinolizidine alkaloids. Quinolizidine alkaloids are widely distributed among the legumes, which use them as a defense against predators because high concentrations of these toxins produce a bitter taste, limiting their consumption. The genus *Lupinus* is the richest of this type of alkaloid. In general, lupines have been used as fodder, green manure, and feed; in folk medicine, they are used as antitumor, antidiabetic, antioxidant and antifungal agents (Galasso et al. 2006).

Cytisine is a quinolizidine alkaloid found in an ornamental tree commonly known as the Golden Chain or Golden Rain (*Laburnum anagyroides*) and in plants of the genus *Cytisus* (Cheeke & Shull 1985), and their seeds have caused intoxications in humans and horses characterized by excitation, incoordination, seizures, coma, and death by asphyxia (Kingsbury 1964).

Lupines contain the quinolizidine alkaloid anagryne, which is known to cause skeletal defects and cleft palate in calves when their dams ingested *Lupinus* sp. during days 40 to 70 of gestation, shown to be a teratogenic alkaloid (Keeler 1978).

A case study of skeletal abnormalities in a child from California, USA, revealed that the primary source of milk consumed by the mother during pregnancy was from goats that had grazed lush pastures of lupine (Ortega & Lazerson 1987). Among the herd, several goats spontaneously aborted and the fetuses showed skeletal defects similar to those of the calf disease reported by Keeler (1978). The mother recalled being clinically ill on many occasions after ingestion of the goats' milk. Further evidence implicating the milk from goats that ingested *Lupinus* was suggested after several puppies born to a bitch fed the same goats' milk during gestation showed similar malformations (Ortega & Lazerson 1987).

Glucosinolates. Glucosinolates are glycosides of β -D-thioglucose that yield isothiocyanate, nitrite, thiocyanate or similar chemicals upon hydrolysis. They occur in the Cruciferae family, whose most of the glucosinolate-containing crucifers that are important in human or animal nutrition are in the genus *Brassica*, which includes cabbage, broccoli, kale, mustard, and turnips, among others. These compounds are responsible for the desirable taste in condiments such as mustard and horseradish sauces (Panter & James 1990).

Goitrin, a hydrolyzed glucosinolate present in some plant species, inhibits thyroid function and causes thyroid hyperplasia and hypertrophy, inhibiting the incorporation of iodine into thyroxine precursors, interfering with the secretion of this thyroid hormone, and its anti-thyroid effect cannot be corrected by adding iodine to the diet. (Panter & James 1990). Nitrites formed from glucosinolates are toxic, causing poor growth, liver and kidney lesions, bile duct hyperplasia, and liver necrosis (Paik et al. 1980).

Glucosinolates and their derivatives can be transferred to the milk of lactating animals, causing thyroid enlargement in young animals or in humans ingesting the milk; placental transfer of these compounds may also occur. Throckmorton et al. (1981) observed goiter and altered serum thyroid hormones in newborn

lambs from ewes fed *Limnanthes alba* meal. In another study, White & Cheeke (1983) goats fed *L. alba* 12 to 13 weeks prior to delivery. At birth, the lambs showed serum levels of thyroid hormones similar to those of animals in the control group; however, at weaning, these lambs presented lower serum levels of thyroid hormones and significantly lower body growth compared with the control animals. Also in this experiment, milk from the goats that ingested the plant was used as feed for growing rabbits, which showed heavier thyroid glands and higher mortality rate than those that received milk from the goats in the control group.

Tremetol (or tremetone). This toxin is found in white snakeroot, *Eupatorium rugosum* (Panter & James 1990), a perennial herbaceous plant that usually grows in low and moist areas, close to streams and in open forests. During the dry season, when other forage lessens, animals are likely to move onto wooded areas and consume this plant. Quite often, this plant turns green and juicy in late autumn because it is protected from frost in its forest habitat (Cheeke 1998).

After ingesting *E. rugosum* for several days, cattle develop apathy and a condition known as “trembles”, characterized by muscle tremors around the neck, shoulders and legs. The animals adopt a curved position; there is often the smell of acetone in their breath, wheezing, urinary incontinence, and constipation. Affected animals may recover, although there may be a long period of inappetence and muscle weakness. In severe poisoning, prostration evolving to death may occur (Cheeke 1998).

Tremetol can be transferred via the milk. Lactating animals seem to be less susceptible to intoxication than non-lactating ones because of their ability to eliminate such toxin in milk (Das et al. 2007).

The toxin can be transferred to humans via cow's milk, producing a serious debilitating disease referred to as “milk sickness”, whose symptoms are weakness, nausea, prostration, ketosis, delirium, coma, and death. In the past, milk sickness sometimes reached epidemic proportions, and whole villages were abandoned because of it. It is said to be responsible for the death of Abraham Lincoln's mother, remaining unknown for many years until the early 1900's, when it was found to be due to the toxin present in *E. rugosum* (Cheeke 1998).

Clinical signs typically begin two days to three weeks after plant ingestion. Although modern techniques of milk treatment and pasture management have drastically reduced the risk of milk sickness, occasional cases can occur with milk produced in small farms without proper treatment or lack of weed control (Beier & Norman 1990).

The same toxic constituents are also found in *Haplopappus heterophyllus* (Kingsbury 1964), which is a perennial, tall and erect plant commonly found in dry pastures in the southern USA states of Colorado, Texas, New Mexico, and Arizona, usually growing along irrigation canals, ditches, and river valleys. Significant losses of grazing animals have occurred in several parts of southwestern USA as a result of consumption of this plant. The clinical signs are as in intoxication by *E. rugosum*, with predominance of tremors. Intake of the green plant in the proportion of 1-1.5% of body weight for 1-3 weeks can cause toxicity in horses, cattle and sheep (Cheeke 1998).

Sodium monofluoroacetate. Sodium monofluoroacetate is a potent naturally occurring toxin in more than 50 plant species in Africa, Australia, and South America (Lee et al. 2014). In Brazil, 12 species are known, belonging to the families Rubiaceae, Bignoniaceae, and Malpighiaceae, causing sudden death syndrome associated with exercise (Carvalho et al. 2009), and sodium monofluoroacetate was identified as the toxic agent that causes this syndrome. Owing to their severe toxicity, the *Palicourea marcgravii*, *Palicourea aeneofusca* and *Amorimia septentrionalis* species are among the most important toxic plants for ruminants in the Country (Tokarnia et al. 2012).

There are reports of intoxication outbreaks by these plants in sheep and goats in the state of Paraíba, Brazil (Vasconcelos et al. 2008). The following clinical signs were observed in experimentally intoxicated goats: dyspnea, tachycardia, and sternal decubitus evolving to lateral decubitus with pedaling movements, followed by death. Some less affected animals recover (Paraguassu 1983). In addition, ingestion of *A. septentrionalis* during pregnancy causes embryonic mortality and abortion in goats (Silva et al. 2017).

After goat and sheep producers mentioned that kids and lambs, born to dams that grazed in areas with plants of the genus *Amorimia* during gestation, died suddenly after colostrum ingestion, suggesting that sodium monofluoroacetate can be excreted by milk, Vasconcelos et al. (2008) fed two pregnant goats with *A. septentrionalis* (2g of fresh plant per kg of body weight) and reported that one kid from one of the goats died 5 min after sucking colostrum. Similarly, Lopes et al. (2018) fed eight pregnant goats (1g of fresh plant per kg of body weight) and reported that four kids, born of two goats, received colostrum and presented with apathy, wheezing, lateral decubitus, bleating, and death after 15 min; and two other kids from two goats that delivered during the night were found dead in the morning and, at necropsy, colostrum was found in the abomasum of both kids, indicating that they died after ingesting it.

Ptaquiloside. Described as the most toxic principle found in *Pteridium arachnoideum* (*P. aquilinum*), a plant popularly known as field fern or simply fern, ptaquiloside can act as a carcinogenic agent (Alvarenga 2015).

Fern is an invading plant with successful adaptation. Its only limitation regards distribution in extremely low temperatures (Rasmussen 2003). In Brazil, pastures invaded by fern have been reported in all states (Tokarnia et al. 2012).

Ptaquiloside is identified as a nor-sesquiterpene glycoside, amorphous, colorless compound, of formula $C_{20}H_{20}O_8$, found in all structures of the plant, with the shoot apex as the aerial part of the plant with the largest content of that compound (Niwa et al. 1983). Ptaquiloside crosses the cell membranes and, in the nucleus, is able to associate with the proteins with exposed amino terminals, such as DNA (Alonso-Amelot & Avendaño 2002), provoking permanent changes in genes that encode the activation of others, or that present regulatory function such as apoptosis regulation and tumor suppression (Santos et al. 1992).

The toxic and/or carcinogenic effect of fern varies according to the amount ingested, ingestion time, and the animal species involved (Tokarnia et al. 2012), and may cause hemorrhagic diathesis, bovine enzootic hematuria, and upper digestive tract carcinomas; occasionally, occurrence of thiamine deficiency,

intestinal tumors, and progressive retinal degeneration are observed (Cruz & Bracarense 2004).

Alonso-Amelot et al. (1998) fed six lactating cows fern (6kg/animal/day); 38 hours after the animals began to ingest the plant, $8.6 \pm 1.16\%$ of the amount of ptaquiloside contained in the plant was identified in the milk. Alvarenga (2015) used 30% of the cheese produced from the milk of cows that had ingested 6g/kg/day of fern in the production of feed provided to rats for 176 days. At the end of the experiment, histopathology revealed that the animals that received feed containing cheese from the cows that ingested fern developed pre-neoplastic lesions (hyperplasia/dysplasia) in the stomach, intestines, and bladder, suggesting that the indirect consumption of ptaquiloside via the milk may induce carcinogenesis.

Studies have demonstrated that, even after pasteurization, part of the amount of ptaquiloside remains in the milk, and that there is positive correlation between the higher prevalence of gastric cancer in humans and permanence in areas invaded by fern (Alonso-Amelot & Avendaño 2002).

Indole diterpene. *Ipomoea asarifolia* R. et Schult. is a prostrate or climbing herbaceous plant belonging to the family Convolvulaceae (Tokarnia et al. 2012), native of South and Central America. In Brazil, it is very commonly found in the Amazon and Northeast regions, as well as along the coast, from the Northeast to the Southeast regions (Kissmann & Groth 1992).

Ipomoea asarifolia provokes intoxication in cattle, sheep, goats, and buffaloes, especially during the dry season, when there is shortage of forage. The main clinical sign in the different species is muscular tremor that, beginning at the head, is characterized by continuous lateral movements (intention tremors). When animals are restless or scared, the tremors increase and become widespread in the body, with possible loss of balance and falls (Riet-Correa et al. 2003).

In sheep, poisoning was observed in suckling lambs that remained confined, without ingesting the plant, while their dams ingested the plant in the field, but without showing signs of intoxication, suggesting that the plant active principle was eliminated in the milk (Araújo et al. 2008). Initially, this hypothesis was confirmed in mice, when newly-born females began consuming feed containing 20% *I. asarifolia* and, after two days, the suckling pups presented with tremors characterized mainly by lateral movements of the head, intensified when they were restless. The pups recovered 4 to 7 days after weaning (Lopes et al. 2014). Lucena et al. (2014) also demonstrated, but in sheep, that the toxic principle of *I. asarifolia* is excreted via milk of lactating females, when they observed that 50% of the lambs, although kept under confinement, showed tremors after ingesting the milk from ewes grazing in areas where the plant was present, or receiving 20% dry leaves of *I. asarifolia* in their feed. The clinical signs disappeared 1-4 weeks after the end of plant consumption by the dams of the suckling lambs.

Recently, it has been demonstrated that *Ipomoea asarifolia* contains indole diterpenes (terpendol C; terpendol K; 6,7-dehydro-11-hydroxy-12,13-epoxy-terpendol A; paxilline) that cause tremors in mice (Gardner et al. 2018).

Other phytotoxins. Several other phytotoxins or their metabolites are excreted via milk, namely, myosin (*Leucaena leucocephala*) (Ghosh et al. 2007), selenium

compounds (Kenneth & McConnell 1948), colchicine (Hale 1999), indole, and 3-methylindole (Eisele 1986), but they pose little risk because the amount eliminated is insufficient to cause intoxication.

Phytotoxins not yet identified

***Chrysocoma ciliata* L. (Asteraceae).** Described as a dense, perennial shrub, approximately 500mm in height, *C. ciliata* presents a rounded shape and numerous small leaves that are slightly sticky and bitter; it has been referred to as "bitter bush". *C. ciliata* is native of southern Africa, and is considered an invasive plant; dense concentrations of this plant indicate occurrence of overgrazing and pasture degradation. A herd will graze large amounts of bitter shrub only if more palatable species are not available (Vahrmeijer 1981).

This plant is associated with alopecia in lambs whose dams ingested the plant during gestation. The exact amount of plant that has to be ingested by sheep for their offspring present with alopecia is still unknown (Steyn 1931).

Steyn (1931) reported that a lamb, whose dam was fed 12.5kg dry *C. ciliata* for 35 days developed alopecia and fatal diarrhea.

The unidentified toxin is transmitted via milk to the young, and this explains why the affected lambs or kids have normal hair at birth: because twins, which consume less milk, are less susceptible to alopecia than the individual offspring, and because the risk of alopecia can be reduced by partial emptying of the udders. Lambs or kids develop alopecia approximately three to 14 days after birth, rarely later. The first signs are pruritus and scratches or bites on the affected areas, often accompanied by swallowing of the hair. The hair becomes creepy and the fleece tufts can be easily pulled. Hair loss begins on the shoulders and sides of the body until eventually only remain at the ends (tail, lower limbs, ears) and at the top of the head. As a result of sun exposure, the hairless areas become reddish and tender, followed by cutaneous exudation and crust formation. In lambs, another common complication is bronchopneumonia, caused by exposure to cold winds. No primary gross lesions were found on the skin of the hairless areas. The non-pigmented skin is notably more easily affected than the pigmented parts. Morbidity in affected farms can range from 1% in dry years to 100% in extremely rainy years. Unless lambs are properly treated, more than 50% mortality can be expected (Steyn 1931).

The most appropriate treatment for affected animals is protection against sun rays and wind, with the use of emollient solution on the hairless areas to avoid hardening of the skin; and as a preventive measure, overgrazing in areas where the plant is present should be avoided (Steyn 1931).

CONCLUSIONS

Elimination of phytotoxins via milk, in addition to being a problem for production animals, is a public health concern, because it can directly or indirectly affect the health of humans.

Knowledge about the risk of exposure to these substances via milk and its dissemination are important for veterinary and human health.

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REFERENCES

- Alonso-Amelot M.E. & Avendaño M. 2002. Human carcinogenesis and bracken fern: review of the evidence. *Current Med. Chem.* 9(6):675-686. <http://dx.doi.org/10.2174/0929867023370743> <PMid:11945131>
- Alonso-Amelot M.E., Castillo U., Smith B.L. & Lauren D.R. 1998. Excretion, through milk, of ptaquiloside in bracken-fed cows, a quantitative assessment. *Lait* 78(4):413-423. <http://dx.doi.org/10.1051/lait:1998440>
- Alvarenga T.M.P. 2015. Avaliação clinicopatológica de ratos alimentados com queijo de leite de vacas que consumiram *Pteridium aquilinum*. Master's Thesis, Universidade Federal de Lavras, Lavras, MG. 88p.
- Araújo J.A.S., Riet-Correa F., Medeiros R.M.T., Soares M.P., Oliveira D.M. & Carvalho F.K.L. 2008. Intoxicação experimental por *Ipomoea asarifolia* (Convolvulaceae) em caprinos e ovinos. *Pesq. Vet. Bras.* 28(10):488-494. <http://dx.doi.org/10.1590/S0100-736X2008001000008>
- Arias H.R. 2000. Localization of agonist and competitive antagonist binding sites on nicotinic acetylcholine receptors. *Neurochem. Int.* 36(7):595-645. <http://dx.doi.org/10.1016/S0197-0186(99)00154-0> <PMid:10771117>
- Barbosa R.C., Riet-Correa F., Medeiros R.M., Lima E.F., Barros S.S., Gimeno E.J., Molyneux R.J. & Gardner E.H. 2006. Intoxication by *Ipomoea sericophylla* and *Ipomoea riedelii* in goats in the state of Paraíba, Northeastern Brazil. *Toxicon* 47(4):371-379. <http://dx.doi.org/10.1016/j.toxicon.2005.11.010> <PMid:16488457>
- Beier R.C. & Norman J.O. 1990. The toxic factor in white snakeroot: identity, analysis and prevention. *Vet. Hum. Toxicol.* 32(Suppl.):81-88. <PMid:2092409>
- Boppré M. 2011. The ecological context of pyrrolizidine alkaloids in food, feed and forage: an overview. *Food Addit. Contam.* 28(3):260-281. <http://dx.doi.org/10.1080/19440049.2011.555085> <PMid:21360373>
- Carvalho G.D., Nunes L.C., Bragança H.B.N. & Porfírio L.C. 2009. Principais plantas tóxicas causadoras de morte súbitas em bovinos no estado do Espírito Santo, Brasil. *Arch. Zootec.* 58:87-98.
- Cheeke P.R. & Shull L.R. 1985. *Natural Toxicants in Feeds and Poisonous Plants*. AVI Publishing Company, Westport, Connecticut. 492p.
- Cheeke P.R. 1998. *Natural Toxicants in Feeds, Forages, and Poisonous Plants*. 2^a ed. Interstate Publishes Inc., Danville, Illinois. 479p.
- Colegate S.M., Dorling P.R. & Huxtable C.R. 1979. A spectroscopic investigation of swainsonine: an α -mannosidase inhibitor isolated from *Swainsona canescens*. *Aust. J. Chem.* 32(10):2257-2264. <http://dx.doi.org/10.1071/CH9792257>
- Cruz G.D. & Bracarense A.P.F.R.L. 2004. Toxicidade da samambaia (*Pteridium aquilinum* (L.) Kuhn) para a saúde animal e humana. *Semina, Ciênc. Agrárias* 25(3):249-258.
- Dantas A.F.M., Riet-Correa F., Gardner D.R., Medeiros R.M., Barros S.S., Anjos B.L. & Lucena R.B. 2007. Swainsonine-induced lysosomal storage disease in goats caused by the ingestion of *Turbina cordata* in Northeastern Brazil. *Toxicon* 49(1):111-116. <http://dx.doi.org/10.1016/j.toxicon.2006.08.012> <PMid:17030054>
- Das S., Manda G.P. & Tyagi A.K. 2007. Milk toxicant of natural plant and feed mold origin: a review. *Indian Dairyman* 59:27-37.
- Driemeier D., Colodel E.M., Gimeno E.J. & Barros S.S. 2000. Lysosomal storage disease caused by *Sida carpinifolia* poisoning in goats. *Vet. Pathol.* 37(2):153-159. <http://dx.doi.org/10.1354/vp.37-2-153> <PMid:10714644>
- Eisele G.R. 1986. Distribution of indole in tissues of dairy cattle, swine, and laying pullets. *Bull. Environ. Contam. Toxicol.* 37(2):246-252. <http://dx.doi.org/10.1007/BF01607757> <PMid:3742078>
- Fink-Gremmels J. 2008. Mycotoxins in cattle feeds and carry-over to dairy milk: a review. *Food Addit. Contam.* 25(2):172-180. <http://dx.doi.org/10.1080/02652030701823142> <PMid:18286407>
- Galasso V., Przybył A.K., Christov V., Kovač B., Asaro F. & Zangrando E. 2006. Theoretical and experimental studies on the molecular and electronic structures of cytosine and unsaturated keto-sparteines. *Chem. Phys.* 325(2-3):365-377. <http://dx.doi.org/10.1016/j.chemphys.2005.12.030>
- Gardner D.R., Welch K.D., Lee S.T., Cook D. & Riet-Correa F. 2018. Tremorgenic indole diterpenes from *Ipomoea asarifolia* and *Ipomoea muelleri* and the identification of 6,7-Dehydro-11-hydroxy-12,13-epoxyterpendole A. *J. Nat. Prod.* 81(7):1682-1686. <http://dx.doi.org/10.1021/acs.jnatprod.8b00257> <PMid:30011204>
- Ghosh M.K., Atreja P.P., Buragohain R. & Bandyopadhyay S. 2007. Influence of short-term *Leucaena leucocephala* feeding on milk yield and its composition, thyroid hormones, enzyme activity, and secretion of mimosine and its metabolites in milk of cattle. *J. Agricult. Sci.* 145(04):407-414. <http://dx.doi.org/10.1017/S0021859607007113>
- Goeger D.E., Cheeke P.R., Schmitz J.A. & Buhler D.R. 1982. Effect of feeding Milk from goats fed tansy ragwort (*Senecio jacobaea*) to rats and calves. *Am. J. Vet. Res.* 43(9):1631-1633. <PMid:7149409>
- Green B.T., Lee S.T., Panter K.E., Welch K.D., Cook D., Pfister J.A. & Kem W.R. 2010. Actions of piperidine alkaloid teratogens at fetal nicotinic acetylcholine receptors. *Neurotoxicol. Teratology* 32(3):383-390. <http://dx.doi.org/10.1016/j.ntt.2010.01.011> <PMid:20116429>
- Hale T.W. 1999. *Medications and Mothers' Milk*. Pharmasoft Medical Publishing. 1096p.
- Heck M.C., Sifuentes dos Santos J., Bogusz Junior S., Costabeber I. & Emanuelli T. 2007. Estimation of children exposure to organochlorine compounds through milk in Rio Grande do Sul, Brazil. *Food Chem.* 102(1):288-294. <http://dx.doi.org/10.1016/j.foodchem.2006.05.019>
- James L.F. & Hartley W.J. 1977. Effects of milk from animals fed locoweed on kittens, calves and lambs. *Am. J. Vet. Res.* 38(8):1263-1265. <PMid:562113>
- Keeler R.F. 1978. Alkaloid teratogens from *Lupinus*, *Conium*, *Veratrum* and related genera, p.397-408. In: Keeler R.F., Van Kampen K.R. & James L.F. (Eds), *Effects of Poisonous Plants on Livestock*. Academic Press, New York. <http://dx.doi.org/10.1016/B978-0-12-403250-7.50045-5>
- Keeler R.F. & Crowe M.W. 1985. Anabasine, a teratogen from the *Nicotiana* species, p.324-333. In: Seawright A.A., Hegarty M.P., James L.F. & Keeler R.F. (Eds), *Plant Toxicology*. Queensland Poisonous Plants Committee, Queensland, Australia.
- Kenneth B.Y. & McConnell P. 1948. Passage of selenium through the mammary glands of the white rat and the distribution of selenium in the milk proteins after subcutaneous injection of sodium selenate. *J. Biol. Chem.* 173(2):653-657. <PMid:18910720>
- Kingsbury J.M. 1964. *Poisonous Plants of the United States and Canada*. Prentice Hall Inc., Englewood Cliffs. 626p. <http://dx.doi.org/10.1097/00010694-196411000-00022>
- Kissmann S. & Groth D. 1992. *Plantas Infestantes e Nocivas*. BASF Brasileira S.A., São Paulo. 978p.
- Kubik M., Refholec J. & Zachoval Z. 1980. Outbreak of hemlock poisoning in cattle. *Veterinarstvi* 30(4):157-158.
- Lee S.T., Cook D., Pfister J.A., Allen J.G., Colegate S.M., Riet-Correa F. & Taylor C.M. 2014. Monofluoroacetate-containing plants that are potentially toxic to livestock. *J. Agricult. Food Chem.* 62(30):7345-7354. <http://dx.doi.org/10.1021/jf500563h> <PMid:24724702>
- Lopes J.R.G., Riet-Correa F., Cook D., Pfister J.A. & Medeiros R.M.T. 2014. Elimination of the tremorgenic toxin of *Ipomoea asarifolia* by milk. *Pesq. Vet. Bras.* 34(11):1085-1088. <http://dx.doi.org/10.1590/S0100-736X2014001100009>
- Lopes J.R.G., Araújo J.A., Pessoa D.A.N., Lee S., Riet-Correa F. & Medeiros R.M.T. 2018. Mortalidade neonatal associada ao monofluoroacetato de sódio em cabritos alimentados com colostro de cabras ingerindo *Amorimia septentrionalis*. *Pesq. Vet. Bras.* (In publication)
- Lucena K.F.C., Rodrigues J.M.N., Campos É.M., Dantas A.F.M., Pfister J.A., Medeiros R.M.T. & Riet-Correa F. 2014. Poisoning by *Ipomoea asarifolia* in lambs by

- the ingestion of milk from ewes that ingest the plant. *Toxicon* 92:129-132. <<http://dx.doi.org/10.1016/j.toxicon.2014.10.019>> <PMid:25448387>
- Lucena R.B., Rissi D.R., Maia L.A., Flores M.M., Dantas A.F.M., Nobre V.M.T., Riet-Correa F. & Barros C.S.L. 2010. Intoxicação por alcaloides pirrolizidínicos em ruminantes e equinos no Brasil. *Pesq. Vet. Bras.* 30(5):447-452. <<http://dx.doi.org/10.1590/S0100-736X2010000500013>>
- Mathews H.B. 1980. Elimination of toxicants and their metabolites, p.175-176. In: Hodgson E. & Gulhrie F.E. (Eds), *Introduction to Biochemical Toxicology*. Elsevier North Holland Inc., New York.
- McLean E.K. 1970. The toxic actions of pyrrolizidine (*Senecio*) alkaloides. *Pharmacol. Rev.* 22(4):429-483. <PMid:4921839>
- Medeiros R.M.T., Gorniak S.L. & Guerra J.L. 1999. Effects of Milk from goat fed *Crotalaria spectabilis* seeds on growing rats. *Braz. J. Vet. Res. Anim. Sci.* 36(2):97-100. <<http://dx.doi.org/10.1590/S1413-95961999000200008>>
- Mendonça F.S., Albuquerque R.F., Evêncio-Neto J., Freitas S.H., Dória R.G.S., Boabaid F.M., Driemeier D., Gardner D.R., Riet-Correa F. & Colodel E.M. 2012. Alfa-mannosidosis in goats caused by the swainsonina-containing plant *Ipomoea verbascoidea*. *J. Vet. Diagn. Invest.* 24(1):90-95. <<http://dx.doi.org/10.1177/1040638711425948>> <PMid:22362938>
- Molyneux R.J. & James L.F. 1982. Loco intoxication: Indolizidine alkaloids of spotted locoweed (*Astragalus lentiginosus*). *Science* 216(4542):190-191. <<http://dx.doi.org/10.1126/science.6801763>> <PMid:6801763>
- Niwa H., Ojika M., Wakamatsu K., Yamada K., Hirono I. & Matsushita K. 1983. Ptaquiloside, a novel norsesquiterpene glucoside from bracken, *Pteridium aquilinum* var. *latiusculum*. *Tetrahedron Letters* 24(38):4117-4120. <[http://dx.doi.org/10.1016/S0040-4039\(00\)88276-3](http://dx.doi.org/10.1016/S0040-4039(00)88276-3)>
- Oliveira C.A., Barbosa J.D., Duarte M.D., Cerqueira V.D., Riet-Correa F., Tortelli F.P. & Riet-Correa G. 2009. Intoxicação por *Ipomoea carnea* subsp. *fistulosa* (Convolvulaceae) em caprinos na Ilha de Marajó, Pará. *Pesq. Vet. Bras.* 29(7):583-588. <<http://dx.doi.org/10.1590/S0100-736X2009000700014>>
- Ortega J.A. & Lazerson J. 1987. Anagryne-induced red cell aplasia, vascular anomaly, and skeletal dysplasia. *J. Pediatr.* 111(1):87-89. <[http://dx.doi.org/10.1016/S0022-3476\(87\)80349-9](http://dx.doi.org/10.1016/S0022-3476(87)80349-9)> <PMid:3110390>
- Paik I.K., Robblee A.R. & Clandinin D.R. 1980. Products of the hydrolysis of rapeseed glucosinolates. *Can. J. Anim. Sci.* 60(2):481-493. <<http://dx.doi.org/10.4141/cjas80-057>>
- Panter K.E. & James L.F. 1990. Natural plant toxicants in milk: a review. *J. Anim. Sci.* 68(3):892-904. <<http://dx.doi.org/10.2527/1990.683892x>> <PMid:2180885>
- Panter K.E., Bunch T.D. & Keeler R.F. 1988. Maternal and fetal toxicity of poison hemlock (*Conium macularum*) in sheep. *Am. J. Vet. Res.* 49(2):281-283. <PMid:3348538>
- Panter K.E., Gardner D.R. & Molyneux R.J. 1998. Teratogenic and fetotoxic effects of two piperidine alkaloid-containing lupines (*L. formosus* and *L. arbustus*) in cows. *J. Nat. Toxins* 7(2):131-140. <PMid:9678187>
- Panter K.E., James L.F. & Gardner D.R. 1999. Lupines, poison-hemlock and Nicotiana spp. toxicity and teratogenicity in livestock. *J. Nat. Toxins* 8(1):117-134. <PMid:10091132>
- Paraguassu A.A. 1983. Intoxicação experimental por *Mascagnia rigida* Grisebach (Malpighiaceae) em caprinos no Nordeste do Brasil. Master's Thesis, Universidade Federal Rural do Rio de Janeiro, Seropédica, RJ. 65p.
- Pereira D.A. 2011. Fatores impactantes na qualidade do leite de tanques comunitários na microrregião de Juiz de Fora-MG. Master's Thesis, Universidade Federal de Juiz de Fora, Juiz de Fora, MG. 112p.
- Prakash A.S., Pereira T.N., Reilly P.E. & Seawright A.A. 1999. Pyrrolizidine alkaloids in human diet. *Mutation Res.* 443(1/2):53-67. <[http://dx.doi.org/10.1016/S1383-5742\(99\)00010-1](http://dx.doi.org/10.1016/S1383-5742(99)00010-1)> <PMid:10415431>
- Radostits O.M., Gay C.C., Blood D.C., Hinchliff K.W., Arundel J.H., Figueiredo C., Jacobs D.E., Leslie B.O., Mckenzie R.A. & Bildfell R.J. 2002. Clínica veterinária: um tratado de doenças de bovinos, ovinos, suínos, caprinos e equinos. Guanabara, Rio de Janeiro. 1737p.
- Rasmussen L.H. 2003. Ptaquiloside an environmental hazard? Occurrence and fate of a Bracken (*Pteridium* sp.) toxin in terrestrial environments. Doctoral Dissertation, The Royal Veterinary and Agricultural University, Denmark. 124p.
- Reynolds T. 2005. Hemlock alkaloids from Socrates to poison aloes. *Phytochemistry* 66(12):1399-1406. <<http://dx.doi.org/10.1016/j.phytochem.2005.04.039>> <PMid:15955542>
- Riet-correa F., Medeiros R.M.T., Pfister J.A., Schild A.L. & Danta S.A.F.M. 2009. Poisonings by plants, mycotoxins and related substances in Brazilian livestock. Pallotti, Santa Maria. 246p.
- Riet-Correa F., Tabosa I.M., Azevedo E.O., Medeiros R.M.T., Simões S.V.D., Dantas A.F.M., Alves C.J., Nobre V.M.T., Athayde A.C.R., Gomes A.A. & Lima E.F. 2003. Doenças dos ruminantes e equinos no semi-árido da Paraíba. *Semi-árido em Foco*, Patos, 1(1):2-86.
- Santos J.C.A., Riet-Correa F., Simões S.V.D. & Barros C.S.L. 2008. Patogênese, sinais clínicos e patologia das doenças causadas por plantas hepatotóxicas em ruminantes e eqüinos no Brasil. *Pesq. Vet. Bras.* 28(1):1-14. <<http://dx.doi.org/10.1590/S0100-736X2008000100001>>
- Santos R.C., Brasileiro Filho G. & Silva M.E. 1992. Tumorigenicity of boiling water extract of bracken fern (*Pteridium aquilinum* (L.) Kuhn). *Ciênc. Tecnol. Aliment.* 12:72-76.
- Schep L.J., Slaughter R.J. & Beasley D.M. 2009. Nicotinic plant poisoning. *Clin. Toxicol.* 47(8):771-781. <<http://dx.doi.org/10.1080/15563650903252186>> <PMid:19778187>
- Silva L.C.A., Pessoa D.A.N., Lopes J.R.G., Santos J.R.S., Olinda R.G. & Riet-Correa F. 2017. Embryonic death and abortions in goats caused by the ingestion of *Amorimia septentrionalis*. *Pesq. Vet. Bras.* 37(12):1401-1404. <<http://dx.doi.org/10.1590/s0100-736x2017001200007>>
- Smith R.L. & Bababunmi E.A. 1980. *Toxicology in the Tropics*. Taylor and Francis Ltd., London. 280p.
- Steyn D.G. 1931. Investigations into the cause of alopecia (kaalsiekte) in kids and lambs. 17th Report of the Director of Veterinary Services and Animal Industry, p.729-756.
- Taylor J.B. & Strickland J.R. 2002. Appearance and disappearance of swainsonine in serum and milk of lactating ruminants with nursing young following a singledose exposure to swainsonine (locoweed; *Oxytropis sericea*). *J. Anim. Sci.* 80(9):2476-2484. <PMid:12350025>
- Throckmorton J.C., Cheeke P.R., Patton N.M., Arscott G.H. & Jolliff G.D. 1981. Evaluation of meadowfoam (*Limnanthes alba*) meal as a feedstuff for broiler chicks and weanling rabbits. *Can. J. Anim. Sci.* 61(3):735-742. <<http://dx.doi.org/10.4141/cjas81-088>>
- Tokarnia C.H., Brito M.F., Barbosa J.D., Peixoto P.V. & Dobereiner J. 2012. *Plantas Tóxicas do Brasil para Animais de Produção*. 2ª ed. Helianthus, Rio de Janeiro, p.29-71.
- Vahrmeijer J. 1981. *Poisonous Plants of Southern Africa that Cause Stock Losses*. Tafelberg, Cape Town. 168p.
- Valsechi O.A. 2001. O leite e seus derivados. Postgraduate Program in Production Engineering, Universidade Federal de São Carlos, Araras, SP. 35p.
- Vasconcelos J.S., Riet-Correa F., Dantas A.F.M., Medeiros R.M.T., Galiza G.J.N., Oliveira D.M. & Pessoa A.F.A. 2008. Intoxicação por *Mascagnia rigida* (Malpighiaceae) em ovinos e caprinos. *Pesq. Vet. Bras.* 28(10):521-526. <<http://dx.doi.org/10.1590/S0100-736X2008001000013>>
- White R.D. & Cheeke P.R. 1983. Meadowfoam (*Limnanthes alba*) meal as a feedstuff for dairy goats and toxicologic activity of the milk. *Can. J. Anim. Sci.* 63(2):391-398. <<http://dx.doi.org/10.4141/cjas83-048>>

Experimental reproduction of cyanogenic poisoning by star grass (*Cynodon nlemfuensis* Vanderyst var. *nlemfuensis* cv. "Florico") in cattle¹

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ABSTRACT- Molossi F.A., Ogliari D., Melchiorretto E., Hugen G.F.G.P., Quevedo L.S., Vettori J.M. & Gava A. 2019. **Experimental reproduction of cyanogenic poisoning by star grass (*Cynodon nlemfuensis* Vanderyst var. *nlemfuensis* cv. "Florico") in cattle.** *Pesquisa Veterinária Brasileira* 39(4):238-243. Laboratório de Patologia Animal, Departamento de Medicina Veterinária, Centro de Ciências Agroveterinárias, Universidade do Estado de Santa Catarina, Av. Luiz de Camões 2090, Lages, SC 88520-000, Brazil. E-mail: aldo.gava@udesc.br

We experimentally reproduced star grass (*Cynodon nlemfuensis* Vanderyst var. *nlemfuensis* cv. "Florico") poisoning in cattle. Star grass was planted in four shaded (1, 2, 3, and 4) and three sunny paddocks (5, 6, and 7) and were fertilized with the equivalent of 0, 750, 200, or 100 kg/ha urea. Fresh plant leaves were collected from each paddock and fed to 8 calves aged 5 months to 1 year at 10g/kg live weight. Clinical examinations were performed pre- and post-feeding. Fresh leaves were also subjected to sodium picrate paper and diphenylamine tests to evaluate hydrocyanic acid and nitrate concentration, respectively. Clinical signs of poisoning by the star grass cv. "Florico" in cattle were observed 5-15 minutes after ingestion; these included wobbling, muscle tremors, frequent and/or dripping urination, tachycardia, ruminal atony, depression, red ocular mucous membranes, and bright red venous blood. Three animals became severely ill, 4 manifested mild to moderate clinical signs, and 1 did not become ill. The 3 severely ill bovines received a thiosulfate and sodium nitrite solution at a dose of 40ml/100 kg live weight, intravenously (IV). Florico star grass poisoned bovines and the reaction to the sodium picrate paper and diphenylamine tests was more significant for samples cultivated in the shade and with high nitrogen fertilization. Florico grass utilization should be avoided in a silvopastoral system.

INDEX TERMS: Experimental reproduction, cyanogenic poisoning, star grass, *Cynodon nlemfuensis*, Florico cultivar, cattle, hydrocyanic acid, shadow, bovine.

RESUMO.- [Reprodução experimental de intoxicação cianogênica por grama estrela (*Cynodon nlemfuensis* Vanderyst var. *nlemfuensis* cv. "Florico") em bovinos.]

Descreve-se a reprodução experimental da intoxicação cianogênica por grama estrela (*Cynodon nlemfuensis* Vanderyst var. *nlemfuensis* cv. "Florico") em bovinos. A grama estrela foi plantada em quatro piquetes em área sombreada (1, 2, 3 e 4) e em três piquetes em área ensolarada (5, 6 e 7) e foram adubados com o equivalente a 0, 750, 200 ou 100kg/ha de ureia. Folhas

da planta foram coletadas de cada piquete e imediatamente fornecidas na dose de 10g/Kg de peso vivo para 8 bezerros com idades entre 5 meses a 1 ano. Exames clínicos foram realizados antes e durante fornecimento da planta. Folhas verdes de estrela africana foram submetidas ao teste do papel picro-sódico para verificar a presença de ácido cianídrico e ao teste da difenilamina para avaliar a concentração de nitrato. Sinais clínicos de intoxicação pela grama estrela cv. "Florico" nos bovinos foram observados entre 5 e 15 minutos após o término da ingestão, estes incluíram andar cambaleante, tremores musculares, micção frequente e/ou em gotejamento, taquicardia, atonia ruminal, depressão, mucosas oculares e sangue venoso de coloração vermelho-vivo. Três animais adoeceram gravemente, quatro manifestaram sinais clínicos leves a moderados e um não adoeceu. Os 3 bovinos que adoeceram gravemente receberam solução de tiosulfato e

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nitrito de sódio na dose de 40ml/100kg de peso vivo, por via endovenosa (IV). Grama estrela florico produziu intoxicação em bovinos e a reação ao teste do papel picro-sódico e difenilamina foi mais significativa para as amostras cultivadas à sombra e com alta adubação nitrogenada. Utilização de grama florico deve ser evitada em sistema silvipastoril.

TERMOS DE INDEXAÇÃO: Reprodução experimental, intoxicação cianogênica, grama estrela, *Cynodon nlemfuensis*, Florico cultivar, bovinos, ácido cianídrico, sombra, grama estrela.

INTRODUCTION

Grasses from the genus *Cynodon* have been widely used in cattle feeding as it is a good quality forage, and can be used for grazing or as hay. Many studies have reported on means to improve the forage potential of plants of this genus (Vilela & Alvim 1998).

The *Cynodon* genus is divided in two groups: the Bermuda grasses (*Cynodon dactylon* (L) Pers.), which have rhizomes and stolons, and the star grasses (*Cynodon nlemfuensis* Vanderyst, *Cynodon aethiopicus*, Clayton e Harlan), which have only stolons. This characteristic confers advantages to the first group, as for example, better resistance to grazing (Nascimento et al. 2002) and resistance to moderately cold winters (Vilela & Alvim 1998). Among the Bermuda grass hybrids, the best known are the Tiftons, while among the star hybrids, one of the most used cultivars is the Florico (Vilela & Alvim 1998).

The Florico star grass cultivar has a large potential cyanogenic glycoside accumulation, mainly under high nitrogen fertilization, and principally during the initial stages of the development of the plant. However, during 16 years of tests in Ona, Florida, no poisoning by hydrocyanic acid (HCN) was observed in cattle grazing on Florico grass (Mislevy et al. 1993). In Brazil, the presence of HCN in the cultivar Florico was correlated with the cutting age of this grass, but no poisoning was observed (Castro 1998).

In Brazil, the integration between agriculture, livestock, and forest, or the silvopastoral system, has been studied since 1970. Some grasses that have shown good results in this system are *Panicum maximum*, *Brachiaria decumbens*, and *Brachiaria brizanta* (Garcia et al. 2013). In Santa Catarina State, the silvopastoral system has been used because the shade provides greater comfort to dairy cattle. Star grasses have been recommended in this context, because they have strong stolons that grow very rapidly (Hanna & Sollenberger 2007). The use of cv. Florico star grass cultivated in the shade provided the accumulation of hydrocyanic acid in the plant and its consumption by the bovines resulted in cyanogenic poisoning (Molossi 2019).

The current study aimed to evaluate the effect of sunlight and nitrogen fertilization on the HCN production and nitrate accumulation in *Cynodon nlemfuensis* Vanderyst var. *nlemfuensis* cv. "Florico", and to verify the cyanogenic poisoning potential of this plant in cattle.

MATERIALS AND METHODS

Star grass seedlings cv. "Florico" were collected from one of the farms where the hydrocyanogenic poisoning was encountered, and were planted in a shaded 200m² paddock, (under eucalyptus trees - Eucalyptus), in 2016. This Paddock (1) received proportionally 750 kg/ha of urea.

In 2017, another six paddocks were planted with Florico star grass from the same origin; three of these were in a shaded area (under eucalyptus trees - Eucalyptus; Paddocks 2, 3, and 4, each 53m² in size) and three other paddocks were in a sunny area (Paddocks 5, 6, and 7, each 100 m² in size). Paddocks 2 and 5 and Paddocks 3 and 6 received, respectively, the equivalent of 200 and 100 kg/ha of urea, while Paddocks 4 and 7 did not receive urea fertilization. With the exception of Paddock 1, the amount of nitrogen fertilization was applied following the Brazilian Society of Soil Science (2004) manual, which recommends an amount of 100-200 kg/ha to tropical perennial grasslands. All paddocks were planted in CAV-UDESC lands, in Lages/SC.

For experimental reproduction, the animals were weighed, fasted for 24 hours, and placed in individual stalls with water *ad libitum*. Green star grass leaves were collected from each paddock and immediately used to feed 8 crossbred bovines, aged from 5 months to 1 year (1 animal per paddock, except for Paddock 1, where 2 animals were used), at a dose of 10g/kg of live weight.

The experiment was performed following procedures approved by the Committee of Ethics in Animal Experimentation of the State University of Santa Catarina (CETEA-UDESC), protocol number 9925244116.

Clinical examinations were made before, during, and after feeding the animals the star grass. Behavioral and postural alterations, as well as the color of the mucous membranes and of the blood, the corporal temperature, gastrointestinal motility (ruminal movements), and cardiac and respiratory frequencies were evaluated. The intensity of these clinical signs of poisoning was classified as mild, moderate, and severe.

In cases of mild and moderate poisoning, the animals were observed until they had recovered. When cyanogenic poisoning was severe, the bovines were treated with a solution containing 30g of sodium thiosulphate and 20g of sodium nitrite, dissolved in 500ml of distilled water, at a dose of 40ml/100 kg of live weight, intravenously. The animals were considered to have recovered when they started eating again.

For qualitative confirmation of cyanogenic glycosides, the sodium picrate paper test was performed, as described by Henrici (1926) cited by Tokarnia et al. (2012). This test involves the utilization of white paper strips bathed in a solution of 5g of sodium carbonate and 0.5g of picric acid, both dissolved in 100ml of distilled water. Then, sixty grams of green Florico leaves were collected from each paddock; these were macerated and placed in glass jars with a lid. The paper strips were fixed to the lids, remaining suspended over the vegetal material; the glass jars remained in a vertical position. The reaction to the sodium picrate paper test was classified in terms of the intensity of the brick-red color that had developed in the paper by 20 minutes after beginning the test. The reaction was considered to be strong when the color changed to a brick-red color; moderate when the color was intermediate, and mild when it only turned orange.

For qualitative evaluation of nitrate in all star grass paddocks, the diphenylamine test was used. A solution composed of 0.5g of diphenylamine, 20ml of distilled water, and sulfuric acid to 100ml (Radostits et al. 2002) was prepared. The test involved combining 1 drop of vegetal extract from the green leaves of the grass, obtained by manual pressure, with 3 drops of the reagent. The reaction was considered positive when an intense blue color could be observed in less than 10 seconds. The reaction was classified as negative, mild, moderate, and accentuated according to the intensity of the blue color.

RESULTS

The clinical signs of experimental poisoning by star grass cv. "Florico" in cattle were observed from 5 to 15 minutes after the end of ingestion and showed a variation in intensity that

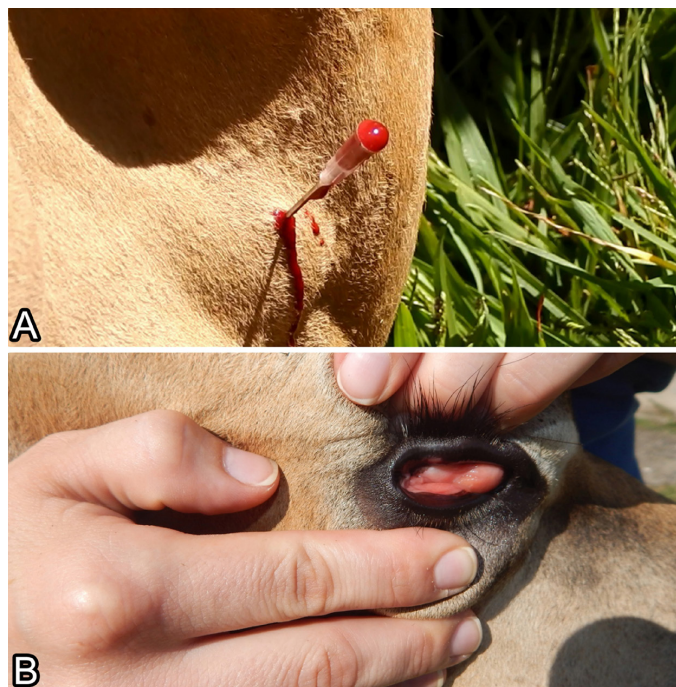


Fig.1. Experimental poisoning with star grass cv. "Florico" in Bovine 1. (A) Venous blood and (B) ocular mucous membranes; both were a bright red color.

depended on the sunlight exposure and the fertilization used. The animals that ingested the plant cultivated in a shaded area (Bovine 1, Paddock 1, 750 kg urea/ha; and Bovine 3, Paddock 2, 200 kg urea/ha) and in a sunny area (Bovine 6, Paddock 5, 200 kg urea/ha), showed a severe cyanogenic poisoning, with clinical signs characterized by wobbling, muscle tremors, frequent and/or dripping urination, tachycardia, ruminal atony, depression, and ocular mucous membranes and venous blood of a bright red color (Fig.1). The clinical condition of Bovine 1 evolved into lateral recumbency with intense dyspnea 10 minutes after ingesting the plant. Bovines 3 and 6 showed a less severe clinical condition that lasted for a longer period and evolved into sternal recumbency, although they did manage to get up. The antidote solution was used in these 3 bovines, and the clinical condition was reversed successfully. Bovine 2 (Paddock 1, in a shaded area, 750 kg urea/ha), Bovine 5 (Paddock 4 without fertilization, in a shaded area), Bovine 7 (Paddock 6, in a sunny area, 100 kg urea/ha), and Bovine 8 (Paddock 7, in a sunny area, without fertilization), showed frequent and dripping urination, alert behavior, tachycardia, ruminal atony, and spontaneously stopped eating, consuming smaller doses than the bovines that became severely ill. Bovine 4 did not become ill at all. The data relative to the amount of plant provided, amount of plant ingested, consummation time, and onset, intensity, and duration of the clinical signs are shown in Table 1.

As shown in Table 2, star grass from shaded paddocks that had received more fertilizer demonstrated a stronger reaction in the sodium picrate and diphenylamine tests. Figure 2 shows the representative results of a strong and a mild reaction in the sodium picrate paper test and a strong as well as a negative reaction in the diphenylamine test.

Table 1. Details of star grass "Florico" supply, intensity, and evolution of the clinical signs in bovines

Bovine	Weight (kg)	Paddock	Supply		Clinical signs				Recovery
			Dose supplied (g/kg)	Dose consumed (g/kg)	Ingestion time (min.)	Min. after administration	Intensity	Duration of the clinical signs (min.)	Recovery time after treatment (min.)
1	160	1	15	10	20	10	+++	10	10
2	168	1	10	6,43	14	10	++	30	NT
3	106	2	10	10	15	5	+++	45	10
4	83	3	10	3	5	ASC	-	-	NT
5	107	4	10	10	9	15	++	60	NT
6	62	5	10	10	10	15	+++	60	10
7	95	6	10	6,31	17	15	+	45	NT
8	92	7	10	5,43	11	10	+	35	NT

Intensity: + mild, ++ moderate, +++ strong; ASC = absence of clinical signs, NT = not treated.

Table 2. Results of the sodium picrate paper test and the diphenylamine test in star grass "Florico" cultivated in shaded and sunny areas, with and without nitrogen fertilization

Fertilization (kg urea/ha)	Shadow (Paddocks 1, 2, 3, and 4)		Sunny (Paddocks 5, 6, and 7)	
	Intensity of reaction of the sodium picrate paper test	Intensity of reaction of the diphenylamine test	Intensity of reaction of the sodium picrate paper test	Intensity of reaction of the diphenylamine test
750	+++	+++	Not used	Not used
200	+++	+++	++	++
100	+++	+++	+	+
WF	++	-	+	-

- Negative reaction, + mild reaction, ++ moderate reaction, +++ strong reaction; WF = without fertilization.

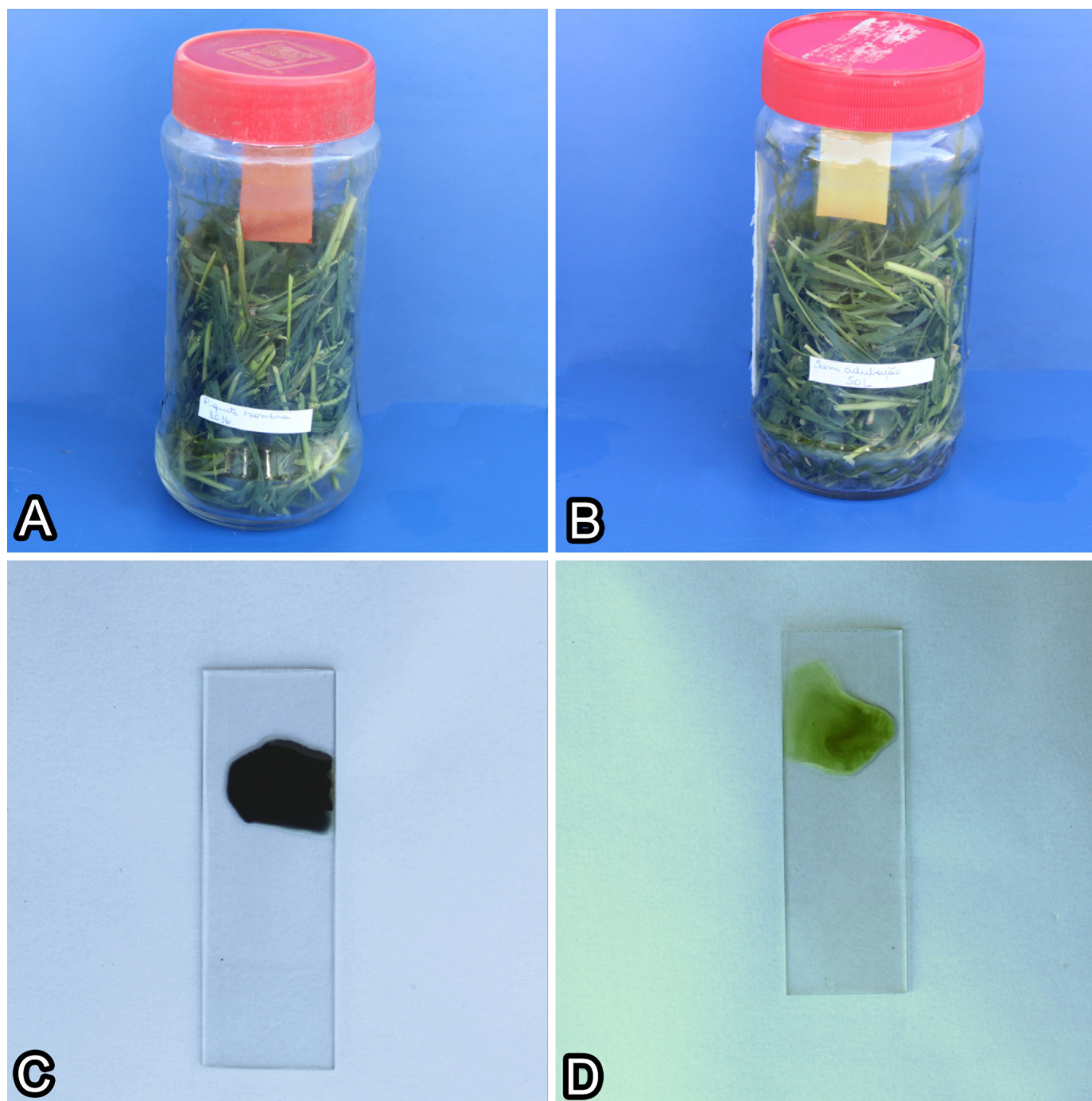


Fig.2. Reactions to the sodium picrate paper test and the diphenylamine test in star grass cv. "Florico". (A) Strong reaction to sodium picrate paper test. (B) Mild reaction to sodium picrate paper test. (C) Strong reaction to diphenylamine test. (D) Negative reaction to diphenylamine test.

DISCUSSION

The clinical condition of the experimental poisoning by star grass cv. "Florico" was marked by the rapid (5-15 min) appearance of muscle tremors, dyspnea, ruminal atony, bloat, wobbling, frequent and/or dripping urination, tachycardia, depression, ocular mucous membranes and venous blood of a bright red color, and sternal or lateral recumbency. This clinical condition is similar to that described in relation to cyanogenic plants by other authors (Saad & Camargo 1967, Canella et al.

1968, Kellerman et al. 1988, Gava et al. 1992, Tokarnia et al. 1994a, 1994b, 1999, Gava et al. 1997, Radostits et al. 2002, Youssef & Maxie 2004, Amorim et al. 2005, Nóbrega Junior et al. 2006, Riet-Correa & Mendez 2007, Oliveira et al. 2008, Carvalho et al. 2011, Juffo et al. 2012, Galindo et al. 2017).

In the current study, the bovines that ate star grass cv. "Florico" from paddocks in a shaded area, and that had received good fertilization, were most severely clinically affected. Similarly, the paddocks in a shaded area with more

fertilization resulted in a stronger reaction to the sodium picrate paper test. This is in accordance with what occurs in the spontaneous poisoning described by Molossi (2019). Vetter & Haraszti (1977), observed that the HCN production in *Sorghum* spp. gradually decreased during its vegetative growing, while the photosynthesis intensity increases, in other words, as photosynthesis decreased, more HCN was produced. Higher HCN levels are due to many factors; among these are a young age of the plants, a rapid growth rate, high nitrogen fertilization, low growing periods followed by rain, wilting, plants burnt by cold weather, and resprouting (Harris & Shearer 1994, Radostits et al. 2002).

According to Vetter (2000), a greater availability of nitrogen in the soil promotes cyanogenic glycosides production. Tapper & Reay (1973) described that nitrogen is part of the chemical composition of the known cyanogenic glycosides. Mislevy et al. (1993) affirmed that the hydrocyanic potential of star grass "Florico" is high under heavy nitrogen fertilization, especially during the initial stages of development of the plants.

Cruz (1997) observed that the nitrogen content increased in shaded pastures. This may occur because the soil in a shaded area tends to have greater humidity, associated with the moderate temperature, resulting in more rapid nitrogen mineralization, decomposition of organic matter, and recycling of this matter (Belsky et al. 1993). According to Wilson Junior & Ludlow (1991), the greater amount of nitrogen in the leaves is a compensatory mechanism that results in more CO₂ assimilation, leading to a more efficient use of solar radiation in shaded pastures. In shade, plants invest less than half of the nitrogen in their leaves in photosynthesis, which thus increases the nitrogen proportion in leaves (Evans 1993). This is also in agreement with the observations of the current study, in relation to nitrate presence, where the diphenylamine test showed a tendency for higher nitrate levels in star grass cv. "Florico" cultivated in a shaded area and/or with a higher levels of fertilization. Poisoning by nitrate does not occur in this situation, as the HCN present in the plants has a rapid action, which hampers ingestion of the larger amounts that are necessary for nitrate poisoning.

The response to the sodium picrate paper test with the star grass cv. "Florico", which was observed after 20 minutes, was different from that obtained from green and tender leaves of *Prunus sellowii*, which occurred from 3 to 5 minutes after the maceration of this plant (Gava et al. 1992). This may be due to the higher fibrous content of this grass, which makes the trituration of the vegetal matter more difficult, and thus affects the reaction between the enzyme and the glycosides. Some plants that are potentially dangerous to animals always have a rapid effect, while the effect of others are slower because of the glycoside stability (some are less volatile) and the enzymes available for hydrolysis (Tokarnia et al. 1999). The plants with slower effects have less toxicity and the related clinical conditions demonstrate a longer, slower evolution (Amorim et al. 2005, Tokarnia et al. 1999). In the current study, 4 of 8 bovines spontaneously ceased star grass ingestion. This indicates that some animals, when ingesting the plant more slowly, perceive that it made them feel indisposed and therefore rejected the plant food, avoiding aggravation of their condition. Bovine 1 ingested the star grass greedily and manifested severe cyanogenic poisoning signs. A solution containing sodium thiosulfate and sodium nitrite was used

successfully to treat the experiment animals, as previously described by Radostits et al. (2002) and Galindo et al. (2017).

CONCLUSIONS

External factors, such as shade and fertilization promote HCN accumulation in star grass (*Cynodon nlemfuensis* Vanderyst var. *nlemfuensis* cv. "Florico") and cause cyanogenic poisoning in bovines when more than 5g/kg live weight is ingested.

Florico grass utilization should be avoided in a silvopastoral system.

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REFERENCES

- Amorim S.L., Medeiros R.M.T. & Riet-Correa F. 2005. Intoxicação experimental por *Manihot glaziovii* (Euphorbiaceae) em caprinos. *Pesq. Vet. Bras.* 25(3):179-187. <<http://dx.doi.org/10.1590/S0100-736X2005000300009>>
- Belsky A.J., Mwonga S.M., Amundson R.G., Duxbury J.M. & Ali A.R. 1993. Comparative effects of isolated trees on their under canopy environments in high- and low-rainfall savannas. *J. Appl. Ecol.* 30(1):143-155. <<http://dx.doi.org/10.2307/2404278>>
- Brazilian Society of Soil Science 2004. Manual de Adubação e Calagem para os Estados do Rio Grande do Sul e Santa Catarina. Química do Solo e Comissão de Fertilidade, Sociedade Brasileira de Ciência do Solo. 10^a ed. Porto Alegre, p.155-160.
- Canella C.F.C., Dobereiner J. & Tokarnia C.H. 1968. Experimental poisoning by maniçoba (*Manihot glaziovii* Muell. Arg.) in bovines. *Pesq. Agropec. Bras.* 3:347-350.
- Carvalho F.K.L., Medeiros R.M.T., Araujo J.A.S. & Riet-Correa F. 2011. *Intoxicação experimental por Passiflora foetida* (Passifloraceae) em caprinos *Pesq. Vet. Bras.* 31(6):477-481. <<http://dx.doi.org/10.1590/S0100-736X2011000600003>>
- Castro F.G.F. 1998. Effect of cutting age on yield, bromatological chemical composition, in vitro dry matter and organic matter digestibility, and hydrocyanic acid content of *Cynodon nlemfuensis* Vanderyst var. *nlemfuensis* cv. "Florico". Master's Thesis, Universidade de São Paulo, São Paulo, SP. 150p.
- Cruz P. 1997. Effect of shade on the carbon and nitrogen allocation in a perennial tropical grass, *Dichanthium aristatum*. *J. Experimental Botany* 48(1):15-24. <<http://dx.doi.org/10.1093/jxb/48.1.15>>
- Evans J.R. 1993. Photosynthetic acclimation and nitrogen partitioning within a lucerne canopy. I. Canopy characteristics. *Austr. J. Plant Physiol.* 20:55-67.
- Galindo C.M., Hemckmeier D., Biondo N., Parizotto L.H., Ogliari D. & Gava A. 2017. Intoxicação espontânea e experimental por tifton 68 (*Cynodon nlemfuensis* Vanderyst) em bovinos. *Pesq. Vet. Bras.* 37(5):441-446. <<http://dx.doi.org/10.1590/s0100-736x2017000500003>>
- Gava A., Pilati C., Cristani J., Simoes J. & Simoes L. 1997. Cyanogenic intoxication in cattle fed with Tifton (*Cynodon* sp.). *Anais VIII Ciclo de Atualização em Medicina Veterinária (CAMEV)*, UDESC, Lages, SC, p.119.
- Gava A., Stolf L., Neves D.S., Stolf O., Varaschim M.S. & Ferreira E.M.M. 1992. Intoxicação experimental por *Prunus sellowii* (Rosaceae) em bovinos. *Pesq. Vet. Bras.* 12(1/2):1-4.
- Hanna W.W. & Sollenberger L.E. 2007. Tropical and subtropical grasses, p.245-255. In: Barnes R.F., Nelson C.J., Moore K.J. & Collins M. (Eds), *Forages the Science of Grassland Agriculture*. Vol.2. 6th ed. Wiley, New Jersey.
- Harris B. & Shearer K.J. 1994. Nitrate, prussic acid (HCN) and grass tetany problems in cattle feeding. *Dairy Sci.* 6:1-3.

- Juffo G.D., Pavarini S.P., Wouters F., Oliveira L.G.S., Antoniassi N.A.B., Cruz C.E.F. & Driemeier D. 2012. Intoxicação espontânea por *Sorghum sudanense* em bovinos leiteiros no Rio Grande do Sul. *Pesq. Vet. Bras.* 32(3):217-220. <<http://dx.doi.org/10.1590/S0100-736X2012000300006>>
- Kellerman T.S., Coetzer J.A.W. & Naudé T.W. 1988. Haemopoietic system, p.193-213. In: Ibid. (Eds), *Plant Poisonings and Mycotoxinoses of Livestock in Southern Africa*. Oxford University Press, Cape Town.
- Mislevy P., Brown W.F., Caro-Costas R., Vicente-Chandler J., Dunavin L.S., Hall D.W., Kalmbacher R.S., Overman A.J., Ruelke O.C., Sonoda R.M., Sotomayor-Rios A., Stanley Junior R.L. & Williams M.J. 1993. Registration of Florico stargrass. *Crop Sci.* 33(2):358-359. <<http://dx.doi.org/10.2135/cropsci1993.0011183X003300020044x>>
- Molossi F.A., Ogliari D., Morais R.M., Wicpolt N.S., Gheller E., Weber L. & Gava A. 2019. Cyanogenic poisoning by spontaneous ingestion of star grass (*Cynodon nlemfuensis* var. *nlemfuensis* cv. 'Florico') in cattle. *Pesq. Vet. Bras.* 39(1):20-24.
- Nascimento M.P.S.C.B., Nascimento H.T.S. & Leal J.A. 2002. Comportamento de cultivares de *Cynodon* em Piauí. Comunicado Técnico 146, Embrapa-CNPMN, Teresina. 3p.
- Nóbrega Junior J.E., Riet-Correa F., Medeiros R.M.T. & Dantas A.F.M. 2006. Intoxicação por *Sorghum halepense* (Poaceae) em bovinos semi-áridos. *Pesq. Vet. Bras.* 26:201-204.
- Oliveira D.M., Pimentel L.A., Araújo J.A.S., Rosane M.T.M., Dantas A.F.M. & Riet-Correa F. 2008. Intoxicação por *Cnidioscolus phyllacanthus* (Euphorbiaceae) em cabras. *Pesq. Vet. Bras.* 28(1):36-42. <<http://dx.doi.org/10.1590/S0100-736X2008000100006>>
- Radostits O.M., Gay C.C., Blood D.C. & Hinchcliff K.W. 2002. Doenças causadas por toxinas de plantas, fungos, cianófitas, clavibactérias e venenos de carrapatos e animais vertebrados, p.1472-1547. In: Ibid. (Eds), *Clínica Veterinária: um tratado sobre doenças de bovinos, ovinos, caprinos, suínos e equinos*. 9ª ed. Guanabara Koogan, Rio de Janeiro.
- Riet-Correa F. & Mendez M.C. 2007. Poisoning by plants and mycotoxins, p.177-181. In: Riet-Correa F., Schild A.L., Lemos R.A.A. & Borges J.R.J. (Eds), *Ruminantes e Doenças Equinas*. Vol.2. 3ª ed. Pallotti, Santa Maria.
- Saad A.D. & Camargo W.V.A. 1967. Intoxicação cianídrica em animais domésticos. *Arqs Inst. Biológico*, São Paulo, 33:211-220.
- Tapper B.A. & Reay P.F. 1973. Cyanogenic glycosides and glucosinolates. p.447-476. In: Butler C.W. & Bailey R.W. (Eds), *Chemistry and Biochemistry of Herbage*. Vol.1. Academic Press, London, New York.
- Tokarnia C.H., Dobereiner J. & Peixoto P.V. 1994a. Aspectos clínico-patológicos complementares da intoxicação por algumas plantas tóxicas brasileiras. *Pesq. Vet. Bras.* 14:111-122.
- Tokarnia C.H., Peixoto P.V. & Dobereiner J. 1994b. Intoxicação experimental por *Piptadenia macrocarpa* (Leg.Mimosoideae) em bovinos. *Pesq. Vet. Bras.* 14:57-63.
- Tokarnia C.H., Peixoto P.V., Brito M.F., Duarte M.D. & Brust L.A.C. 1999. Estudos experimentais com plantas cianogênicas em bovinos. *Pesq. Vet. Bras.* 19(2):84-90. <<http://dx.doi.org/10.1590/S0100-736X1999000200006>>
- Tokarnia C.H., Brito M.F., Barbosa J.D., Peixoto P.V. & Dobereiner J. 2012. *Plantas Tóxicas do Brasil: para animais de produção*. 2ª ed. Helianthus, Rio de Janeiro, p.443-459.
- Vetter J. 2000. Plant cyanogenic glycosides. *Toxicon* 38(1):11-36. <[http://dx.doi.org/10.1016/S0041-0101\(99\)00128-2](http://dx.doi.org/10.1016/S0041-0101(99)00128-2)> <PMid:10669009>
- Vetter J. & Haraszti E. 1977. Changes in the hydrogen cyanide content of Sudan grass (*Sorghum sudanense*) and broomcorn (*Sorghum bicolor* var. technicum) during the growing season. *Acta Agron. Sci. Hung.* 26:15-22.
- Vilela D. & Alvim M.J. 1998. Manejo de pastagens do gênero *Cynodon*: introdução, caracterização e evolução do uso no Brasil. Anais XV Simpósio sobre Manejo de Pastagens, Fundação de Estudos Agrários "Luiz de Queiroz" (FEALQ), Piracicaba, p.23-54.
- Wilson Junior & Ludlow M.M. 1991. The environment and potential growth of herbage under plantations, p.10-24. In: Shelton H.M. & Stur W.W. (Eds), *Forage for Plantation Crops*. XXXII ACIAR Proceedings, Australian Centre for International Agricultural Research, Canberra.
- Youssef S. & Maxie M.G. 2004. Nervous system, p.347-348. In: Ibid. (Eds), *Jubb, Kennedy and Palmer Pathology of Domestic Animals*. Vol.3. 5th ed. Academic Press, London.

Neurological diseases in cattle caused by plants and mycotoxins in Santa Catarina state, Brazil¹

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ABSTRACT.- Morais R.M., Wicpolt N.S., Molossi F.A., Ogliari D., Mori A.M., Surkamp V. & Gava A. 2019. **Neurological diseases in cattle caused by plants and mycotoxins in Santa Catarina state, Brazil.** *Pesquisa Veterinária Brasileira* 39(4):244-250. Laboratório de Patologia Animal, Centro de Ciências Agroveterinárias, Universidade do Estado de Santa Catarina, Av. Luiz de Camões 2090, Bairro Conta Dinheiro, Lages, SC 88520-000, Brazil. E-mail: aldo.gava@udesc.br

This study described the epidemiological and clinical-pathological aspects of 25 outbreaks of neurological diseases in cattle caused by plants and mycotoxins in Santa Catarina state. Six of them were due to *Sida carpinifolia* poisoning, five to *Solanum fastigiatum*, five to *Phalaris angusta*, three to *Claviceps paspali*, three to *Claviceps purpurea*, and three outbreaks were of unknown etiology. The clinical signs observed in the affected cattle were mild to severe and characterized by generalized muscle tremors, incoordination, hypermetria, wide-based stance, intentional head tremors, dull staring eyes, and frequent ear twitching, with convulsions in some cases. At necropsy, lesions were observed only for *P. angusta* poisoning, characterized by gray-greenish discoloration in thalamus and midbrain. Microscopically, rarefaction and/or disappearance of Purkinje neurons with substitution by Bergmann cells were observed for *S. carpinifolia* and *S. fastigiatum* poisoning. For *P. angusta* poisoning, thin granular brown-yellowish pigment was observed in the cytoplasm of some neurons. Gross and microscopic findings were not observed in three outbreaks of tremorgenic disease of unknown etiology. Experiments conducted with leaves, flowers and seeds of *Ipomoea indivisa* and *Ipomoea triloba*, as well as with maize and soybean residues contaminated with *Ipomoea* spp. did not reproduced clinical signs.

INDEX TERMS: Neurological diseases, cattle, mycotoxins, Santa Catarina, Brazil, plant poisoning, neuropathology, diseases of cattle, toxicoses.

RESUMO.- [Enfermidades neurológicas em bovinos causadas por plantas e micotoxinas no estado de Santa Catarina.] Descrevem-se os aspectos epidemiológicos e clínico-patológicos de 25 surtos de enfermidade neurológica em bovinos no estado de Santa Catarina causadas por plantas e micotoxinas. Destes, seis corresponderam a intoxicação

por *Sida carpinifolia*, cinco por *Solanum fastigiatum*, cinco por *Phalaris angusta*, três por *Claviceps paspali*, três por *Claviceps purpurea* e três surtos de etiologia não definida. Os sinais clínicos observados nos bovinos afetados eram de intensidade leve a acentuada e caracterizados por tremores musculares generalizados, incoordenação, hipermetria, aumento da base de sustentação, balanço contínuo de cabeça, olhar atento e movimentos frequentes de orelhas, e em alguns surtos convulsões. Por meio de necropsia foram observadas alterações somente na intoxicação por *P. angusta* as quais caracterizaram por coloração cinza-esverdeada no tálamo e mesencéfalo. Na histologia, rarefação e/ou desaparecimento de neurônios de Purkinje com substituição por células de Bergmann foram observadas na intoxicação por *S. carpinifolia* e *S. fastigiatum*. Na intoxicação por *P. angusta* foi observado no citoplasma de alguns neurônios do tronco encefálico com pigmentação finamente granular marrom-amarelada. Nos três surtos de enfermidade tremorgênica com etiologia

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não definida não foram observadas lesões macroscópicas e microscópicas. Experimentos com folhas, flores e sementes de *Ipomoea indivisa* e *Ipomoea triloba* e resíduos de milho e soja contaminados com sementes destas duas plantas não produziram alterações clínicas.

TERMOS DE INDEXAÇÃO: Doenças neurológicas, bovinos, micotoxinas, Santa Catarina, intoxicações por plantas, neuropatologia, doenças de bovinos, toxicoses.

INTRODUCTION

Bovine nervous system diseases caused by plants and/or mycotoxins are neurotoxicoses characterized by depression, muscle and head tremors, incoordination, ataxic gait with hypermetria or dysmetria, wide-based stance, and alertness with dull staring eyes. In some cases, when cattle are moved, clinical signs become exacerbated and animals tend to present decubitus with seizures and nystagmus. Deaths are rare and most often due to trauma resulting from falls or emaciation (Radostits et al. 2007).

In the South region of Brazil, *Solanum fastigiatum* (Riet-Correa et al. 1983b, Rech et al. 2006) and *Sida carpinifolia* (Furlan et al. 2009, Pedroso et al. 2010) are plants that cause storage disease and, consequently, severe neurological signs. *Phalaris angusta* (Gava et al. 1999) and *Claviceps paspali* (Riet-Correa et al. 1983a) have been described as causes of tremorgenic syndrome. *Aspergillus clavatus* can produce toxins that cause neurological disease when ingested by cattle (Loretti et al. 2003, Bezerra Junior et al. 2009). In the North and Northeast regions of the Country, *Ipomoea carnea* subs. *fistulosa* (Armién et al. 2007), *Turbina cordata* (Dantas et al. 2007), *Ipomoea riedelli*, *Ipomoea sericophylla* (Barbosa et al. 2007), and *Solanum paniculatum* (Guaraná et al. 2011) have also been reported as causing storage disease in ruminants. *Ipomoea asarifolia* has been described as responsible for causing tremorgenic syndrome in ruminants (Döbereiner et al. 1960, Medeiros et al. 2003, Tortelli et al. 2008). Pessoa et al. (2010) described eight outbreaks in cattle, sheep, horses, and mules that developed tremorgenic neurological signs after grazing pastures with predominance of the *Enteropogon mollis* and *Chloris* spp. species.

At necropsy, bovine carriers of these diseases do not usually show lesions, except in poisoning by *P. angusta*, in which gray-greenish discoloration in thalamus and midbrain is observed (Gava et al. 1999), and by *S. fastigiatum* and *S. paniculatum*, in which cerebellar atrophy may occur (Rech et al. 2006, Guaraná et al. 2011). Microscopically, in poisoning by plants that cause lysosomal accumulation in neurons and by *C. paspali*, degeneration and vacuolization of Purkinje neurons with axonal spheroids in the granular layer of the cerebellum are observed in the central nervous system (CNS) (Riet-Correa et al. 1983b, Rech et al. 2006, Armién et al. 2007, Barbosa et al. 2007, Dantas et al. 2007, Furlan et al. 2008, 2009). In poisoning by *A. clavatus*, histological findings are limited to rarefaction and/or absence of Nissl granules (chromatolysis) and central or peripheral pyknotic nuclei (Loretti et al. 2003).

This study described the epidemiological and clinical-pathological aspects of 25 outbreaks of neurological diseases in cattle caused by plants and/or mycotoxins diagnosed at the Laboratory of Animal Pathology of the “Centro de Ciências

Agroveterinárias” (CAV-UDESC) and evaluates the toxicity of *Ipomoea triloba* and *Ipomoea indivisa* as a possible cause of three tremorgenic disease outbreaks in cattle.

MATERIALS AND METHODS

Spontaneous disease. Surveys of 25 outbreaks of neurological diseases in cattle caused by plants and/or mycotoxins diagnosed from 1987 to 2017 were obtained from the Laboratory of Animal Pathology, “Centro de Ciências Agroveterinárias” of the “Universidade do Estado de Santa Catarina” (CAV-UDESC). In 22 of these outbreaks, diagnoses were performed based on epidemiological data, clinical-pathological aspects, and observation of the feed used in the farms and the grazing areas where the cattle were kept. Three of these outbreaks were of unknown etiology, and green leaves and seeds of *Ipomoea triloba* and *Ipomoea indivisa* provided to the animals were collected.

Twenty-five clinically ill head of cattle were necropsied and samples were collected from the CNS, liver, lungs, heart, spleen, lymph nodes, forestomach, abomasum, intestines, kidneys, parotid, thyroid, pancreas, and skeletal muscle. In addition to the samples collected at necropsy, CNS samples were also collected from four head of cattle with tremorgenic disease that had been referred to slaughter. All samples were fixed in 10% formalin, routinely processed, and stained using the hematoxylin-eosin (HE) method for reading under light microscopy.

Experimental study. Three bovines, one black-and-white Holstein Friesian and two Jersey, were used in the experimental study. Green leaves, flowers and seeds of *Ipomoea indivisa* (Aiea morning glory) collected in the municipalities of Concórdia and Abelardo Luz, Santa Catarina state, were provided *ad libitum* to the first bovine. The second bovine was fed maize (*Zea mays*) contaminated with seeds of *Ipomoea indivisa* and *Ipomoea triloba* collected in Concórdia. The third bovine was fed, *ad libitum*, green leaves, flowers and seeds of *Ipomoea triloba* (Aiea morning glory) and soybean (*Glycine max*) residue contaminated with seeds of *Ipomoea triloba* and *Ipomoea indivisa* collected in the municipality of Canoinhas. At the end of the afternoon, the calves were released for grazing in a paddock of kikuyu grass (*Pennisetum clandestinum*) with water *ad libitum*. Clinical examinations, which prioritized behavioral and locomotion assessment, were performed before and after administration of the plants and residues. Table 1 shows the experimental design.

Samples of green leaves, flowers and seeds of *Ipomoea* spp. were sent for botanical identification. This study was approved by the Animal Research Ethics Committee of the “Universidade do Estado de Santa Catarina” (CEUA-UDESC) under protocol no. 4893241116.

RESULTS

Epidemiology

Of the 25 outbreaks of neurological diseases in cattle associated with poisoning by plants and/or mycotoxins monitored by the Laboratory of Animal Pathology of the CAV-UDESC in Santa Catarina state, six were due to *Sida carpinifolia* poisoning, five to *Solanum fastigiatum*, five to *Phalaris angusta*, three to *Claviceps paspali*, three to *Claviceps purpurea*, and three outbreaks were of unknown etiology with suspicion of ingestion of leaves, flowers and seeds of *Ipomoea indivisa* and *Ipomoea triloba*.

Poisoning by *Sida carpinifolia* was observed in six farms located in the Alto Vale do Itajaí region from 2003 to 2005. The disease occurred in small properties where there was

Table 1. Experimental design with *Ipomoea indivisa*, *Ipomoea triloba*, and residues of maize and soybean contaminated with *Ipomoea* spp. seeds

Bovine	Breed	Age (months)	Weight (Kg)	<i>Ipomoea indivisa</i>	<i>Ipomoea triloba</i>	Maize residue	Soybean residue
				g/Kg bw day/ consumption days	g/Kg bw day/ consumption days	g/Kg bw day/ consumption days	g/Kg bw day/ consumption days
1	BWHF ^{a,b}	12	170	-	-	18.58/6	-
2	Jersey ^b	12	160	86.53/22	-	-	-
3	Jersey ^b	12	160	-	113.03/6	-	22.13/5

^a Black-and-white Holstein Friesian, ^b male.

invasion of *S. carpinifolia*, which, in many cases, was the predominant vegetation. In some properties, the cattle were removed from the areas invaded by the plant, and the clinical signs regressed, but upon return the condition worsened. Poisoning by *Solanum fastigiatum* was observed in five rural properties located in the following municipalities and years: Erval Velho in 1997 and 2007, Chapecó in 2003, Água Doce in 2006, and Herval do Oeste in 2005 and 2007. The affected cattle grazed areas infested by large amounts of *S. fastigiatum*.

Poisoning by *Phalaris angusta* was reported in five farms located in the following municipalities and years: Capinzal in 1993, Canoinhas in 1996, Xanxerê in 1997 and 1998, and Porto União in 2010. In these outbreaks, the cattle grazed areas of soybean and/or maize stubble, and/or associated with pasture of common oat (*Avena sativa*) with presence of *P. angusta*.

The three outbreaks of poisoning by *Claviceps paspali* occurred in the municipalities of São Joaquim in pastures with *Paspalum dilatatum* in 1987 and 1988 and Otacílio Costa in pastures with *Paspalum paniculatum* in 2014. The affected cattle showed clinical signs two days after introduction in paddocks of *Paspalum* spp. in the seeding phase and infected with *C. paspali* sclerotia. Falls in steep areas and ditches were frequent in severe cases. When infestation with contaminated *Paspalum* spp. was low, the signs were mild and only ataxia and constant ear twitching were observed.

Outbreaks of poisoning by *Claviceps purpurea* in the neurological form have occurred in the municipalities of Água Doce and Lages in 2015 and Campos Novos in 2016. In the first two outbreaks, the cattle were grazing perennial ryegrass (*Lolium multiflorum*) pasture in the seed maturation phase, whereas in the third outbreak, the bovines were confined and fed pre-dried common oat (*Avena sativa*) in the seed maturation phase.

Three outbreaks of tremorgenic syndrome monitored from 2015 to 2017 were of unknown etiology. In the outbreak observed in Concórdia, 30 bovines aged 24 months were kept under semi-intensive management and received residues from maize dryer with large amounts of seeds of *I. triloba* and *I. indivisa*. They also had access to maize stubbles with large amounts of these seeds (Fig.1) and, according to the farm owner, the cattle ingested a large amount of this plant. In the outbreak observed in Abelardo Luz in April 2016, 10 crossbred cattle aged 18 months showed muscle tremors, frequent ear twitching, and ataxic gait. According to the farm owner, the disease began after introduction of the cattle in an area of maize remains contaminated by large amount of *I. indivisa*, which was in the phase of flowering and seed maturation (Fig.2). In the outbreak occurred in Canoinhas in May 2017, a batch of eight crossbred 12-month-old cattle showed clinical



Fig.1. *Ipomoea triloba* (Aiea morning glory or little bell) collected in the municipality of Concórdia, Santa Catarina state.



Fig.2. *Ipomoea indivisa* (Aiea morning glory or little bell) in maize stubble in the municipality of Abelardo Luz, Santa Catarina state.

signs identical to those described in the outbreak observed in Abelardo Luz; however, these cattle were kept in native field paddocks with no *Paspalum* spp. and, additionally, received dryer residue containing remnants of soybean grains and seeds of *I. triloba* and *I. indivisa*.

Tables 2 and 3 show the main epidemiological data and clinical findings (necropsy and histology), respectively.

Table 2. Epidemiological data relative to the outbreaks of neurological diseases in cattle caused by plants and/or mycotoxins diagnosed at the Laboratory of Animal Pathology, CAV-UDESC, Santa Catarina, from 1987 to 2017

Plant	Number of outbreaks	Number of bovines at risk/ natural death	Age	Time of year	Morbidity (%)	Mortality (%)	Clinical outcome
<i>Sida carpinifolia</i>	6	120/20	2-4 years	Year-round	Up to 90%	Up to 10%	Chronic
<i>Solanum fastigiatum</i>	5	100/4	>4 years	Year-round	Up to 50%	Up to 4%	Chronic
<i>Phalaris angusta</i>	5	150/2	>1 year	Jul./Sep.	10 - 100%	Up to 1.33% ^a	Acute
<i>Claviceps paspali</i>	3	50/0	>1 year	Mar./Apr.	30 - 100%	0	Acute
<i>Claviceps purpurea</i>	3	40/5	1-3 years	Oct./Nov.	3 - 10%	12.5%	Acute
Unknown etiology	3	48/0	1-2 years	Apr./May	80%	0	Acute

^a Traumas.**Table 3. Main clinical findings (necropsy and histopathology) for outbreaks of neurological diseases in cattle caused by plants and/or mycotoxins diagnosed in the Laboratory of Animal Pathology, CAV-UDESC, Santa Catarina state, from 1987 to 2017**

	Clinical signs	Necropsy findings	Histopathology findings
<i>Sida carpinifolia</i>	Dull staring eyes, continuous head and neck tremor; incoordination; convulsion and falls during moving in severe cases.	Unchanged	Thin granular vacuolization in the neurons of the central and peripheral nervous system; engorged Purkinje neurons, eosinophilic cytoplasm and irregular cytoplasmic borders; rarefaction of Purkinje neurons and proliferation of Bergman cells; axonal spheroids.
<i>Solanum fastigiatum</i>	Dull staring eyes, incoordination, varying degrees of hypermetria, wide-based stance, falls followed by generalized muscle tremors lasting few minutes, opisthotonus, and nystagmus.	Unchanged	Vacuolization of soma, necrosis and rarefaction of Purkinje neurons with Bergmann cell replacement.
<i>Phalaris angusta</i>	Frequent head tremors and ear twitching, marching gait, ataxia, varying degrees of hypermetria, convulsions, colliding against fences and walls, and/or falls in steep areas. Traumatic pectoral edema observed in one of the outbreaks.	Gray-greenish discoloration in thalamus and midbrain; increased volume of the ventral thoracic region with large amounts of fibrin and edema.	Some brainstem neurons with intracytoplasmic brown-yellowish granular pigment.
<i>Claviceps paspali</i>	Alertness with erect ears, hyperexcitability, rigid thoracic and pelvic limbs, wide-based stance, head and neck tremors, seizures, frequent exposure of the penis in bulls.	Unchanged	Unchanged
<i>Claviceps purpurea</i>	Dull staring eyes with alertness, incoordination, wide-based stance, convulsions with falls, diarrhea.	Unchanged	Unchanged
Unknown etiology	Dull staring eyes with head tremors, incoordination accentuated when animals were moved, wide-based stance, sternal decubitus.	Unchanged	Unchanged

Experiments conducted with green leaves, flowers and seeds of *I. triloba* and *I. indivisa*, as well as with maize and soybean residues contaminated with seeds of these two plants did not reproduce clinical signs.

DISCUSSION

In this study, *Sida carpinifolia* was the main plant responsible for causing neurological diseases in cattle in Santa Catarina state, followed by *Solanum fastigiatum*, *Phalaris angusta*, *Claviceps paspali*, and *Claviceps purpurea*. The outbreaks of *S. carpinifolia* occurred in small properties where the plant invaded the paddocks and was predominant in some situations, which, associated with hunger, were essential factors for occurrence of the diseases. These conditions were

also reported by Colodel et al. (2002), Furlan et al. (2009), and Bassuino et al. (2017) in poisoning in goats, cattle, and horses, respectively. The clinical signs observed in bovines in outbreaks of *S. carpinifolia* and *S. fastigiatum* are characteristic of cerebellar lesion. Because these diseases result in lysosomal accumulation and mainly affect the Purkinje neurons, the clinical signs were mainly head and neck tremors, ataxic gait, and frequent falls. One of the characteristics of poisoning by *S. fastigiatum* in the described outbreaks was convulsive episodes, lasting few minutes and with fast recovery. These characteristics have been described by Riet-Correa et al. (1983b) and Rech et al. (2006) in poisoning by *S. fastigiatum* and by Guaraná et al. (2011) in poisoning by *S. paniculatum*.

In poisoning by plants that cause lysosomal storage disease, especially those containing swainsonine toxins, it is

described that animals tend to be avid for these plants even when other feed is available. Tokarnia et al. (2012) interprets this avidity of the animals as a neurophysiological dependence due to neurological lesions, with loss of selective instinct and indiscriminate consumption of the plant. In poisoning by *S. carpinifolia*, ingestion was resumed even in areas with good feed availability, which demonstrates that cattle acquire the habit of ingesting the plant.

Deaths were recorded in cattle regarding poisoning by *S. fastigiatum*, *S. carpinifolia*, *P. angusta*, and *C. purpurea*. These deaths were associated with the lesions produced in the CNS, which are always present in poisoning by *S. carpinifolia* and *S. fastigiatum*. Some deaths observed in poisoning by *P. angusta* were always accidental, when cattle had access to steep areas. In the deaths caused by *Claviceps purpurea* in the neurological form, gross and microscopic lesions were not found, and no association with accidents was observed.

Outbreaks caused by ingestion of *P. angusta* have occurred from July to September. This plant presents an annual cycle and grows well in winter, when it is found invading other pastures, mainly of common oat, as well as in areas where maize or soybean had been cultivated in the summer. Thus, the diseases caused by this plant were observed only in winter and early spring. The onset of clinical signs may occur within the first days of plant ingestion, and their intensity depends on the soil conditions and moisture and stage of development of the plant (Gava et al. 1999), whereas in poisoning by *S. carpinifolia* and *S. fastigiatum*, the disease has occurred in different times of the year and, according to Riet-Correa et al. (1983b) and Furlan et al. (2008, 2009), clinical signs begin a few weeks after ingestion.

Poisoning by *C. paspali* was observed in cattle that ingested seeds of *Paspalum dilatatum* and/or *P. paniculatum* contaminated by the mycotoxin after the first days of introduction in the pasture. These outbreaks occurred in autumn, coinciding with periods of low rainfall with maturation of seeds of the genus *Paspalum* and proliferation of *C. paspali*. In the Southern Hemisphere, the described outbreaks have occurred in times of low rainfall between the end of February and the beginning of June (Riet-Correa et al. 1983a, Lopez et al. 1985, Botha et al. 1996, García et al. 2017).

The three outbreaks of nervous ergotism occurred in late spring, and the diagnoses were performed based on epidemiological data and clinical-pathological aspects. The cattle showed clinical signs while grazing ryegrass pasture (2 outbreaks) and being fed common oat with seeds contaminated by a fungus morphologically similar to *C. purpurea* in trough (1 outbreak). In Chile, cattle showed similar clinical signs after 30 days in a pasture of *Lolium perenne* and *Dactylis glomerata* contaminated by *C. purpurea* sclerotia (Poo & Araya 1989). The neurological or convulsive form is little observed in cattle, and occurs when consumption of *C. purpurea* sclerotia is relatively high (Clegg 1959).

Comparing the severity of the clinical condition manifested by the cattle, more severe clinical signs are verified in poisoning by *P. angusta*, often leading the animals to hurl themselves against obstacles. This explains why, in one of the outbreaks, due to the daily stalling of cattle, the animals had edemas of traumatic origin in the pectoral region. In poisoning by *S. fastigiatum*, the clinical signs were less severe than in poisoning by *P. angusta*; however, the clinical condition

worsened mainly when cattle had access to feed in the trough. This fact can be explained by Purkinje neuron lesion, which occurs in lysosomal storage diseases. As for tremorgenic disease caused by *C. paspali*, clinical signs ranged from mild to severe depending on the amount of *Paspalum* spp. in pasture.

In poisoning caused by *S. carpinifolia*, *S. fastigiatum*, *C. paspali*, and *C. purpurea* in the CNS, no gross lesions other than those resulting from traumas were observed. In *P. angusta* poisoning, the gross lesions observed included gray-greenish discoloration in thalamus and midbrain, which have also been previously reported by other authors (Gava et al. 1999, Sousa & Irigoyen 1999). In the present study, significant histological changes were observed in the CNS of bovines poisoned with *S. carpinifolia* and *S. fastigiatum*. Degeneration and vacuolization of the pericardium of neurons, especially of Purkinje cells of the cerebellum, are findings particularly common in lysosomal storage diseases (Riet-Correa et al. 1983b, Antoniassi et al. 2007, Barbosa et al. 2007, Dantas et al. 2007, Furlan et al. 2008, 2009). In both diseases, the cattle presented rarefaction and/or disappearance of Purkinje neurons, some with substitution by Bergmann cells - a finding that corroborates those of other studies and characterizes the chronicity of poisoning (Riet-Correa et al. 1983b, Rech et al. 2006, Furlan et al. 2008, 2009). In the present study, of all the bovines poisoned with *P. angusta*, only one animal showed pigmented brainstem neurons. According to Sousa & Irigoyen (1999) and Alden et al. (2014), poisoning by *P. angusta* and *P. aquatica* microscopically shows thin granular brownish pigment in the cytoplasm of neurons, mainly in the cerebellum and brainstem.

In western Santa Catarina state, invasive plants such as *Ipomoea* spp. are commonly observed in plantations of maize and other cultures, and this plant is described by farmers as palatable for cattle of the region. In the epidemiological study, the main suspicion was that poisoning was caused by the plant and/or seeds, because in the three outbreaks of unknown etiology, the bovines showed neurological clinical signs after ingesting a large amount of leaves, flowers and seeds of *Ipomoea* spp. Nevertheless, experimental administration did not result in clinical changes. In experiments conducted with ruminants using *I. sericophylla*, *I. riedellii*, *I. asarifolia*, and *I. carnea* subsp. *fistulosa*, the clinical signs were chronic, being observed at least one week after consumption (Barbosa et al. 2007, Araújo et al. 2008, Ríos et al. 2012).

For the diagnosis of neurological diseases in cattle associated with plant and/or mycotoxin poisoning, the geographical distribution and seasonality, severity of clinical signs, and presence of one of the plants and/or fungi responsible for these diseases should be considered. Clinical signs such as muscle tremors, ataxia, and alertness are also observed in poisoning by the *Aspergillus clavatus* mycotoxin (Loretto et al. 2003, Bezerra Junior et al. 2009); however, this disease has not yet been diagnosed in Santa Catarina state, and in none of the outbreaks analyzed in the present study was there consumption of brewery residues. In Brazil, other diseases produce neurological clinical signs in cattle, such as poisoning by *I. asarifolia* (Döbereiner et al. 1960), *I. carnea* subsp. *fistulosa* (Antoniassi et al. 2007), *I. riedellii*, *I. sericophylla* (Barbosa et al. 2007), *I. verbascoidea* (Lima et al. 2013), *S. paniculatum* (Garaná et al. 2011), and *Turbina cordata* (Dantas et al. 2007). In other countries, *Swainsonina*, *Oxitropis*, and *Astragalus* have been described as causing lysosomal storage

diseases (James et al. 1981, Smith 2006). These plants were not found in the farms of the present study. For differential diagnosis, gross and microscopic changes should also be considered. Blue-greenish coloration is observed only in poisoning by *P. angusta*, and cerebellar atrophy is found in poisoning by *S. fastigiatum* and *S. paniculatum*. Significant microscopic lesions occur in poisoning by *S. carpinifolia*, *S. fastigiatum*, *S. paniculatum*, *I. carnea* subsp. *fistulosa*, *I. riedelii*, *I. sericophylla*, *I. verbascoidea*, *S. paniculatum*, *Turbina cordata*, and *A. clavatus*, but are not observed in poisoning by *C. paspali*, *C. purpurea*, *P. angusta*, and *I. asarifolia*.

CONCLUSIONS

In Santa Catarina state, *Sida carpinifolia*, *Solanum fastigiatum*, *Phalaris angusta*, *Claviceps paspali*, and *Claviceps purpurea* are responsible for causing neurological diseases in cattle.

In experiments conducted with cattle, *Ipomoea triloba* and *Ipomoea indivisa* did not reproduce clinical signs.

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REFERENCES

- Alden R., Hackney B., Weston L.A. & Quinn J.C. 2014. Phalaris toxicoses in Australian livestock production systems: prevalence, aetiology and toxicology. *J. Toxins* 1(1):7.
- Antoniassi N.A.B., Ferreira E.V., Santos C.E.P., Arruda L.P., Campos J.L.E., Nakazato L. & Colodel E.M. 2007. Intoxicação espontânea por *Ipomoea carnea* subsp. *fistulosa* (Convolvulaceae) em bovinos no Pantanal Matogrossense. *Pesq. Vet. Bras.* 27(10):415-418. <<http://dx.doi.org/10.1590/S0100-736X2007001000005>>
- Araújo J.A.S., Riet-Correa F., Medeiros R.M.T., Soares M.P., Oliveira D.M. & Carvalho F.K.L. 2008. Intoxicação experimental por *Ipomoea asarifolia* (Convolvulaceae) em caprinos e ovinos. *Pesq. Vet. Bras.* 28(10):488-494. <<http://dx.doi.org/10.1590/S0100-736X2008001000008>>
- Armién A.G., Tokarnia C.H., Peixoto P.V. & Frese K. 2007. Spontaneous and experimental glycoprotein storage disease of goats induced by *Ipomoea carnea* subsp. *fistulosa* (Convolvulaceae). *Vet. Pathol.* 44(2):170-184. <<http://dx.doi.org/10.1354/vp.44-2-170>> <PMid:17317794>
- Barbosa R.C., Riet-Correa F., Lima E.F., Medeiros R.M.T., Guedes K.M.R., Gardner D.R., Molyneux R.J. & Melo L.E.H. 2007. Experimental swainsonine poisoning in goats ingesting *Ipomoea sericophylla* and *Ipomoea riedelii* (Convolvulaceae). *Pesq. Vet. Bras.* 27(10):409-414. <<http://dx.doi.org/10.1590/S0100-736X2007001000004>>
- Bassuino D.M., Konradt G., Bianchi M.V., Reis M.O., Pavarini S.P. & Driemeier D. 2017. Spontaneous poisoning by *Sida carpinifolia* (Malvaceae) in horses. *Pesq. Vet. Bras.* 37(9):926-930. <<http://dx.doi.org/10.1590/S0100-736X2017000900005>>
- Bezerra Junior P.S., Raymundo D.L., Spanemberg A., Corrêa A.M.R., Bangel Junior J.J., Ferreira L. & Driemeier D. 2009. Neurotoxicose em bovinos associada ao consumo de bagaço de malte contaminado por *Aspergillus clavatus*. *Pesq. Vet. Bras.* 29(3):220-228. <<http://dx.doi.org/10.1590/S0100-736X2009000300006>>
- Botha C.J., Kellerman T.S. & Fourie N. 1996. A tremorgenic mycotoxicosis in cattle caused by *Paspalum distichum* (L.) infected by *Claviceps paspali*. *J. S. Afr. Vet. Assoc.* 67(1):36-37. <PMid:8786617>
- Clegg F.G. 1959. A convulsive syndrome in sheep. *Vet. Rec.* 71:824-827.
- Colodel E.M., Driemeier D., Loretto A.P., Gimeno E.J., Traverso S.D., Seitz A.L. & Zlotowski P. 2002. Aspectos clínicos e patológicos da intoxicação por *Sida carpinifolia* (Malvaceae) em caprinos no Rio Grande do Sul. *Pesq. Vet. Bras.* 22(2):51-57. <<http://dx.doi.org/10.1590/S0100-736X2002000200004>>
- Dantas A.F.M., Riet-Correa F., Gardner D.R., Medeiros R.M.T., Barros S.S., Anjos B.L. & Lucena R.B. 2007. Swainsonine-induced lysosomal storage disease in goats caused by the ingestion of *Turbina cordata* in Northeastern Brazil. *Toxicon* 49(1):111-116. <<http://dx.doi.org/10.1016/j.toxicon.2006.08.012>> <PMid:17030054>
- Döbereiner J., Tokarnia C.H. & Canella C.F.C. 1960. Intoxicação experimental por "salsa" (*Ipomoea asarifolia* R. et Schult.) em ruminantes. *Arqs Inst. Biol. Anim.*, Rio de Janeiro, 3:39-57.
- Furlan F.H., Lucio J., Veronezi L.O., Traverso S.D. & Gava A. 2008. Intoxicação experimental por *Sida carpinifolia* (Malvaceae) em bovinos. *Pesq. Vet. Bras.* 28(1):57-62. <<http://dx.doi.org/10.1590/S0100-736X2008000100009>>
- Furlan F.H., Lucio J., Veronezi L.O., Medeiros A., Barros S.S., Traverso S.D. & Gava A. 2009. Spontaneous lysosomal storage disease caused by *Sida carpinifolia* (Malvaceae) poisoning in cattle. *Vet. Pathol.* 46(2):343-347. <<http://dx.doi.org/10.1354/vp.46-2-343>> <PMid:19261649>
- García J.A., Cantón G.J., García B.L., Micheloud J.F., Campero C.M., Späth E.J.A. & Odriozola E.R. 2017. Retrospective analysis of cattle poisoning in Argentina (2000-2013). *Pesq. Vet. Bras.* 37(3):210-214. <<http://dx.doi.org/10.1590/S0100-736X2017000300002>>
- Gava A., Sousa R.S., Deus M.S., Pilati C., Cristani J., Mori A. & Neves D.S. 1999. *Phalaris angusta* (Gramineae) como causa de enfermidade neurológica em bovinos no estado de Santa Catarina. *Pesq. Vet. Bras.* 19(1):35-38. <<http://dx.doi.org/10.1590/S0100-736X1999000100005>>
- Guaraná E.L.S., Riet-Correa F., Mendonça C.L., Medeiros R.M.T., Costa N.A. & Afonso J.A.B. 2011. Intoxicação por *Solanum paniculatum* (Solanaceae) em bovinos. *Pesq. Vet. Bras.* 31(1):59-64. <<http://dx.doi.org/10.1590/S0100-736X2011000100009>>
- James L.F., Hartley W.J. & Van-Kampen K.R. 1981. Syndromes of *Astragalus* poisoning in livestock. *J. Am. Vet. Med. Assoc.* 178(2):146-150. <PMid:7204239>
- Lima D.D.C.C., Albuquerque R.F., Rocha B.P., Barros M.E.G., Gardner D.R., Medeiros R.M.T., Riet-Correa F. & Mendonça F.S. 2013. Doença de depósito lisossomal induzida pelo consumo de *Ipomoea verbascoidea* (Convolvulaceae) em caprinos no semiárido de Pernambuco. *Pesq. Vet. Bras.* 33(7):867-872. <<http://dx.doi.org/10.1590/S0100-736X2013000700007>>
- Lopez T.A., Odriozola E. & Mutti G. 1985. Características de la intoxicación de bovinos con *Paspalum dilatatum* poir (pasto miel) contaminado con *Claviceps paspali* Stevens et Hall. *Revta Vet. Argentina* 3(29):863-870.
- Loretto A.P., Colodel E.M., Driemeier D., Corrêa A., Bangel Junior J.J. & Ferreira L. 2003. Neurological disorder in dairy cattle associated with consumption of beer residues contaminated with *Aspergillus clavatus*. *J. Vet. Diagn. Invest.* 15(2):123-132. <<http://dx.doi.org/10.1177/104063870301500206>> <PMid:12661722>
- Medeiros R.M., Barbosa R.C., Riet-Correa F., Lima E.F., Tabosa I.M., de Barros S.S., Gardner D.R. & Molyneux R.J. 2003. Tremorgenic syndrome in goats caused by *Ipomoea asarifolia* in northeastern Brazil. *Toxicon* 41(7):933-935. <[http://dx.doi.org/10.1016/S0041-0101\(03\)00044-8](http://dx.doi.org/10.1016/S0041-0101(03)00044-8)> <PMid:12782095>
- Pedroso P.M.O., Oliveira L.G.S., Cruz C.E.F., Soares M.P., Barreto L.R.B. & Driemeier D. 2010. Doença do armazenamento lisossomal induzida pelo consumo de *Sida carpinifolia* em bovinos do Rio Grande do Sul. *Pesq. Vet. Bras.* 30(10):833-838. <<http://dx.doi.org/10.1590/S0100-736X2010001000005>>
- Pessoa C.R.M., Medeiros R.M.T., Pessoa A.F.A., Dantas A.F.M., Oliveira O.F. & Riet-Correa F. 2010. Doença tremorgênica em ruminantes e equídeos no semiárido da Paraíba. *Pesq. Vet. Bras.* 30(7):541-546. <<http://dx.doi.org/10.1590/S0100-736X2010000700005>>
- Poo M.V. & Araya O. 1989. Ergotismo convulsivo en novillos debido a ingestión de gramíneas infectadas por *Claviceps purpurea*. *Arch. Med. Vet.* 21:66-68.

- Radostits O.M., Gay C.C., Blood D.C., Hinchcliff K.W. & Constable P.D. 2007. Veterinary Medicine. 10th ed. W.B. Saunders, London, p.1889-1912.
- Rech R.R., Rissi D.R., Rodrigues A., Pierezan F., Piazer J.V.M., Kommers G.D. & Barros C.S.L. 2006. Intoxicação por *Solanum fastigiatum* (Solanaceae) em bovinos: epidemiologia, sinais clínicos e morfometria das lesões cerebelares. Pesq. Vet. Bras. 26(3):183-189. <<http://dx.doi.org/10.1590/S0100-736X2006000300010>>
- Riet-Correa F., Schild A.L., Méndez M.C., Tavares A.S. & Rodrigues J.O. 1983a. Intoxicação por *Claviceps paspali* em bovinos no Rio Grande do Sul. Pesq. Vet. Bras. 3(2):59-65.
- Riet-Correa F., Mendez M.C., Schild A.L., Summers B.A. & Oliveira J.A. 1983b. Intoxication by *Solanum fastigiatum* var. *fastigiatum* as a cause of cerebelar degeneration in cattle. Cornell Vet. 73(3):240-256. <PMid:6411429>
- Ríos E.E., Cholic L.A., Gimeno E.J., Guidi M.G. & Acosta de Pérez O.C. 2012. Experimental poisoning of goats by *Ipomoea carnea* subsp. *fistulosa* in Argentina: a clinic and pathological correlation with special consideration on the central nervous system. Pesq. Vet. Bras. 32(1):37-42. <<http://dx.doi.org/10.1590/S0100-736X2012000100007>>
- Smith M.O. 2006. Doenças do sistema nervoso, p.872-1018. In: Smith B.P. (Ed.), Tratado de Medicina Interna de Grandes Animais. Manole, São Paulo.
- Sousa R.S. & Irigoyen L.F. 1999. Intoxicação experimental por *Phalaris angusta* (Gramineae) em bovinos. Pesq. Vet. Bras. 19(3/4):116-122. <<http://dx.doi.org/10.1590/S0100-736X1999000300005>>
- Tokarnia C.H., Brito M.F., Barbosa J.D., Peixoto P.V. & Döbereiner J. 2012. Plantas Tóxicas do Brasil para Animais de produção. 2ª ed. Helianthus, Rio de Janeiro, p.223-294.
- Tortelli F.P., Barbosa J.D., Oliveira C.M.C., Duarte M.D., Cerqueira V.D., Oliveira C.A., Correa F.R. & Correa G.R. 2008. Intoxicação por *Ipomoea asarifolia* em ovinos e bovinos na Ilha de Marajó. Pesq. Vet. Bras. 28(12):622-626. <<http://dx.doi.org/10.1590/S0100-736X2008001200011>>

High frequency of cutaneous squamous cell carcinoma in Friesian Milchschaaf sheep in Uruguay¹

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ABSTRACT.- Costa R.A., Schild C., Caffarena D., Giannitti F., Banchemo G., DelPino L. & Riet-Correa F. 2019. **High frequency of cutaneous squamous cell carcinoma in Friesian Milchschaaf sheep in Uruguay.** *Pesquisa Veterinária Brasileira* 39(4):251-254. Instituto Nacional de Investigación Agropecuaria, Estación Experimental INIA La Estanzuela, Ruta 50 Km 11, Colonia del Sacramento, Colonia, Uruguay. E-mail: frcorrea@inia.org.uy

This study described five cases of squamous cell carcinoma (SCC) in the pinnae, eyelids, and eyes of Friesian Milchschaaf sheep from two flocks raised under extensive conditions in Uruguay. The prevalence values for both flocks were 2.12% and 2.53%, respectively. Macroscopically, single or multiple, nodular, verrucous, or plaque-like neoplasms, with occasional ulceration and bleeding, were observed in the affected areas. Histologically, well- and moderately-differentiated SCCs were diagnosed in three and two cases, respectively. The Milchschaaf breed is native of Germany, and its individuals present white non-pigmented skin with the face and legs clear of fleece, thus adapted to areas with lower solar radiation. Additionally, in the German breeding systems, animals remain indoors for long periods. The increased exposure time to higher levels of solar radiation of the Uruguayan extensive production systems may be a predisposing factor for the development of SCC in this sheep breed. In Uruguay, there is more solar radiation and the production systems provide less protection against sunlight compared with the German production system. The high frequency of SCC should be considered prior to introducing European breeds with non-pigmented skin in Uruguay and other countries of the region.

INDEX TERMS: Cutaneous carcinoma, squamous cell carcinoma, Friesian Milchschaaf, sheep, Uruguay, tumors, neoplasm, sheep diseases, skin diseases, dermatopathology, pathology.

RESUMO.- [Alta frequência de carcinoma cutâneo de células escamosas em ovinos da raça Frisona Milchschaaf no Uruguai.] Descreveram-se carcinomas de células escamosas (CCE) nas orelhas, pálpebras e olhos em cinco ovinos da raça Frisona Milchschaaf em dois rebanhos de criação extensiva no Uruguai. A prevalência nos rebanhos foi de 2,12% e 2,53%. Macroscopicamente se observaram lesões focais ou multifocais, com aumento de volume, nodulares, verrucosas ou em placas, com ocasionais ulcerações e

hemorragias. Apresentavam aspecto verrucoso, por vezes com superfície ulcerada e firmes ao corte. De acordo com as características histológicas da neoplasia, as lesões foram classificadas em CCEs bem diferenciados em três casos e moderadamente diferenciados em dois casos. Esta raça é nativa da Alemanha, caracterizada pela face deslanada e pele branca despigmentada. Na Alemanha, há menores níveis de radiação solar do que no Uruguai e seu sistema de produção é mais intensivo, com permanência dos animais confinados durante longos períodos. O aumento do tempo de exposição a níveis mais elevados de radiação solar pode ser um fator predisponente para o desenvolvimento de CCEs nesta raça em sistemas extensivos de produção no Uruguai. Neste País há mais radiação solar e os sistemas produtivos fornecem menos proteção contra a luz solar, em relação ao sistema de produção alemão. A alta frequência de CCE deveria ser levada em consideração para quem decide introduzir no Uruguai e outros países da região, raças europeias com a face despigmentada.

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TERMOS DE INDEXAÇÃO: Carcinoma cutâneo, células escamosas, ovinos, Frisona Milchschaft, Uruguai, tumores, neoplasma, doenças de ovinos, doenças de pele, dermatopatologia, patologia.

INTRODUCTION

Squamous cell carcinomas (SCCs) are malignant neoplasms of epithelial origin with keratinocyte differentiation (Ramos et al. 2007, Goldschmidt & Goldschmidt 2016). They are most common in horses, cattle, dogs and cats, uncommon in sheep, and rare in goats (Barbosa et al. 2009). Although these neoplasms present higher incidence in senile animals, they may occur in young animals, especially when associated with other risk factors such as exposure of areas with poor pigmentation and little hair to ultraviolet (UV) rays (Goldschmidt & Goldschmidt 2016, Mauldin & Peters-Kennedy 2015). *Ovis aries* Papillomavirus 3 infection (Alberti et al. 2010, Vitiello et al. 2017) as well as traumatic skin lesions (Goldschmidt & Goldschmidt 2016) may also be risk factors for the emergence of neoplasms in sheep.

Although SCC can develop in any anatomical region of the skin, it is predominant in mucocutaneous junctions such as eyelids, conjunctiva, vulva, and perineum in cattle (Fernandes 2007, Ramos et al. 2007, Carvalho et al. 2014, Mauldin & Peters-Kennedy 2015), whereas the face is the most commonly affected region in sheep, especially the pinna (Mauldin & Peters-Kennedy 2015), followed by the muzzle, lips, eyelids, and eyes (Ramos et al. 2007, Carvalho et al. 2014). Traumatic cutaneous lesions may also be a risk factor for the emergence of neoplasms in sheep (Goldschmidt & Goldschmidt 2016), and the diagnosis should be based on histology.

This study aimed to report the occurrence of SCC in Friesian Milchschaft sheep from two flocks in Uruguay and discuss the possible causes of the high prevalence of this disease in some sheep breeds.

MATERIALS AND METHODS

During technical visits to the production farms, all the sheep under study were clinically assessed and epidemiological data were collected. Biopsy was performed in the affected regions of two animals and three other sheep were necropsied. The samples were fixed in 10% formalin, dehydrated, paraffin embedded, cut into 4-5µm sections, and stained using the hematoxylin-eosin (HE) technique.

Ultraviolet (UV) radiation in the region of origin of this breed - East and North Friesland (53°N), Germany, were compared with those recorded in La Estanzuela (34°S), Colonia, and Rincón del Colorado (34°S), Canelones, in Uruguay, which are areas located near the farms where the cases occurred. The World Health Organization (WHO 2017) UV classification scale, which ranges from 1 to 12 according to the levels of UV radiation in various capital cities of the world, was used. According to this scale, UV radiation levels are classified as low (1 and 2), moderate (3, 4 and 5), high (6 and 7), very high (8, 9 and 10), and extreme (>10). The latitudes 34°S and 52°N were used as reference to compare La Estanzuela and Rincón del Colorado, in Uruguay, and East and North Friesland, in Germany.

RESULTS

The first farm, located in La Estanzuela, Colonia (34°47'13"S, 57°84'41"O), was visited in July 2015. In this property, 481 adult sheep (282 Texel, 80 Finnish Landrace, and 79 Friesian Milchschaft, and their respective crosses) were raised in an

area of 80 ha, with moderate shade cover from native or planted trees mainly distributed near the fences between the paddocks. During 2013 and 2014, the flock was composed of approximately 600 sheep, and 15 deaths associated with tumors on the face were recorded in the Friesian Milchschaft and Finnish Landrace breeds.

In the second farm, located in Rincón del Colorado, Canelones (34°69'20"S 56°37'74"O), the technical visit occurred in November 2017. The farm held 312 sheep, approximately 94 Friesian Milchschaft and 218 Finnish Landrace, grazing in an area of 120 ha with little shade cover not available in every paddock. The shade was provided by eucalyptus trees usually located near the fences. The animals grazed on improved pasture and received feed supplement before lambing. From 2013 to 2017, 18 deaths associated with tumors on the face were registered in the Friesian Milchschaft and Finnish Landrace breeds.

During flock inspection in the first property, tumor lesions located in the eyelids infiltrating into the eyes were identified in two (2.53%) Friesian Milchschaft sheep aged 2½ and 3 years, whereas herd inspection in the second farm identified similar lesions in three animals (2.12%) of this same breed aged 4 to 6 years: two of them showed neoplasms in the pinna and one in the eye and eyelid. The animals with eyelid and ocular lesions showed abundant lacrimation, apathy, and weight loss.

The tumors were locally infiltrative, nodular, approximately 2-3 cm in diameter, verrucous, firm, with dark surface, and whitish on cut section, with multifocal, yellowish granules. In the sheep with eyelid and/or ocular lesions, crusty epidermal proliferation was observed along the outer surface of the eyelids. Some of the sheep showed cavitory, plaque-like lesions (Fig.1A), and one ewe had intratumoral myiasis in the eye (Fig.1B). The eyelid and/or ocular masses were ulcerative, bled easily, and had deformed the upper and lower eyelids.

Microscopically, the neoplasms were classified as moderately-differentiated SCCs in one tumor affecting the eyelid and the eye (first property) and one tumor affecting the pinna (second property); the other three cases were defined as well-differentiated SCCs.

In the well-differentiated SCCs, multifocal-to-coalescing areas of concentrically arranged lamellar large keratin islands (keratin pearls) were observed, which were often associated with infiltrates of viable and degenerate neutrophils. The lesion was infiltrating, poorly delimited and nonencapsulated. The neoplastic cells were arranged in nests and supported by moderate fibrovascular stroma (Fig.1C). The cells were polygonal with partially distinct borders, prominent intercellular bridges, abundant homogeneous eosinophilic cytoplasm, with round-to-oval, central to paracentral nucleus and coarse basophilic chromatin with one to two prominent nucleoli. Mild anisocytosis and anisokaryosis was observed. The more superficial layer of the neoplastic mass showed multifocal areas of ulceration, hemorrhage, and neutrophilic infiltrate.

In the tumors classified as moderately-differentiated SCCs, a densely cellular, infiltrating, nonencapsulated mass was observed. The neoplastic cells were arranged in cords and nests supported by moderate fibrovascular stroma. The cells showed polygonal to spindle shape with partially distinct borders, moderate amount of homogeneous eosinophilic cytoplasm, round to oval central nucleus with coarse or

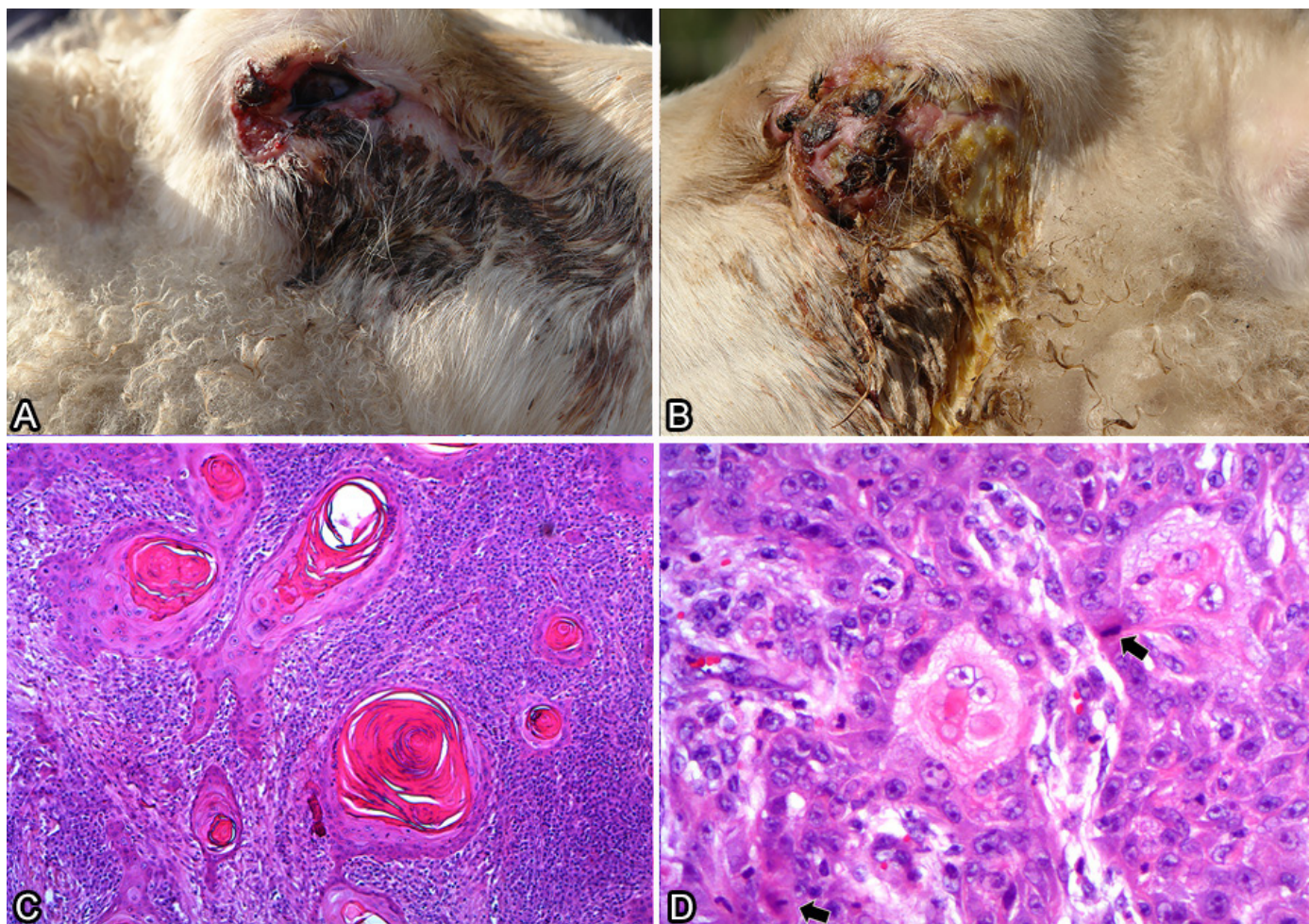


Fig.1 (A) Cavitory, plaque-like neoplasm in the lower eyelid. Ovine cutaneous squamous cell carcinoma. (B) Neoplastic proliferation in the eyelid with invasion of the eyeball and intratumoral myiasis. Ovine cutaneous squamous cell carcinoma. (C) Multiple keratin pearls surrounded by keratinocytes in the middle of the neoplastic tissue and abundant inflammatory infiltrate. Well-differentiated ovine cutaneous squamous cell carcinoma. HE, obj.5x. (D) Neoplastic cells of irregular arrangement, with occasional keratin formations, and frequent mitoses (arrows). Moderately-differentiated ovine cutaneous squamous cell carcinoma. HE, obj.20x.

vesicular chromatin, and one to two nucleoli; although cells with 3-5 nucleoli were occasionally observed. Rare keratin pearls, multinucleated cells, and individual keratinization were also observed (Fig.1D). Anisocytosis and anisokaryosis were moderate and there were large numbers of mitotic figures. In addition, multifocal neutrophilic inflammatory infiltrate and superficial ulcerated bleeding areas were found.

DISCUSSION

The diagnoses of SCCs were based on macroscopic and histological examination of the lesions in all cases, which were similar to those described by other authors in different sheep breeds such as Santa Inês and their crosses, Bergamacia (Macêdo et al. 2008), Corriedale, Ideal, and Ile de France (Ramos et al. 2007). Cases involving the Friesian Milchschaaf breed have also been reported in Uruguay (García et al. 2018).

These neoplasms can emerge anywhere in the animal's body, but they predominate in non-pigmented areas clear of fleece where there is less protection from UV rays. Sheep of the Milchschaaf breed have the head clear of fleece, the face

is covered by pink whitish skin with no dark pigmentation and thin hair (ARCO 2017).

Most of the neoplastic masses were macroscopically classified as nodular tumors; however, some animals showed plaque-like neoplastic lesions. The tumors assessed were verrucous and papillary, resembled a cauliflower, and bled easily, and the plaque-like lesions were characterized mainly by the presence of ulcerated, cavitory areas (Fernandes 2007, Ramos et al. 2007, Goldschmidt & Goldschmidt 2016).

In order to categorize the SCCs according to their differentiation, several histological characteristics were evaluated based on the classification criteria described by Goldschmidt & Goldschmidt (2016), which classify these neoplasms as well-differentiated, moderately-differentiated and poorly-differentiated. Because keratinocytes are the cellular origin of this neoplasm, it is mainly characterized by keratin formation. Well-differentiated tumors produce large amounts of keratin, deposited in the form of concentric islands, known as keratin pearls, and well-differentiated cellular bridges. In moderately-differentiated lesions, keratin pearls are scarce, and their intercellular bridges are more difficult

to identify. In poorly-differentiated neoplasms, individual cell keratinization is observed. In addition to the keratinocyte characteristics, other histological findings associated with SCC are squamous differentiation, cellular pleomorphism, mitotic activity, and invasiveness.

Solar radiation is the sum of visible light plus ultraviolet (UV) radiation. Approximately 95% of UV radiation is UVA and 5% of it is UVB; the latter being more efficient in causing neoplasms. Severity of solar radiation depends on several factors, namely, exposure time; reflection of the Sun's rays on the surface (sand, snow, water, etc.); meteorological conditions; altitude and latitude (Young 2009). UV radiation causes mutations in the p53 gene, which is an important regulator of cell proliferation and growth, involved in carcinogenesis (Bruins et al. 2004). According to the classification of the World Health Organization (WHO 2017) for latitude 52°N (a region close to the East and North Friesland), the incidence of UV rays ranges from 2 to 5 (low-to-moderate level) in spring, and 3 to 7 (moderate-to-high level) in summer, whereas the levels for latitude 34°S (La Estanzuela and Rincón del Colorado) are 5 to 9 (moderate to very high in spring, and 7 to 10 (high to very high) in summer. The differences observed in the UV radiation levels indicate a greater potential for damage to the skin and conjunctiva, sites where the tumors occurred, at latitude 34°S than at the place of origin of the breed (latitude 52°N).

The high relative prevalence of tumors in Milchschaaf sheep in Uruguay is possibly associated with poor adaptation of these animals to the local climate and breeding system. In Germany, where this breed was developed, sheep are raised under intensive or semi-intensive system, for dairy production, in humid temperate climate, and remain confined for long periods. In contrast, in Uruguay, they are bred under extensive systems, intended for meat production, in temperate climate, and subjected to higher UV levels. These factors potentially intensify the exposure of animals to UV rays, which together with the morphological characteristics of the Milchschaaf breed (white, non-pigmented skin and face clear of fleece), increased the frequency of neoplasms compared with other breeds in Uruguay (García et al. 2018).

A study conducted in Rio Grande do Sul state, Brazil, described high prevalence (9.1%) of SCC in the perineum in a flock of Ile de France sheep (Schild et al. 1995). The authors suggest that this high prevalence was due to the greater susceptibility of this breed to the disease associated with several factors, including lack of skin pigmentation and mucocutaneous junction of the vulva, small amount of fleece in the perineum, and very short sectioning of the tail (Schild et al. 1995). In this case, the higher susceptibility to the disease could also be related to the fact that the Ile de France breed comes from regions with lower levels of radiation incidence than Rio Grande do Sul state.

CONCLUSIONS

The high prevalence of squamous cell carcinoma (SCC) should be considered by farmers intending to introduce European sheep breeds with non-pigmented, clear of fleece skin in Uruguay and other countries in the region.

Reducing exposure of animals to solar radiation, providing larger shaded areas, and crossing them with pigmented breeds could be appropriate measures to reduce the risk of SCC.

REFERENCES

- Alberti A., Pirino S., Pintore F., Addis M.F., Chessa B., Cacciotto C., Cubeddu T., Anfossi A., Benenati G., Coradduzza E., Lecis R., Antuofermo E., Carcangiu L. & Pittau M. 2010. *Ovis aries* papillomavirus 3: a prototype of a novel genus in the family Papillomaviridae associated with ovine squamous cell carcinoma. *Virology* 407(2):352-359. <<http://dx.doi.org/10.1016/j.virol.2010.08.034>> <PMid:20863546>
- ARCO 2017. Padrão Racial East Friesian. Associação Brasileira de Criadores de Ovinos, Bagé. Available at <<http://www.arcoovinos.com.br/index.php/mn-srgo/mn-padroesraciais/46-east-friesian>> Accessed on Nov. 15, 2017.
- Barbosa J.D., Duarte M.D., Oliveira C.M.C., Reis A.B., Peixoto T.C., Brito M.F. & Peixoto P.V. 2009. Carcinoma de células escamosas perineal em cabras no Pará. *Pesq. Vet. Bras.* 29(5):421-427
- Bruins W., Zwart E., Attardi L.D., Iwakuma T., Hoogervorst E.M., Beems R.B., Miranda B., Oostrom C.T., Berg J., Aardweg G.J., Lozano G., Steeg H., Jacks T. & Vries A. 2004. Increased sensitivity to UV radiation in mice with a p53 point mutation at Ser389. *Mol. Cell. Biol.* 24(20):8884-8894. <<http://dx.doi.org/10.1128/MCB.24.20.8884-8894.2004>> <PMid:15456863>
- Carvalho F.K.L., Dantas A.F., Riet-Correa F., Andrade R.L., Nóbrega Neto P.I., Miranda Neto E.G., Simoes S.V.D. & Azevedo S.S. 2014. Estudo retrospectivo das neoplasias em ruminantes e equídeos no semiárido do Nordeste Brasileiro. *Pesq. Vet. Bras.* 34(3):211-216. <<http://dx.doi.org/10.1590/S0100-736X2014000300003>>
- Fernandes C.G. 2007. Neoplasmas em ruminantes e equinos, p.650-656. In: Riet-Correa F., Schild A.L., Lemos R.A.A. & Borges J.R.J. (Eds), *Doenças de Ruminantes e Equídeos*. Vol.2. 3ª ed. Pallotti, Santa Maria.
- García J.A., Quinteros C., Romero A. & Dutra F. 2018. Occurrence of squamous cell carcinoma in Milchschaaf sheep in Uruguay. *Ciência Rural* 48(1):e20170406. <<http://dx.doi.org/10.1590/0103-8478cr20170406>>
- Goldschmidt M.H. & Goldschmidt K.H. 2016. Epithelial and melanocytic tumors of skin, p.88-141. In: Meuten D.J. (Ed.), *Tumors in Domestic Animals*. 5th ed. John Wiley and Sons, Ames, Iowa. <<http://dx.doi.org/10.1002/9781119181200.ch4>>
- Macêdo J.T., Riet-Correa F., Dantas A.F.M. & Simões S.V.D. 2008. Doenças da pele em caprinos e ovinos no semi-árido brasileiro. *Pesq. Vet. Bras.* 28(12):633-642. <<http://dx.doi.org/10.1590/S0100-736X2008001200013>>
- Mauldin E.A. & Peters-Kennedy J. 2015. Integumentary system, p.509-736. In: Maxie G. (Ed.), *Jubb, Kennedy and Palmer's Pathology of Domestic Animals*. Vol.1. 6th ed. W.B. Saunders, Philadelphia.
- Ramos A.T., Norte D.M., Elias F. & Fernandes C.G. 2007. Carcinoma de células escamosas em bovinos, ovinos e equinos: estudo de 50 casos no sul do Rio Grande do Sul. *Braz. J. Vet. Res. Anim. Sci.* 44(Suppl.):5-13. <<http://dx.doi.org/10.11606/issn.1678-4456.bjvras.2007.26583>>
- Schild A.L., Riet-Correa F., Mendez M.C. & Ferreira J.L.M. 1995. Boletim do Laboratório Regional de Diagnóstico. Número 15, Gráfica Universitária, Pelotas, RS, p.18-20.
- Vitiello V., Burrai G.P., Agus M., Anfossi A.G., Alberti A., Antuofermo E., Rocca S., Cubeddu T. & Pirino S. 2017. *Ovis aries* papillomavirus 3 in ovine cutaneous squamous cell carcinoma. *Vet. Pathol.* 54(5):775-782. <<http://dx.doi.org/10.1177/0300985817705171>> <PMid:28494708>
- WHO 2017. Ultraviolet Radiation (UV). World Health Organization, Geneva. Available at <http://www.who.int/uv/intersunprogramme/activities/uv_index/en/> Accessed on Dec. 11, 2017.
- Young C. 2009. Solar ultraviolet radiation and skin cancer. *Occup. Med.* 59(2):82-88. <<http://dx.doi.org/10.1093/occmed/kqn170>> <PMid:19233827>

Diagnosis of canine leptospirosis: evaluation of two PCR assays in comparison with the microagglutination test¹

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ABSTRACT.- Martin P.L., Stanchi N.O., Brihuega B.F., Bonzo E., Galli L. & Arauz M.S. 2019. **Diagnosis of canine leptospirosis: evaluation of two PCR assays in comparison with the microagglutination test.** *Pesquisa Veterinária Brasileira* 39(4):255-262. Servicio Central de Laboratorio, Hospital Escuela, Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata, Av. 60 y 118, CC 296, La Plata, CP 1900, Buenos Aires, Argentina. E-mail: plmartin@fcv.unl.edu.ar

Canine leptospirosis is definitely diagnosed by demonstrating seroconversion in paired serum samples from the acute and convalescent period by the microagglutination test (MAT). However, the application of a polymerase chain reaction (PCR) assay can provide earlier confirmation of suspected cases. The objective of this study was to evaluate two PCR assays used in diagnosis of human leptospirosis (*lipL32* real-time PCR and *rrs* conventional PCR) in cultured microorganisms and experimentally contaminated samples (whole blood, serum, urine), and investigate their applicability in clinical samples from dogs with presumptive diagnosis of leptospirosis by using the MAT as a reference. The analytical sensitivity of the *lipL32* real-time PCR was 1 genome equivalent per reaction, whereas that for the *rrs* conventional PCR was 10 genome equivalents per reaction. Both assays amplified the pathogenic strains but were negative when evaluating the DNA of other microorganisms that may be present in clinical samples. The *lipL32* real-time PCR detected 100 bacteria/mL in whole blood samples, 1000 bacteria/mL in serum samples and 10 bacteria/mL in urine samples, whereas the *rrs* conventional PCR detected 1000 bacteria/mL in whole blood and serum samples and 100 bacteria/mL in urine samples. Seven out of the 51 samples from dogs with presumptive diagnosis of leptospirosis were considered as confirmed cases. The *lipL32* real-time PCR detected positive results in six of the seven confirmed cases, whereas the *rrs* conventional PCR detected four. The PCR assays evaluated proved to be useful diagnostic tools in the confirmation of canine leptospirosis when used together with the MAT.

INDEX TERMS: Canine leptospirosis, PCR, microagglutination test, diagnosis, leptospirosis, dogs, bacterioses.

RESUMO.- [Diagnóstico de leptospirose canina: avaliação de dois ensaios de PCR em comparação com o teste de microaglutinação.] O diagnóstico definitivo da leptospirose canina é geralmente realizado demonstrando a seroconversão

em amostras do paciente no período agudo e de convalescença por serologia. No entanto, a aplicação de técnicas de PCR pode contribuir para a confirmação de casos suspeitos num período de tempo mais curto. O objetivo deste estudo foi avaliar dois ensaios de PCR publicados em humanos (PCR-*lipL32* em tempo real e PCR-*rrs* convencional) em culturas puras e em amostras de sangue com anticoagulante, soro e urina experimentalmente contaminados. Posteriormente, investigamos a utilidade de ambos os ensaios de PCR em amostras clínicas de cães com suspeita de leptospirose tomando a técnica de microaglutinação (MAT) como referência. A sensibilidade analítica foi de 1 e 10 genoma equivalente por reação para PCR-*lipL32* em tempo real e para PCR-*rrs* convencional, respectivamente. Ambos os ensaios amplificaram

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corretamente as 14 estirpes patogênicas, mas foram negativos para avaliar o ADN de outros microrganismos que poderiam estar presentes em amostras clínicas. Em nas amostras experimentalmente contaminadas PCR-*LipL32* em tempo real detectou 100 bactérias/mL em sangue total, 1000 bactérias/mL em soro e 10 bactérias/mL em urina. Enquanto o PCR-*rrs* convencional detectou 1000 bactérias/mL em sangue total e soro e 100 bactérias/mL na urina. Dos 51 cães suspeitos, sete foram considerados casos confirmados pela MAT. O PCR-*lipL32* em tempo real detectou seis dos sete casos confirmados, enquanto o PCR-*rrs* convencional foi positivo em quatro deles. As técnicas de PCR avaliadas provaram ser uma ferramenta de diagnóstico útil na confirmação de casos clínicos caninos quando utilizados em conjunto com a técnica MAT.

TERMOS DE INDEXAÇÃO: Leptospirose canina, PCR, teste de microaglutinação, caninos, diagnóstico, reação em cadeia da polimerase, bacterioses.

INTRODUCTION

Leptospirosis is a zoonotic disease caused by pathogenic species of the genus *Leptospira*, which are transmitted directly or indirectly from animals to humans. Canine leptospirosis has been associated mainly with the serovars Canicola and Icterohaemorrhagiae, but may also be associated with a wide range of serovars or genotypes (Rojas et al. 2010, Sykes et al. 2011, Löffler et al. 2014). The clinical presentation of acute canine leptospirosis can range from mild to severe, partly depending on the infecting serovar (Brihuega & Martiarena 1996, Townsend et al. 2006, Kohn et al. 2010). The resolution of acute infection may lead to asymptomatic shedding. In addition, clinically normal dogs can be chronic carriers of infection, shedding *Leptospira* spp. to the environment (Harkin et al. 2003b). Early and accurate diagnosis of suspected cases in dogs is essential to alter the course of the disease. Therefore, validated diagnostic tools should be available to confirm a clinical suspicion (Fraune et al. 2013).

Definitive diagnosis of leptospirosis in dogs is currently achieved either by isolation of the bacterium from tissue or body fluids or by the use of the microagglutination test (MAT). The isolation of *Leptospira* spp. provides proof of infection, but culturing may take months and is not suitable to detect acute disease (Ahmed et al. 2012). On the other hand, the MAT requires the maintenance of a panel of live cultured microorganisms and the use of samples from the acute and convalescent period to confirm the diagnosis (Faine 1982). In contrast with these methods, polymerase chain reaction (PCR) assays have revolutionized the diagnosis of different diseases due to their sensitivity, making isolation and culture of the infecting microorganism not often necessary for a confirmatory result. Conventional or real-time PCR assays previously studied to detect *Leptospira* spp. target genes common to all *Leptospira* spp., including the *rrs*, *gyrB*, *flaB* and *secY* genes, or pathogen-specific genes, including the *lipL32*, *ligA* and *ligB* genes (Mérien et al. 1992, Levett et al. 2005, Palaniappan et al. 2005, Slack et al. 2006, Ahmed et al. 2009, 2012, Rojas et al. 2010, Monte et al. 2012, Koizumi et al. 2013, Xu et al. 2014). The advantage of a conventional PCR assay based on the detection of the *rrs* gene is its low cost, which allows using it in routine clinical diagnosis. In addition, the use of these primers increases the analytical sensitivity

of the assay (Mérien et al. 1992, 2005) because the *rrs* gene has double copies on the chromosome of *Leptospira* species (Nascimento et al. 2004). On the other hand, the real-time PCR assay based on the detection of the *lipL32* gene provides a specific method to detect pathogenic *Leptospira* and has several advantages compared to the conventional PCR assay, including a shorter turnaround time, increased specificity when 5' nuclease assay probes are used, as well as a substantially less likelihood of cross-contamination. Both the *lipL32* real-time PCR and the *rrs* conventional PCR assays have been demonstrated to be suitable tools to confirm clinical diagnosis of human leptospirosis (Levett et al. 2005, Stoddard et al. 2009, Villumsen et al. 2012). However, PCR assays standardized to be used in human clinical samples might not have the same performance when applied to dog samples (Schuller et al. 2015).

In dogs, these assays have been used to monitor renal colonization in experimental infection (Ahmed et al. 2012), to evaluate urinary shedding of *Leptospira* spp. in dogs (Harkin et al. 2003b, Rojas et al. 2010, Fink et al. 2015, Gentilini et al. 2015), and to detect *Leptospira* spp. in suspected patients (Harkin et al. 2003a, Fraune et al. 2013, Koizumi et al. 2013, Xu et al. 2014, Recavarren et al. 2015). Nevertheless, there is limited published information regarding the analytical sensitivity of conventional and real-time PCR assays in whole blood, serum and urine experimentally contaminated samples (Branger et al. 2005), the incorporation of internal amplification controls in the PCR assays (Fink et al. 2015, Recavarren et al. 2015) and the evaluation of different PCR assays using canine whole blood, serum and urine samples from suspected cases in comparison with the MAT (Xu et al. 2014, Fink et al. 2015). Moreover, the utility of the PCR assays in diagnosis of suspected dogs is still under debate (Harkin et al. 2003a, Fraune et al. 2013). Based on the above, the objectives of this study were to evaluate the *lipL32* real-time PCR and the *rrs* conventional PCR with reference strains and experimentally contaminated samples and investigate their applicability in the diagnosis of leptospirosis in comparison with the MAT as a reference method, in whole blood, serum and urine samples from dogs with presumptive diagnosis of leptospirosis.

MATERIALS AND METHODS

Bacterial strains. Fifteen *Leptospira* strains (Table 1) and 16 other microorganisms (*Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli*, *Enterobacter aerogenes*, *Shigella dysenteriae*, *Proteus vulgaris*, *Proteus mirabilis*, *Morganella morganii*, *Staphylococcus aureus*, *S. pseudointermedius*, *S. epidermidis*, *Streptococcus canis*, *Ehrlichia canis*, *Borrelia burgdorferi*, *Candida albicans* and *Hepatozoon canis*) were included in this study. The *Leptospira* strains were obtained from the collection of the Instituto Nacional de Tecnología Agropecuaria de Castelar (Argentina), and maintained in leptospire-specific medium (Fletcher Medium Base, BD Difco, France) at 28°C. Subcultures in leptospire-specific Ellinghausen-McCullough-Johnson-Harris (EMJH) medium (Leptospira Medium Base EMJH, BD Difco, France) were incubated at 28°C for 7 days before DNA extraction. The genomic DNA from the other microorganisms tested was gifted by colleagues from the Instituto de Zoonosis Luis Pasteur (Buenos Aires, Argentina), Instituto Malbrán (Buenos Aires, Argentina), and Laboratorio de Microbiología of the Facultad de Ciencias Veterinarias of the Universidad Nacional de La

Plata (La Plata, Argentina). The non-*Leptospira* strains included in this study can be present in clinical samples (whole blood, serum and urine) and may cause unexpected cross-reactivity (nonspecific amplification). They were selected based on previous studies (Rojas et al. 2010, Gentilini et al. 2015).

DNA extraction. The DNA from *Leptospira* strains was extracted using a QIAamp DNA minikit (QIAGEN, Germany) according to the manufacturer's instructions. DNA concentration was determined by NanoVue Plus Spectrophotometer (GE Healthcare, USA). The number of Genomic Equivalents (GE), assuming one genome copy per *Leptospira*, was estimated based on a genomic size of 4.659 Mb (Ren et al. 2003, Nascimento et al. 2004, Miotto et al. 2017).

lipL32 real-time PCR assay. The *lipL32* real-time PCR assay to detect pathogenic *Leptospira* was conducted according to the method described by Villumsen et al. (2012), with some modifications. The probe was labeled with 6-carboxy-fluorescein (FAM) at the 5' end and Iowa Black FQ (IBFQ) at the 3' end (Integrated DNA Technologies, USA). The probe incorporates a new internal ZEN quencher that allows producing double-quenched probes with less background and more signal. The final volume of the assay was 25 µL and consisted of 1X of KAPPA PROBE FAST qPCR Master Mix (2X) ABI Prism (KAPA Biosystems, MA, USA) 900 nM of each primer (*lipL32Fw* and *lipL32Rv*), 250 nM of probe and 5 µL of template DNA. The sequences of these primers and probe are shown in Table 2. The following conditions were used for amplification: 95°C for 3 minutes, 40 cycles at 95°C for 15 seconds, 60°C for 1 minute.

Table 1. *Leptospira* strains used in this study

Species	Serogroup	Serovar	Strain
Pathogenic			
<i>L. interrogans</i>	Canicola	Canicola	Hond Utrecht IV
	Icterohaemorrhagiae	Copenhageni	M20
	Pomona	Pomona	Pomona
	Pyrogenes	Pyrogenes	Salinem
	Pomona	Pujato	Pujato
	Sejroe	Hardjo	Hardjoprajitno
	Sejroe	Wolffi	3705
	Sejroe	Sejroe	M84
<i>L. borgpetersenii</i>	Ballum	Castellonis	Castellon 3
<i>L. borgpetersenii</i>	Tarassovi	Tarassovi	Perepelitsin
<i>L. noguchii</i>	Pomona	Proechimys	1161 U
<i>L. weilii</i>	Sarmin	Sarmin	Sarmin
<i>L. kirschneri</i>	Grippotyphosa	Grippotyphosa	Moskva V
Non-pathogenic			
<i>L. meyeri</i>	Ranarum	Ranarum	ICF
<i>L. biflexa</i>	Semarang	Patoc	Patoc I

The *lipL32* real-time PCR assay was performed in a StepOne Plus Real-Time PCR System (Applied Biosystems, USA) and included a TaqMan Exogenous Internal Positive Control (Applied Biosystems, USA) to assess inhibition or suboptimal reaction conditions. The Internal Positive Control (IPC) included in this study was a heterologous target that included the primers and IPC-probe labeled with VIC® at the 5' end and TAMRA™ Quencher at the 3' end. Ultra-pure water and DNA from *Leptospira interrogans* serovar Canicola were used as negative and positive controls, respectively. Moreover, a DNA negative extraction control was included.

rrs conventional PCR assay. The *rrs* conventional PCR assay to detect *Leptospira* spp. was genus-specific and targeted to the *rrs* gene of *Leptospira* spp. We used a protocol published previously by Mérien et al. (1992), with minor modifications. The reaction mixture was prepared in a volume of 25 µL, containing 200 µM of each deoxynucleotide triphosphate, 2 mM MgCl₂, 1 µM of each primer (A and B), 1 U Taq DNA Polymerase (Productos Bio-Lógicos, Argentina), and 5 µL of template DNA. The sequences of these primers are shown in Table 2. Ultra-pure water and DNA from *L. interrogans* serovar Canicola were used as negative and positive controls, respectively. Moreover, a DNA negative extraction control was included. The *rrs* conventional PCR assay was performed in a Bio-Rad MyCycler Thermal Cycler PCR (Bio-Rad, CA, USA) with an initial denaturation at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 63°C for 60 seconds, extension at 72°C for 60 seconds, and final extension at 72°C for 10 minutes. The amplified products were electrophoresed on a 2% agarose gel in Tris-Borate-EDTA buffer 1× (89 mM Tris, 89 mM of boric acid, 2 mM EDTA, pH 8.3) containing 1 µg/mL of ethidium bromide for 60 minutes at 90 V. The bands were visualized under ultraviolet light (296 nm) and recorded using a Doc-It LS Image Acquisition and Analysis Software (UVP, Upland, CA, USA).

Evaluation of PCR assays with DNA samples of the reference *Leptospira* strains. To assess the analytical specificity of both PCR assays, DNA samples of the reference *Leptospira* strains (Table 1) as well as of the 16 other clinical important microorganisms were subjected to both PCR assays. To investigate the analytical sensitivity of both PCR assays, the DNA from *L. interrogans* Canicola Hond Utrecht IV and Copenhageni M20 was standardized to an initial concentration of 1×10^6 GE/reaction, to perform serial 10-fold dilutions in a DNA buffer consisting of Tris-EDTA buffer (10 mM Tris pH 8.0, 1 mM EDTA) until reaching 1×10^0 GE/reaction. All PCRs were run in triplicate. For *lipL32* PCR real-time, the dynamic range, R² coefficient and amplification efficiency for each strain were determined from the standard curve.

Evaluation of PCR assays in spiked whole blood, serum and urine samples. To evaluate the sensitivity of both PCR assays in biological samples, whole blood, serum and urine samples were spiked with the *L. interrogans* serovar Canicola strain Hond Utrecht IV and subjected to amplification. The concentration of *Leptospira* spp.

Table 2. Primers and probe used in this study

Primers or probe	Sequence (5'3')	Reference
<i>lipL32Fw</i>	AGAGGTCTTTACAGAATTTCTTCTACTACCT	Villumsen et al. 2012
<i>lipL32Rv</i>	TGGGAAAAGCAGACCAACAGA	
<i>lipL32PFAM-ZEN-IBFQ</i>	AAGTGAAAGGATCTTTCGTTGC	
Primer A	GGCGGCGCGTCTTAAACATG	Mérien et al. 1992
Primer B	TTCCCCCATTTGAGCAAGATT	

was determined using a Petroff-Hausser Counting Chamber (Hausser Scientific, USA). Counting was performed in duplicate in two separate aliquots and averaged.

The blood and urine samples used for the spiking experiments were acquired from healthy dogs. Blood was collected in K3 EDTA tubes and plastic serum tubes, whereas urine was collected in sterile urine collection cups. To confirm that the donor was not serologically positive for leptospirosis, MAT was performed following standard procedures (OIE 2008).

The appropriate amount of *Leptospira* spp. was spiked in whole blood, serum and urine samples so that the final concentration was 1×10^6 *Leptospira* spp./mL. Blood in serum tubes was spiked before clotting and then centrifuged. After spiking the whole blood, serum and urine samples, serial 10-fold dilutions of 1×10^6 down to 1×10^0 /mL were made. The DNA from each dilution (whole blood, serum and urine samples) was extracted using a High Pure PCR Template Preparation Kit (Roche Diagnostics, Germany) according to the manufacturer's instructions. The eluted DNA was kept at -20°C until PCR assays. All PCRs were run in triplicate. For *lipL32* PCR real-time, the dynamic range, R^2 coefficient and amplification efficiency for each spiked sample were determined from the standard curve.

Application of both PCR assays and the MAT in clinical samples. This study included blood, serum and urine samples from 51 dogs with presumptive diagnosis of leptospirosis from the cities of Berisso, Ensenada and La Plata (Argentina) collected from July 2015 to December 2016. All samples came from dog patients cared for at the Hospital Escuela, Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata, Buenos Aires, Argentina, according to animal welfare programs and with the informed consent of owners. The samples were analyzed at the Servicio Central de Laboratorio, Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata.

Blood, serum and urine samples were examined by both PCR assays and an aliquot of serum was examined by MAT. Blood was collected in K3 EDTA tubes and plastic serum tubes, whereas urine was collected in sterile urine collection cups. Hematological, biochemical and urine analyses were performed within 12 hours of collection. Subsequently, whole blood and serum samples were stored at -20°C until DNA extraction. Urine samples were processed according to the protocol described previously by Lucchesi et al. (2004) and kept at -20°C until DNA extraction. DNA was extracted from aliquots using a High Pure PCR Template Preparation Kit (Roche Diagnostics, Germany) according to the manufacturer's instructions. The eluted DNA was kept at -20°C before the PCR assays. All PCRs were run in triplicate. Samples with two or more positive reactions were scored as PCR positive.

The MAT was performed according to the standard method (OIE 2008). Briefly, the antigens were a panel of six live *Leptospira* reference strains representing the serovars most frequently found in dogs of Argentina: Castellonis, Canicola, Grippotyphosa, Copenhageni, Pomona

and Pyrogenes. The strains selected were grown in EMJH medium at 28°C and serum samples were screened initially at a dilution of 1:100. The samples with positive reactions were subsequently serially diluted to the final titer. The end point was determined by the presence of ~50% free unagglutinated leptospires compared with the control suspension.

The following case definition was adopted: a) confirmed leptospirosis case: patients with epidemiological, clinical and laboratory findings compatible with leptospirosis, where seroconversion to MAT (increase of at least four times in the titer for a serovar) was demonstrated or a single positive sample for more than one serovar (coagglutination) with at least one titer greater than or equal to 1/800; with no history of receiving recent antileptospiral vaccine; b) non-leptospirosis case: patients with negative MAT in two samples collected at different stages of the disease or a single positive sample with a titer lower than 1/800 (Goldstein et al. 2006, Ghneim et al. 2007).

Statistical analysis. The concordance between each PCR assay and the MAT was determined by calculating kappa values with 95% confidence intervals. According to kappa values, the concordance between assays was classified as following: 0-0.20 indicated poor agreement, 0.21-0.40 indicated fair agreement, 0.41-0.60 indicated moderate agreement, 0.61-0.80 indicated strong agreement, and 0.81-1 indicated almost perfect agreement (Landis & Koch 1977).

RESULTS

Evaluation of PCR assays with reference strains

The *lipL32* real-time PCR amplified all the pathogenic *Leptospira* but did not amplify the DNA of the saprophytic *L. meyeri* serovar Ranarum and *L. biflexa* serovar Patoc. Conversely, the *rrs* conventional PCR amplified all the pathogenic *Leptospira* and the saprophytic *L. meyeri* serovar Ranarum and *L. biflexa* serovar Patoc. Neither the *lipL32* real-time PCR nor the *rrs* conventional PCR was able to amplify the DNA of other microorganisms. The IPC added to the *lipL32* real-time PCR assay was detected in all cases, confirming the absence of inhibitors in the PCR runs. The analytical sensitivity was 1 and 10 GE/reaction for the *lipL32* real-time PCR and the *rrs* conventional PCR respectively. Using the serial dilutions of DNA from *Leptospira interrogans* Canicola Utrecht IV and Copenhageni M20, the *lipL32* real-time PCR assay showed similar values of dynamic range, R^2 coefficient, and PCR efficiency (Table 3 and Fig.1).

Evaluation of PCR assays in spiked whole blood, serum and urine samples

The sensitivity of the *lipL32* real-time PCR assay was 100 *Leptospira* spp./mL in whole blood samples, 1000 *Leptospira* spp./mL in serum samples, and

Table 3. Results of dynamic range, R^2 coefficient and PCR efficiency obtained with the *lipL32* real-time PCR assay from reference strains and spiked whole blood, serum and urine samples

	Dynamic range	R^2 coefficient	Efficiency (%)
Reference strains			
<i>L. interrogans</i> Canicola Hond Utrecht IV	1×10^6 - 1×10^0	0.99	100.88
<i>L. interrogans</i> Copenhageni M20	1×10^6 - 1×10^0	0.99	100
Spiked samples			
Whole blood	1×10^6 - 1×10^2	0.992	99.07
Serum	1×10^6 - 1×10^3	0.99	93.07
Urine	1×10^6 - 1×10^1	0.999	112.5

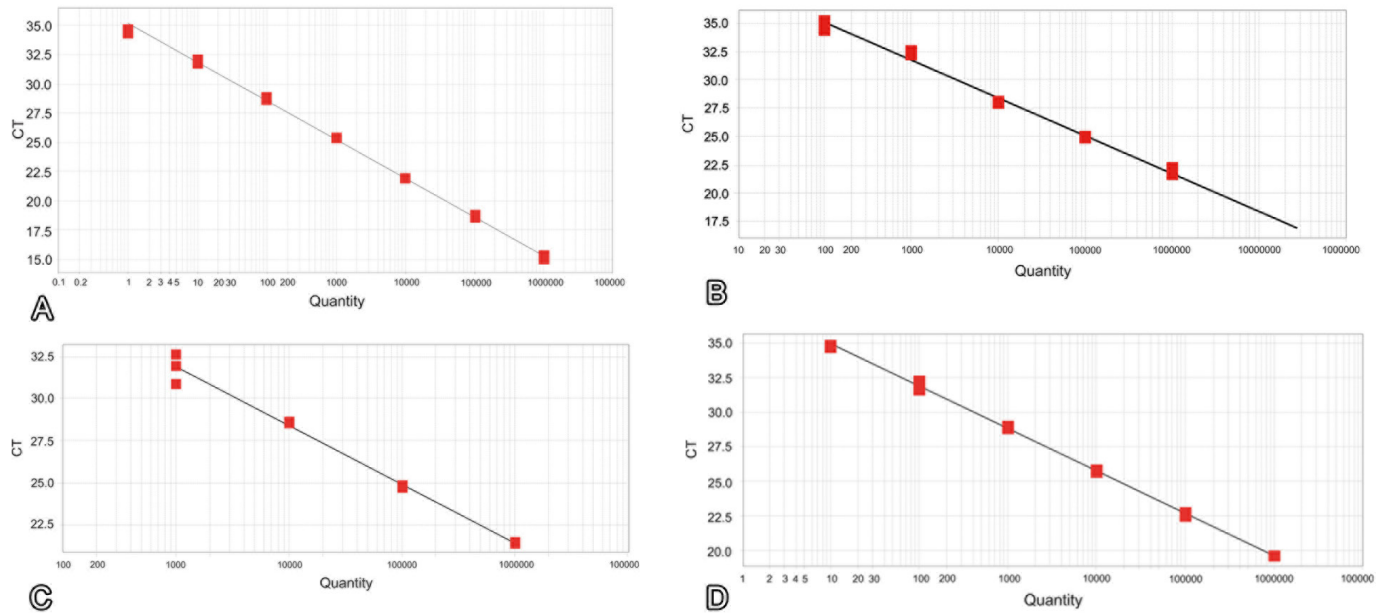


Fig.1. (A) Standard curve of the *lipL32* real-time PCR assay using DNA of *Leptospira interrogans* Canicola Hond Utrecht IV. (B) Standard curve of the *lipL32* real-time PCR assay using DNA of spiked whole blood samples. (C) Standard curve of the *lipL32* real-time PCR assay using DNA of spiked serum samples. (D) Standard curve of the *lipL32* real-time PCR assay using DNA of spiked urine samples.

10 *Leptospira* spp./mL in urine samples. Moreover, no PCR inhibition was identified in any of the reactions. The results of dynamic range, R^2 coefficient and PCR efficiency obtained with the *lipL32* real-time PCR assay from whole blood, serum and urine samples are shown in Table 3 and Figure 1. Regarding the *rrs* conventional PCR, its sensitivity was 1000 *Leptospira* spp./mL in whole blood and serum samples, and 100 *Leptospira* spp./mL in urine samples.

Application of both PCR assays and the MAT in clinical samples

Seven out of the 51 serum samples tested by MAT were considered confirmed leptospirosis cases according to the definition adopted. The three most common serovars were Castellonis, Canicola and Copenhageni. The *lipL32* real-time PCR was positive in six out of the seven confirmed cases, whereas the *rrs* conventional PCR was positive in four of them. The two urine samples positive by the *lipL32* real-time PCR assay but negative by the *rrs* conventional PCR assay showed a threshold cycle (C_t) value of 34.5 with the *lipL32* real-time PCR assay.

Regarding the type of sample, all five patients positive by the *lipL32* real-time PCR assay showed positive results in urine samples with C_t values ranging from 15.9 to 35, whereas only one patient showed positive results in whole blood and serum samples (with C_t values of 29 and 33, respectively). Likewise, the *rrs* conventional PCR assay was positive in the four urine samples of confirmed cases and only one patient was positive in whole blood sample. With regard to non-leptospirosis cases, 42 out of the 44 samples were also negative by both PCR assays, whereas 2 out of the 44 samples were positive in urine samples by both PCR assays. The IPC used in the *lipL32* real-time PCR assay was positive in all samples tested, confirming the absence of inhibitors in the

PCR runs. In addition, the *lipL32* real-time PCR assay showed a strong agreement ($\kappa=0.76$, 95% IC 0.492-1.039) when compared to the MAT. Conversely, the *rrs* conventional PCR assay showed a moderate agreement ($\kappa=0.56$, 95% IC 0.286-0.833).

DISCUSSION

Canine leptospirosis is traditionally diagnosed using the microagglutination test (MAT). However, as mentioned, for a correct interpretation of the results, samples from the acute and convalescent periods are required. In addition, this test does not allow the confirmation of the disease in patients who died prior to blood sampling to evidence seroconversion. In human leptospirosis, several authors have demonstrated the usefulness of PCR as a diagnostic tool (Levett et al. 2005, Mérien et al. 2005, Ahmed et al. 2009, Stoddard et al. 2009, Boonsilp et al. 2011, Bourhy et al. 2011), while in veterinary medicine, particularly in canine leptospirosis, the validity of this technique to diagnose the disease has not been determined yet.

The sensitivity of the PCR assay obtained from cultured microorganisms has been previously determined in studies performed in human clinical samples (Mérien et al. 1992, Villumsen et al. 2012). In our study, both assays showed sensitivity values lower than those reported in these previous studies but similar to those described in other publications (Xu et al. 2014, Fink et al. 2015, Recavarren et al. 2015). On the other hand, all the pathogenic strains were correctly amplified, while a negative result was obtained when evaluating other genera of microorganisms that could be present in the clinical samples and cause nonspecific amplification.

Values of analytical sensitivity in experimentally contaminated whole blood, serum and urine samples differ from those obtained from cultured microorganisms. In our study, the *lipL32* real-time PCR assay showed higher analytical sensitivity

values in whole blood and urine samples than those reported by other authors in rat urine samples (Rojas et al. 2010) and similar to those reported in humans (Stoddard et al. 2009). Regarding serum samples, studies performed in humans (Stoddard et al. 2009, Bourhy et al. 2011) have shown that the analytical sensitivity and efficiency of the PCR are lower than those obtained in whole blood, coinciding with the results obtained in this study. The *rrs* conventional PCR was less sensitive in whole blood and urine samples than the *lipL32* real-time PCR. However, the *rrs* conventional PCR showed values higher than those reported in human urine (10^4 bacteria/mL of urine) and similar to those obtained by other authors in canine urine after using a previous stage of immunomagnetic separation to increase sensitivity or a conventional PCR assay targeted *hap1* gene (Lucchesi et al. 2004, Branger et al. 2005, Monte et al. 2012).

The incorporation of internal amplification controls in the PCR assays has contributed to verifying whether the efficiency of the assay has been affected during the amplification process (Gentilini et al. 2015, Recavarren et al. 2015). In this context, some authors have used primers targeted to canine endogenous gene fragments such as those encoding enzymes of cellular metabolism (glycerol-aldehyde-phosphate dehydrogenase or cytochrome) as the amplification control (Barker et al. 2010, Recavarren et al. 2015). This option has the advantage of evaluating the presence of inhibitors and the DNA extraction procedure. However, it is unsuitable for samples such as serum or urine because the presence of cells in this type of samples is usually poor (Gentilini et al. 2015). Therefore, a negative result for the amplification control may be erroneously analyzed as inhibition. In our study, incorporating an exogenous amplification control allowed avoiding the need to have somatic cells for proper amplification. With this approach, we obtained a positive result of the amplification control (mean C_t value of 28) in all reactions, demonstrating the absence of inhibitors in the runs. In contrast, Recavarren et al. (2015), who used an endogenous amplification control, had to exclude 37 serum samples from their study because the amplification of the control was not adequate (C_t value greater than 35).

Several authors have evaluated the PCR assay in naturally infected dogs, but their results are discrepant (Harkin et al. 2003a, Fraune et al. 2013). Some of these authors (Harkin et al. 2003a) demonstrated the utility of a conventional PCR assay targeted to the gene encoding the 23S ribosomal subunit from urine samples, obtaining sensitivity and specificity values of 100% and 88.3%, respectively. However, in contrast, other researchers (Fraune et al. 2013) failed to detect any positive cases by means of a *lipL32*-based-nested-PCR assay performed from 15 whole blood samples and 10 urine samples from dogs diagnosed by MAT. Likewise, in another study, where the authors used a *flaB*-based-nested PCR assay, the sensitivity obtained was lower (62%) than that obtained with the MAT in paired samples (90.9%) (Koizumi et al. 2013). Although the sensitivity of these nested PCR assays is comparable to that of real-time PCR assays, other reasons could have influenced the results obtained by these authors. For example, the failure of the nested PCR in serologically confirmed cases can be attributed to the absence of the organisms in the sample secondary to the administration of antimicrobials or to the degradation of DNA during prolonged storage of the sample.

In our work, the *lipL32* real-time PCR assay detected six out of the seven leptospirosis cases confirmed by MAT, while the *rrs* conventional PCR assay detected only four. Although the *rrs* conventional PCR assay showed a moderate agreement when compared to the MAT, it failed to detect two positive patients by the *lipL32* real-time PCR assay. This finding could be attributed to the low bacterial concentration in the urine sample. Possibly, these patients positive by the *lipL32* real-time PCR assay were shedding a low number of microorganisms that was below the limit of detection for the conventional PCR assay. Similar findings (patients positive by qPCR but negative by conventional PCR) have been reported by other authors (Fink et al. 2015). In addition, the *lipL32* real-time PCR assay was positive in samples of two dogs before serological conversion was observed, reaffirming the usefulness of the *lipL32* real-time PCR assay for the early confirmation of the disease, as described by other authors (Harkin et al. 2003a). Our *lipL32* real-time PCR assay showed an adequate value of concordance with the MAT and allowed detecting a higher percentage of positive animals than that detected by other authors (Xu et al. 2014). Regarding confirmed cases, studies performed in dogs by Xu et al. (2014) and Fink et al. (2015) have shown a low percentage of positive cases, coinciding with our results. The cause of this finding is possibly due to the continued use of a vaccine for decades, with the consequent reduction of the clinical manifestation of the disease.

With respect to the type of samples used for the DNA search of *Leptospira* spp., in a previous study, Xu et al. (2014) evaluated 207 whole blood samples and 9 urine samples from 207 dogs by using three real-time PCR assays, and obtained three positive cases that showed amplification in urine samples and only one with the simultaneous presence of DNA in whole blood. However, in our study, the highest number of positive samples was obtained in urine samples by the *lipL32* real-time PCR assay. Therefore, to increase the probability of a positive result, it would be advisable to jointly analyze whole blood and urine samples from the same patient (Schuller et al. 2015).

With the two PCR assays evaluated in the present study, two of the 44 patients classified as non-leptospirosis cases according to established criteria showed positive results in urine samples. Unfortunately, a second serum sample could not be obtained to evaluate the appearance of antibodies because the result of the MAT in both patients was non-reactive. However, these animals had epidemiological data, clinical signs and alterations in the parameters of blood biochemistry (increases in urea and creatinine values) compatible with leptospirosis. Our results agree with those described in other publications (Harkin et al. 2003a, Xu et al. 2014, Fink et al. 2015, Recavarren et al. 2015). Previous reports have also shown that serological results may be negative in the acute phase of the disease or inconsistent when the microorganisms are located in protected sites of the immune system (Birnbaum et al. 1998, Harkin & Gartrell 1996). The possible explanations for the finding of seronegative animals in a single sample of the MAT but positive by PCR are multiple. Firstly, they could constitute false-positive results due to an unspecific amplification of DNA from the microorganisms present in urine (Fink et al. 2015). Secondly, animals infected with serovars that were not included in the panel of antigens tested would yield negative results. Finally, they could reflect a higher sensitivity of the PCR than

the serology performed in the acute stage, especially in animals with exposure to risk factors and compatible clinical signs.

Previous studies have also shown negative results with the PCR assay in animals classified as confirmed cases of leptospirosis by serology (Fraune et al. 2013). The main cause of these findings is possibly the establishment of antimicrobial treatment before sample extraction and submission, the inadequate conservation of the sample, or the transient and/or intermittent shedding by dogs of pathogenic *Leptospira* in urine (Fink et al. 2015). In our study, the false-negative result using the real-time PCR assay corresponded to a dog patient that was initially treated in a private clinic, so we cannot rule out the previous administration of antibiotics.

CONCLUSIONS

The results of our study emphasize the importance of carrying out field studies to evaluate diagnostic assays because the tests with strains or experimentally contaminated samples may not be extrapolated to the situations encountered when attempting to diagnose leptospirosis.

PCR assays have been shown to be very useful for diagnosis when serological assays fail to detect antibodies or patients die before the convalescent serum sample can be obtained.

Although the MAT assay remains the most sensitive and standard technique for diagnosis, mainly when patients have been treated with antimicrobials, our results also highlight the utility of PCR assays as tools complementary to serology for the confirmation of leptospirosis cases in dogs.

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

REFERENCES

- Ahmed A., Engelberts M.F., Boer K.R., Ahmed N. & Hartskeerl R.A. 2009. Development and validation of a real-time PCR for detection of pathogenic leptospira species in clinical materials. *PLoS One* 4(9):e7093. <<http://dx.doi.org/10.1371/journal.pone.0007093>> <PMid:19763264>
- Ahmed A., Klaasen H., van der Veen M., Van der Linden H., Goris M. & Hartskeerl R.A. 2012. Evaluation of real-time pcr and culturing for the detection of leptospires in canine samples. *Adv. Microbiol.* 2:162-170. <<http://dx.doi.org/10.4236/aim.2012.22021>>
- Barker E.N., Tasker S., Day M.J., Warman S.M., Woolley K., Birtles R., Georges K.C., Ezeokoli C.D., Newaj-Fyzul A., Campbell M.D., Sparagano O.A., Cleaveland S. & Helps C.R. 2010. Development and use of real-time PCR to detect and quantify *Mycoplasma haemocanis* and "Candidatus *Mycoplasma haematoparvum*" in dogs. *Vet. Microbiol.* 140(1/2):167-170. <<http://dx.doi.org/10.1016/j.vetmic.2009.07.006>> <PMid:19646827>
- Birnbaum N., Barr S.C., Center S.A., Schermerhorn T., Randolph J.F. & Simpson K.W. 1998. Naturally acquired leptospirosis in 36 dogs: serological and clinicopathological features. *J. Small Anim. Pract.* 39(5):231-236. <<http://dx.doi.org/10.1111/j.1748-5827.1998.tb03640.x>> <PMid:9631358>
- Boonsilp S., Thaipadungpanit J., Amornchai P., Wuthiekanun V., Chierakul W., Limmathurotsakul D., Day N.P. & Peacock S.J. 2011. Molecular detection and speciation of pathogenic *Leptospira* spp. in blood from patients with culture-negative leptospirosis. *BMC Infect. Dis.* 11(1):338. <<http://dx.doi.org/10.1186/1471-2334-11-338>> <PMid:22151687>
- Bourhy P., Bremont S., Zinini F., Giry C. & Picardeau M. 2011. Comparison of real-time PCR assays for detection of pathogenic *Leptospira* spp. in blood and identification of variations in target sequences. *J. Clin. Microbiol.* 49(6):2154-2160. <<http://dx.doi.org/10.1128/JCM.02452-10>> <PMid:21471336>
- Branger C., Blanchard B., Fillonneau C., Suard I., Aviat F., Chevallier B. & André-Fontaine G. 2005. Polymerase chain reaction assay specific for pathogenic *Leptospira* based on the gene hap1 encoding the hemolysis-associated protein-1. *FEMS Microbiol. Lett.* 243(2):437-445. <<http://dx.doi.org/10.1016/j.femsle.2005.01.007>> <PMid:15686847>
- Brihuega B. & Martiarena B. 1996. Prevalencia de leptospirosis en insuficiencia renal en caninos. *Revta Vet. Arg.* 13:746-752.
- Faine S. 1982. Guidelines for the control of Leptospirosis. World Health Organization, Geneva. 171p.
- Fink J.M., Moore G.E., Landau R. & Vemulapalli R. 2015. Evaluation of three 5' exonuclease-based real-time polymerase chain reaction assays for detection of pathogenic *Leptospira* species in canine urine. *J. Vet. Diagn. Invest.* 27(2):159-166. <<http://dx.doi.org/10.1177/1040638715571360>> <PMid:25776541>
- Fraune C.K., Schweighauser A. & Francey T. 2013. Evaluation of the diagnostic value of serologic microagglutination testing and a polymerase chain reaction assay for diagnosis of acute leptospirosis in dogs in a referral center. *J. Am. Vet. Med. Assoc.* 242(10):1373-1380. <<http://dx.doi.org/10.2460/javma.242.10.1373>> <PMid:23634681>
- Gentilini F., Zanoni R.G., Zambon E. & Turba M.E. 2015. A comparison of the reliability of two gene targets in loop-mediated isothermal amplification assays for detecting leptospiral DNA in canine urine. *J. Vet. Diagn. Invest.* 29(1):100-104. <<http://dx.doi.org/10.1177/1040638716672503>> <PMid:28074711>
- Ghneim G.S., Viers J.H., Chomel B.B., Kass P.H., Descollonges D.A. & Johnson M.L. 2007. Use of a case-control study and geographic information systems to determine environmental and demographic risk factors for canine leptospirosis. *Vet. Res.* 38(1):37-50. <<http://dx.doi.org/10.1051/vetres:2006043>> <PMid:17074294>
- Goldstein R.E., Lin R.C., Langston C.E., Scrivani P.V., Erb H.N. & Barr S.C. 2006. Influence of infecting serogroup on clinical features of leptospirosis in dogs. *J. Vet. Intern. Med.* 20(3):489-494. <<http://dx.doi.org/10.1111/j.1939-1676.2006.tb02886.x>> <PMid:16734079>
- Harkin K.R. & Gartrell C.L. 1996. Canine leptospirosis in New Jersey and Michigan: 17 cases (1990-1995). *J. Am. Anim. Hosp. Assoc.* 32(6):495-501. <<http://dx.doi.org/10.5326/15473317-32-6-495>> <PMid:8906726>
- Harkin K.R., Roshto Y.M. & Sullivan J.T. 2003a. Clinical application of a polymerase chain reaction assay for diagnosis of leptospirosis in dogs. *J. Am. Vet. Med. Assoc.* 222(9):1224-1229. <<http://dx.doi.org/10.2460/javma.2003.222.1224>> <PMid:12725309>
- Harkin K.R., Roshto Y.M., Sullivan J.T., Purvis T.J. & Chengappa M.M. 2003b. Comparison of polymerase chain reaction assay, bacteriologic culture, and serologic testing in assessment of prevalence of urinary shedding of leptospires in dogs. *J. Am. Vet. Med. Assoc.* 222(9):1230-1233. <<http://dx.doi.org/10.2460/javma.2003.222.1230>> <PMid:12725310>
- Kohn B., Steinicke K., Arndt G., Gruber A.D., Guerra B., Jansen A., Kaser-Hotz B., Klopffleisch R., Lotz F., Luge E. & Nöckler K. 2010. Pulmonary abnormalities in dogs with leptospirosis. *J. Vet. Intern. Med.* 24(6):1277-1282. <<http://dx.doi.org/10.1111/j.1939-1676.2010.0585.x>> <PMid:20738768>
- Koizumi N., Muto M.M., Akachi S., Okano S., Yamamoto S., Horikawa K., Harada S., Funatsumaru S. & Ohnishi M. 2013. Molecular and serological investigation of *Leptospira* and leptospirosis in dogs in Japan. *J. Med. Microbiol.* 62(Pt 4):630-636. <<http://dx.doi.org/10.1099/jmm.0.050039-0>> <PMid:23264455>

- Landis J.R. & Koch G.G. 1977. The measurement of observer agreement for categorical data. *Biometrics* 33(1):159-174. <<http://dx.doi.org/10.2307/2529310>> <PMid:843571>
- Levett P.N., Morey R.E., Galloway R.L., Turner D.E., Steigerwalt A.G. & Mayer L.W. 2005. Detection of pathogenic leptospires by real-time quantitative PCR. *J. Med. Microbiol.* 54(Pt 1):45-49. <<http://dx.doi.org/10.1099/jmm.0.45860-0>> <PMid:15591254>
- Löffler S.G., Passaro D., Samartino L., Soncini A., Romero G. & Brihuega B. 2014. Genotypes of *Leptospira* spp. strains isolated from dogs in Buenos Aires, Argentina. *Revta Arg. Microbiol.* 46(3):201-204. <[http://dx.doi.org/10.1016/S0325-7541\(14\)70073-2](http://dx.doi.org/10.1016/S0325-7541(14)70073-2)> <PMid:25444128>
- Lucchesi P.M., Arroyo G.H., Etcheverría A.I., Parma A.E. & Seijo A.C. 2004. Recommendations for the detection of *Leptospira* in urine by PCR. *Recomendações para detecção de Leptospira em urina pela PCR. Revta Soc. Bras. Med. Trop.* 37(2):131-134. <<http://dx.doi.org/10.1590/S0037-86822004000200003>>
- Mérien F., Amouriaux P., Perolat P., Baranton G. & Saint Girons I. 1992. Polymerase chain reaction for detection of *Leptospira* spp. in clinical samples. *J. Clin. Microbiol.* 30(9):2219-2224. <PMid:1400983>
- Mérien F., Portnoi D., Bourhy P., Charavay F., Berlioz-Arthaud A. & Baranton G. 2005. A rapid and quantitative method for the detection of *Leptospira* species in human leptospirosis. *FEMS Microbiol. Lett.* 249(1):139-147. <<http://dx.doi.org/10.1016/j.femsle.2005.06.011>> <PMid:16006065>
- Miotto B.A., da Hora A.S., Taniwaki S.A., Brandão P.E., Heinemann M.B. & Hagiwara M.K. 2017. Development and validation of a modified TaqMan based real-time PCR assay targeting the *lipL32* gene for detection of pathogenic *Leptospira* in canine urine samples. *Braz. J. Microbiol.* 49(3):584-590. <<http://dx.doi.org/10.1016/j.bjm.2017.09.004>> <PMid:29233483>
- Monte L.G., Jorge S., Luiz J.P., Sinnott F., Seixas K.S., Aleixo J.A., Samartino L.E., Conceição F.R. & Hartleben C.P. 2012. Diagnosis of canine leptospirosis using an immunomagnetic separation-PCR method. *Braz. J. Microbiol.* 43(2):602-605. <<http://dx.doi.org/10.1590/S1517-83822012000200023>> <PMid:24031870>
- Nascimento A.L., Ko A.I., Martins E.A., Monteiro-Vitorello C.B., Ho P.L., Haake D.A., Verjovski-Almeida S., Hartskeerl R.A., Marques M.V., Oliveira M.C., Menck C.F., Leite L.C., Carrer H., Coutinho L.L., Degraive W.M., Dellagostin O.A., El-Dorry H., Ferro E.S., Ferro M.I., Furlan L.R., Gamberini M., Gigliotti E.A., Góes-Neto A., Goldman G.H., Goldman M.H., Harakava R., Jerônimo S.M., Junqueira-de-Azevedo I.L., Kimura E.T., Kuramae E.E., Lemos E.G., Lemos M.V., Marino C.L., Nunes L.R., de Oliveira R.C., Pereira G.G., Reis M.S., Schrieffer A., Siqueira W.J., Sommer P., Tsai S.M., Simpson A.J., Ferro J.A., Camargo L.E., Kitajima J.P., Setubal J.C. & Van Sluys M.A. 2004. Comparative genomics of two *Leptospira interrogans* serovars reveals novel insights into physiology and pathogenesis. *J. Bacteriol.* 186(7):2164-2172. <<http://dx.doi.org/10.1128/JB.186.7.2164-2172.2004>> <PMid:15028702>
- OIE 2008. Leptospirosis, p.251-264. In: Ibid. (Ed.), *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals*. 6th ed. World Organisation for Animal Health, Paris.
- Palaniappan R.U., Chang Y.F., Chang C.F., Pan M.J., Yang C.W., Harpending P., McDonough S.P., Dubovi E., Divers T., Qu J. & Roe B. 2005. Evaluation of lig-based conventional and real time PCR for the detection of pathogenic leptospires. *Mol. Cell. Probes* 19(2):111-117. <<http://dx.doi.org/10.1016/j.mcp.2004.10.002>> <PMid:15680212>
- Recavarren M., Quintana S., Rivero M., Ballesteros B. & Scialfa E. 2015. *Leptospira* diagnosis in serum and urine of dogs by real time PCR. *J. Vet. Sci. Med. Diagn.* 4:2.
- Ren S.X., Fu G., Jiang X.G., Zeng R., Miao Y.G., Xu H., Zhang Y.X., Xiong H., Lu G., Lu L.F., Jiang H.Q., Jia J., Tu Y.F., Jiang J.X., Gu W.Y., Zhang Y.Q., Cai Z., Sheng H.H., Yin H.F., Zhang Y., Zhu G.F., Wan M., Huang H.L., Qian Z., Wang S.Y., Ma W., Yao Z.J., Shen Y., Qiang B.Q., Xia Q.C., Guo X.K., Danchin A., Saint Girons I., Somerville R.L., Wen Y.M., Shi M.H., Chen Z., Xu J.G. & Zhao G.P. 2003. Unique physiological and pathogenic features of *Leptospira interrogans* revealed by whole-genome sequencing. *Nature* 422(6934):888-893. <<http://dx.doi.org/10.1038/nature01597>> <PMid:12712204>
- Rojas P., Monahan A.M., Schuller S., Miller I.S., Markey B.K. & Nally J.E. 2010. Detection and quantification of leptospires in urine of dogs: a maintenance host for the zoonotic disease leptospirosis. *Eur. J. Clin. Microbiol. Infect. Dis.* 29(10):1305-1309. <<http://dx.doi.org/10.1007/s10096-010-0991-2>> <PMid:20559675>
- Schuller S., Francey T., Hartmann K., Hugonnard M., Kohn B., Nally J.E. & Sykes J. 2015. European consensus statement on leptospirosis in dogs and cats. *J. Small Anim. Pract.* 56(3):159-179. <<http://dx.doi.org/10.1111/jsap.12328>> <PMid:25754092>
- Slack A., Symonds M.L., Dohnt M.F. & Smythe L.D. 2006. Identification of pathogenic *Leptospira* species by conventional or real-time PCR and sequencing of the DNA gyrase subunit B encoding gene. *BMC Microbiol.* 6(1):95. <<http://dx.doi.org/10.1186/1471-2180-6-95>> <PMid:17067399>
- Stoddard R.A., Gee J.E., Wilkins P.P., McCaustland K. & Hoffmaster A.R. 2009. Detection of pathogenic *Leptospira* spp. through TaqMan polymerase chain reaction targeting the *LipL32* gene. *Diagn. Microbiol. Infect. Dis.* 64(3):247-255. <<http://dx.doi.org/10.1016/j.diagmicrobio.2009.03.014>> <PMid:19395218>
- Sykes J.E., Hartmann K., Lunn K.F., Moore G.E., Stoddard R.A. & Goldstein R.E. 2011. 2010 ACVIM small animal consensus statement on leptospirosis: diagnosis, epidemiology, treatment, and prevention. *J. Vet. Intern. Med.* 25(1):1-13. <<http://dx.doi.org/10.1111/j.1939-1676.2010.0654.x>> <PMid:21155890>
- Townsend W.M., Stiles J. & Krohne S.G. 2006. Leptospirosis and panuveitis in a dog. *Vet. Ophthalmol.* 9(3):169-173. <<http://dx.doi.org/10.1111/j.1463-5224.2006.00464.x>> <PMid:16634930>
- Villumsen S., Pedersen R., Borre M.B., Ahrens P., Jensen J.S. & Krogfelt K.A. 2012. Novel TaqMan® PCR for detection of *Leptospira* species in urine and blood: pit-falls of in silico validation. *J. Microbiol. Methods* 91(1):184-190. <<http://dx.doi.org/10.1016/j.mimet.2012.06.009>> <PMid:22750039>
- Xu C., Loftis A., Ahluwalia S.K., Gao D., Verma A., Wang C. & Kaltenboeck B. 2014. Diagnosis of canine leptospirosis by a highly sensitive FRET-PCR targeting the *lig* genes. *PLoS One* 9(2):e89507. <<http://dx.doi.org/10.1371/journal.pone.0089507>> <PMid:24586833>

A new experimental model of intrinsic denervation in ileum from wistar rats through intramural microinjections of benzalkonium chloride¹

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ABSTRACT.- Ribeiro J.O., Ferreira M.G.P.A., Marques I.C.S., Alvarenga C., Nardi A.B., Garcia S.B., Montanhim G.L. & Moraes P.C. 2019. **A new experimental model of intrinsic denervation in ileum from wistar rats through intramural microinjections of benzalkonium chloride.** *Pesquisa Veterinária Brasileira* 39(4):263-270. Departamento de Patologia, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Rua Ten. Catão Roxo 2398, Vila Monte Alegre, Ribeirão Preto, SP 14051-140, Brazil. E-mail: juliana.oribeiro@yahoo.com.br

Extensive literature is available about the intrinsic denervation of segments of the digestive tube through the application of CB in the serosa of the viscera. However, this technique has some disadvantages like causing peritonitis, flanges and high mortality, limiting its use in humans. The aim of the present study was to evaluate the feasibility of benzalkonium chloride (CB) to induce intrinsic chemical denervation, through applications of CB in the intramural ileum of wistar rats, as well as deepen the knowledge about the evolution of neuronal injury caused in the process. We used 40 rats, divided into two groups (control-GC and benzalkonium-GB) of 20 animals each, divided into four sub-groups according to the time of postoperative assessment of 24, 48 hours, 30 and 90 days. The animals were submitted to intramural microinjections of sterile saline solution 0.9% (GC) or benzalkonium chloride (GB) in ileal portion, and subsequent histopathological analysis and immunohistochemistry for evaluation of neuronal injury. A significant decrease ($p < 0.05$) was found of the neuronal myenteric count over time in groups, GB3, GB4 and GB2. The specific positive immunolabeling for H2AX and Caspase-3 confirmed the results obtained in the histopathological evaluation, denoting the ignition of irreversible cell injury in 24 hours, evolving into neuronal apoptosis in 48 hours after application of the CB 0.3%. Under the conditions in which this work was conducted, it can be concluded that the application of CB 0.3% by means of microinjections intramural in the ileal wall is able to induce intrinsic chemical denervation of the diverticulum of wistar rats and that the main mechanism of neuronal death is induction of apoptosis.

INDEX TERMS: Experimental model, intrinsic denervation, ileum, wistar rats, intramural microinjections, benzalkonium chloride, apoptosis, myenteric plexus, neurons myenterics, short bowel syndrome, small intestine, rodents, clinics.

RESUMO.- [Um novo modelo experimental de desnervação intrínseca em íleo de ratos wistar através de micro injeções intramurais de cloreto de benzalcônio.] Existe

vasta literatura sobre a desnervação intrínseca de segmentos do tubo digestório através da aplicação de CB na serosa da víscera. Entretanto, essa técnica tem a desvantagem de causar peritonite, formação de bridas e alta mortalidade, não sendo factível para eventuais utilizações em humanos. O objetivo do presente estudo foi avaliar a viabilidade do Cloreto de benzalcônio (CB) induzir desnervação química intrínseca, por meio de aplicações intramurais em íleo de ratos wistar, além de aprofundar o conhecimento sobre a evolução da lesão neuronal causada neste processo. Foram utilizados 40 ratos, distribuídos em dois grupos (controle- GC e benzalcônio- GB) de 20 animais cada, subdivididos em quatro

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subgrupos de acordo com o tempo de avaliação pós-operatória de 24, 48 horas, 30 e 90 dias. Os animais foram submetidos à microinjeções intramurais de solução salina estéril 0,9% (GC) ou de cloreto de benzalcônio (GB) em porção ileal, e posterior análise histopatológica e imuno-histoquímica, para avaliação da lesão neuronal. Houve diminuição significativa ($p < 0,05$) na contagem neuronal mientérica ao longo do tempo nos grupos GB2, GB3 e GB4. A imunomarcagem específica positiva para H2AX e Caspase-3 confirmou os resultados obtidos na avaliação histopatológica, denotando início da lesão celular irreversível em 24 horas, evoluindo para apoptose neuronal em 48 horas após a aplicação do CB 0,3%. Nas condições em que este trabalho foi conduzido, é possível concluir que a aplicação de CB 0,3% por meio de microinjeções intramurais na parede ileal é capaz de induzir desnervação química intrínseca da porção ileal de ratos wistar e que o principal mecanismo de morte neuronal é a indução de apoptose.

TERMOS DE INDEXAÇÃO: Modelo experimental, desnervação intrínseca, íleo, ratos wistar, micro injeção intramural, cloreto de benzalcônio, apoptose, desnervação, neurônios mientéricos, síndrome do intestino curto, plexo mientérico, roedores, clínica.

INTRODUCTION

Chemical denervation by benzalkonium chloride as an experimental model of the digestive tube has been studied in different ways in the pathophysiology enteric coated, for example, for studies of the role of the enteric nervous system in proliferation mucosal epithelial cell and control of peristalsis (Zucoloto et al. 1991, Garcia et al. 1999, Buttow et al. 2003, Carvalho et al. 2006). There is a possibility that the benzalkonium chloride (CB) causes intrinsic denervation of the intestine (Oliveira et al. 1990, Hanani et al. 2003, Laranjeira et al. 2011, Fujimura et al. 2016, Yu et al. 2016) and possibly has a clinical application in situations where denervation is a recommended for specific segments. An example is the denervation of the remaining ileum in Short Bowel Syndrome in experimental rats, in which it has a beneficial effect on animal clinical parameters, therefore constituting a possibility of treatment in the future

(Garcia et al. 1999, Carvalho et al. 2006). However, there is a need to improve the techniques of application of CB, because in the existing model, the application in the intestine serosa through laparotomy can lead to peritonitis and formation of bands, which lead to a high mortality.

The results obtained in this study demonstrated the feasibility and effectiveness of intramural application of CB to get the digestive tube denervation, with a view to greater precision of this method of application of CB, through intramural applications. It was also possible to deepen the knowledge about the evolution of neuronal injury caused in this process, through histopathological analysis and immunohistochemistry by immunolabeling histones specific gamma-H2AX (H2AX) that assesses the presence of oxidative nuclear stress injuries (Berra et al. 2006, Redon et al. 2012) and Caspase-3, which detects apoptosis (Terzian et al. 2007, El-Aal et al. 2016) of the myenteric neurons.

MATERIALS AND METHODS

It was used 40 wistar rats (*Rattus norvegicus albinus* variation), male, weighting from 350 to 450g, taken from the vivarium of the Faculty of Medicine of Ribeirão Preto, University of São Paulo (FMRP-USP). The 40 wistar rats were randomly divided into two groups, GC (Control Group) and GB (Benzalkonium Group), containing 20 animals each. The groups were divided evenly into four sub-groups according to the time: 24, 48 hours, 30 and 90 days, containing five animals each, being GC1 GC2 GC3 GC4, respectively, for the control group and GB1 GB2, GB3 GB4 with same times for the Benzalkonium Group.

The GB rats were submitted to intramural microinjections of benzalkonium chloride 0.3% in the ileal segment. For this, median ventral laparotomy was performed with subsequent exposure and exteriorization of the ileum. Intramural microinjections were applied in 5cm of the ileal segment cranial to the ileum cecum-colic valve, using 1mL syringe with size needle of 13mm x 0.45mm. 1mL of CB 0.3% was applied and distributed in each microinjections Fishbone-shaped (Fig.1A) totalizing 10 microinjections, these applications were distributed in five microinjections on the right side and five on the left side of the ileum, from the antimesenteric toward the mesenteric border (Fig.1A,B). After this procedure, the

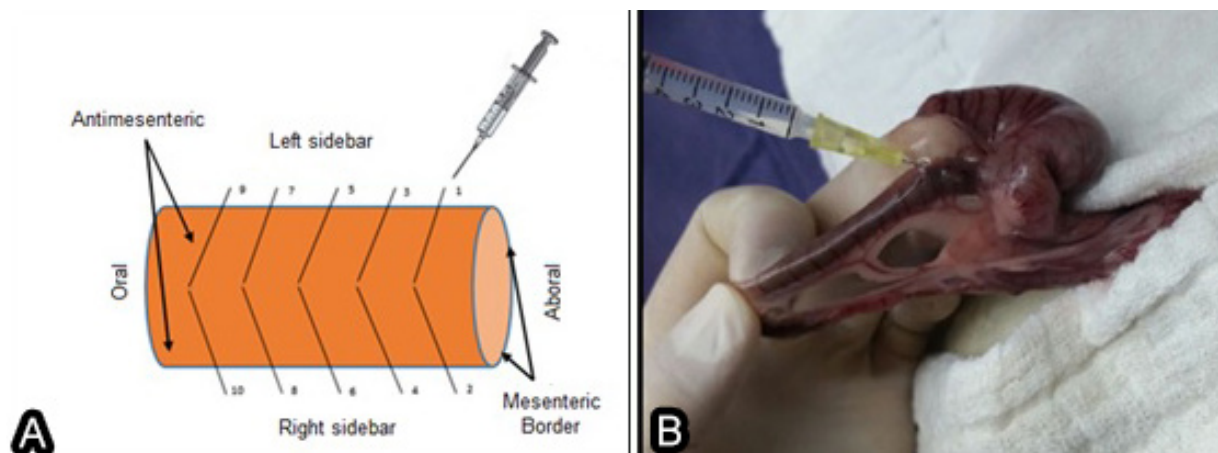


Fig.1. (A) Schematic drawing of microinjections intramural applied in rat ileal segment in the dorso-ventral aspect. Held ten microinjections Fishbone-shaped distributed in five microinjections in right side and five on the left side of the ileum, from antimesenteric edge toward the mesenteric border. (B) Surgical procedure for realization of microinjections intramural of sterile saline or benzalkonium hydrochloride in wistar rats. Exposure and exteriorization of ileal segment, microinjections in muscle layer in 5cm aborais ileal segment.

ileal segment was submitted to washing with profuse application of saline 0.9%. The viscera were reintroduced into the abdomen and the abdominal wall rebuilt through two plans suture. The rats in GC were submitted to the same procedure of GB, except for the application of saline solution 0.9%.

The animals of the GC1 and GB 1 were euthanized 24 hours postoperatively, the GC2 and GB2 48 hours postoperatively, the GC3 and GB3 with 30 days and finally, GC4 and GB4 with 90 days postoperatively. The ileal segment was collected and fixed in formalin (10%) for histopathological and immunohistochemical study with Giemsa staining.

Histopathological evaluation. After routine histological processing and staining for hematoxylin and eosin (HE), histopathological evaluation was performed of the ileal wall and the population of myenteric neurons were counted in objective of 40x and rated by histological cut.

Immuno-istochemical analyses. Immunolabeling evaluation of histones gamma-H2AX (H2AX) and Caspase-3 was made in objective of 40x taking the entire length of each cut for evaluation. For evaluation of cells positively stained for H2AX and occurrence of oxidative stress, assessed the nucleus, since the histone acts as a marker of breaks on dual tape DNA (Berra et al. 2006, Redon et al. 2012). For evaluation of cells positively stained for Caspase-3 and occurrence of apoptosis, assessed the cellular cytoplasm (Terzian et al. 2007).

Statistical analysis. The results of the neuronal counts will be presented as mean $\pm$ standard deviation. The comparison of the count of the number of myenteric neurons made during the histopathological analysis between the GA and GB were performed by using the non-parametric Student t test with significance level established at $p < 0.05$. The analysis of immunolabeling of H2AX and Caspase-3 was made in descriptive form.

Ethics. The project was submitted and approved by the Commission of ethics in the use of Animals (CEUA), College of Agricultural Sciences and Veterinary (FCAV), Universidade Estadual Paulista "Júlio

de Mesquita Filho" (Unesp), Campus de Jaboticabal, SP, executed according to the standards required by CONCEA (National Council for Animal Experimentation control). Protocol no. 18,937/16.

RESULTS

Technical assessment of implementation of CB intramural

Confirmation of the effectiveness of microinjections applied intramural, was accomplished through histopathologic study of the ileal wall. In all rats (both the GC as in GB) it was possible to observe the presence of injury hitting part of the internal muscle layer and part of the submucosal layer (asterisk) in 24 hours (Fig.2B).

Neuronal analysis-counting the number of myenteric neurons

To assess the effectiveness of microinjections of 0.3% in CB to induce intrinsic denervation of the ileal wall, the population of myenteric neurons of ileum wall was estimated through neuronal count. The results were expressed as number of myenteric neurons by histological cut.

There was no significant difference in neuronal count between the GC1 and GB1 (24 hours). However, there was a reduction in the number of neurons in the groups treated GB2 (48 hours), (30 days) and GB3 GB4 (90 days) when compared to the respective Control Groups ($P < 0.05$). Of these, GB3 and GB4 presented the lowest number of neurons in clumps, in which exposure to CB was prolonged (30 and 90 days respectively) ($p < 0.0001$) (Fig. 3).

Analysis of the mechanism of action of neuronal injury caused by CB

The evaluation of myenteric neuron injury by oxidative stress and apoptosis was analyzed by means of histopathological and immunohistochemical study. In the histopathological analysis

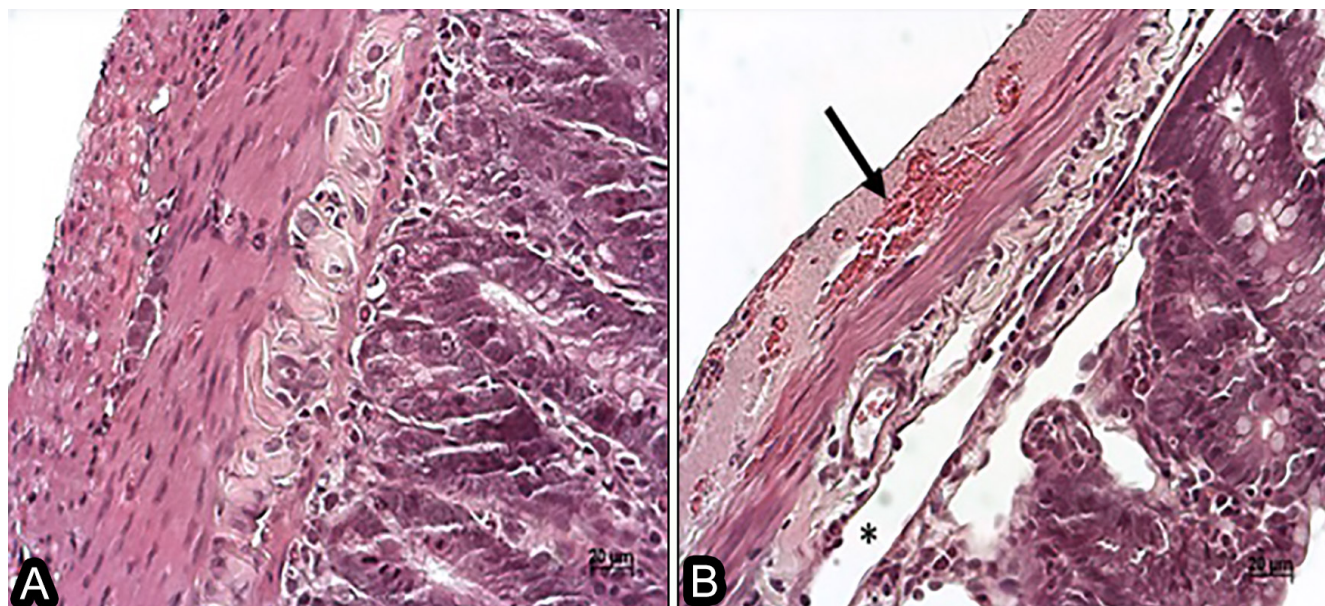


Fig.2. Ileal segment of wistar rat. (A) Normal rat ileal segment. Giemsa stain, obj.40x. (B) Injury that affects part of the submucosal layer and part of the internal muscle layer diverticulum after 24 hours (asterisk). Intraparietal hemorrhage, characterized by large numbers of red blood cells (arrow). Significance level $p < 0.05$ (asterisk). Giemsa stain, obj.40x.

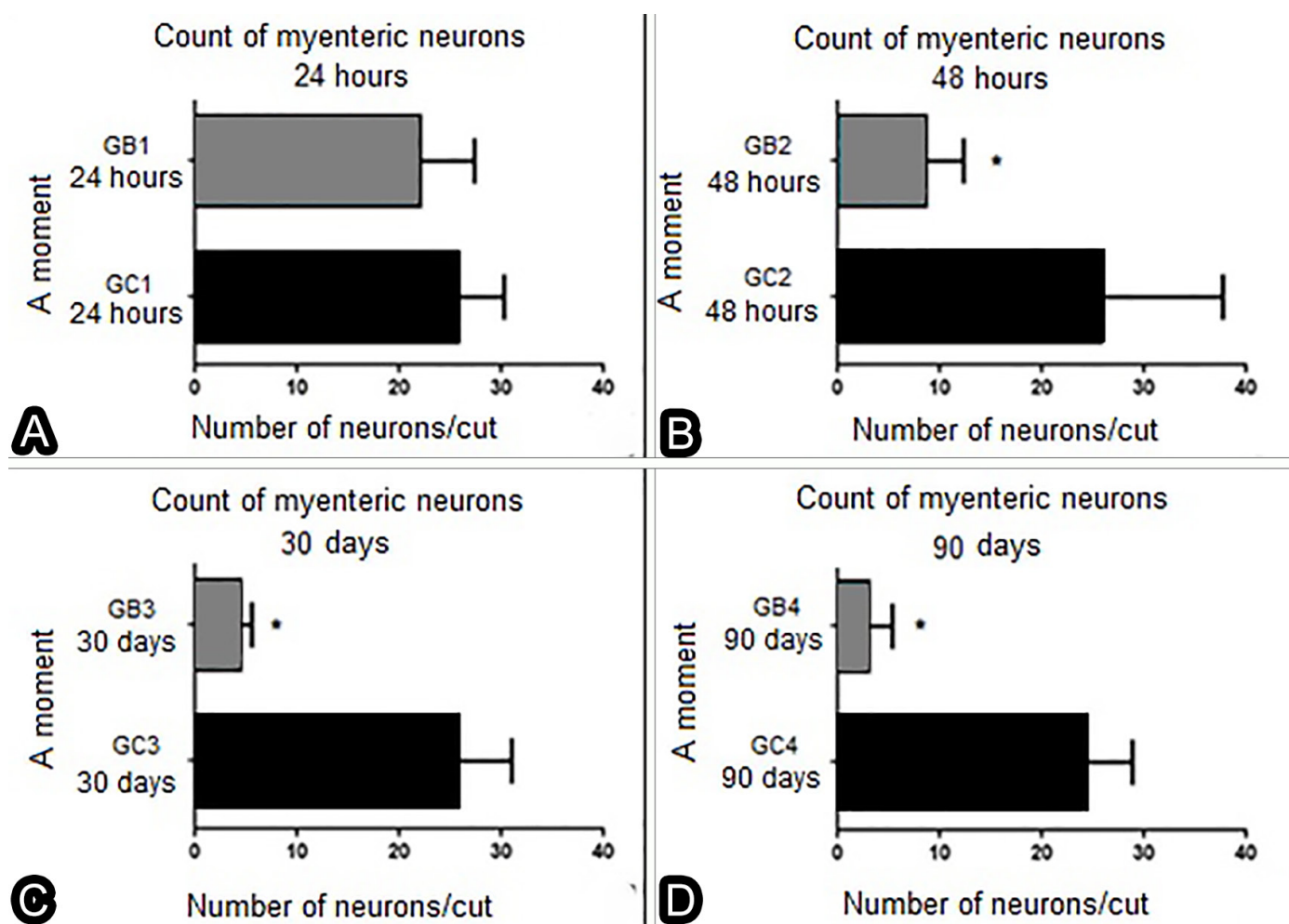


Fig.3. Comparison of myenteric neuronal count in different moments between the GC (Control Group) and GB (Benzalkonium Group). (A) Comparison between the groups GC1 and GB1 in 24 hours. (B) Comparison between the groups GB2 and GC2 in 48 hours. (C) Comparison between the groups GB3 and GC3 in 30 days. (D) Comparison between the groups GC4 and GB4 in 90 days.

there were no significant changes in scores and morphological changes in neurons of the myenteric Control Groups (Fig. 4A). In GB1 (24 hours) myenteric neurons exhibited cytoplasmic vacuolation, indicating the beginning of the apoptotic process (Fig. 4B). In GB2 (48 hours) apoptotic cells and inflammatory were numerous (Fig. 4C). In GB3 (30 days) showed in Figure 4D, and GB4 (90 days) apoptosis cells were not observed. In these (GB3 and GB4) rarefaction of myenteric neurons was observed in areas close to the traumatic injuries of the mucosa. However, it was possible to observe the presence of GB4 hypertrophied residual myenteric neurons (Fig. 4E), possibly as a mechanism of neuroplasticity, the compensatory neuronal loss caused by chemical denervation.

In the Immunohistochemistry analysis, immunolabeling of H2AX was observed from 24 hours (GB1) (Fig. 5A) after application of the CB in the ileal segment, extending for a period of 48 hours (GB2) (Table 1, Fig. 5B).

DISCUSSION

The myenteric nerve block using CB is widely accepted and used in experimental studies by means of technique in which the application is done locally on the serosa of the viscera, using a

Table 1. Expression of positivity of the specific immunomarkers for evaluation of oxidative stress and apoptosis in ileum from wistar rats submitted to ileal myenteric denervation with benzalkonium chloride 0.3% by means of microinjections

Moment	Group	Positivity	
		H2AX	CASPASE-3
24 hours	GB1	***	-
48 hours	GB2	***	***
30 days	GB3	-	-
90 days	GB4	-	-

*** Positive immunolabeling, - absence of immunolabeling; GB = Benzalkonium Group.

gauze soaked with the substance and applied on the intestinal segment for a period of 30 minutes, followed by profuse lavage with saline 0.9% (Zucoloto et al. 1997, Hanani et al. 2003, Laranjeira et al. 2011, Yu et al. 2016). This technique has been applied to produce an aganglionose model, suggesting that these treatment parameters are ideal for the production of hipoganglia (Oliveira et al. 1990, Yu et al. 2016). However,

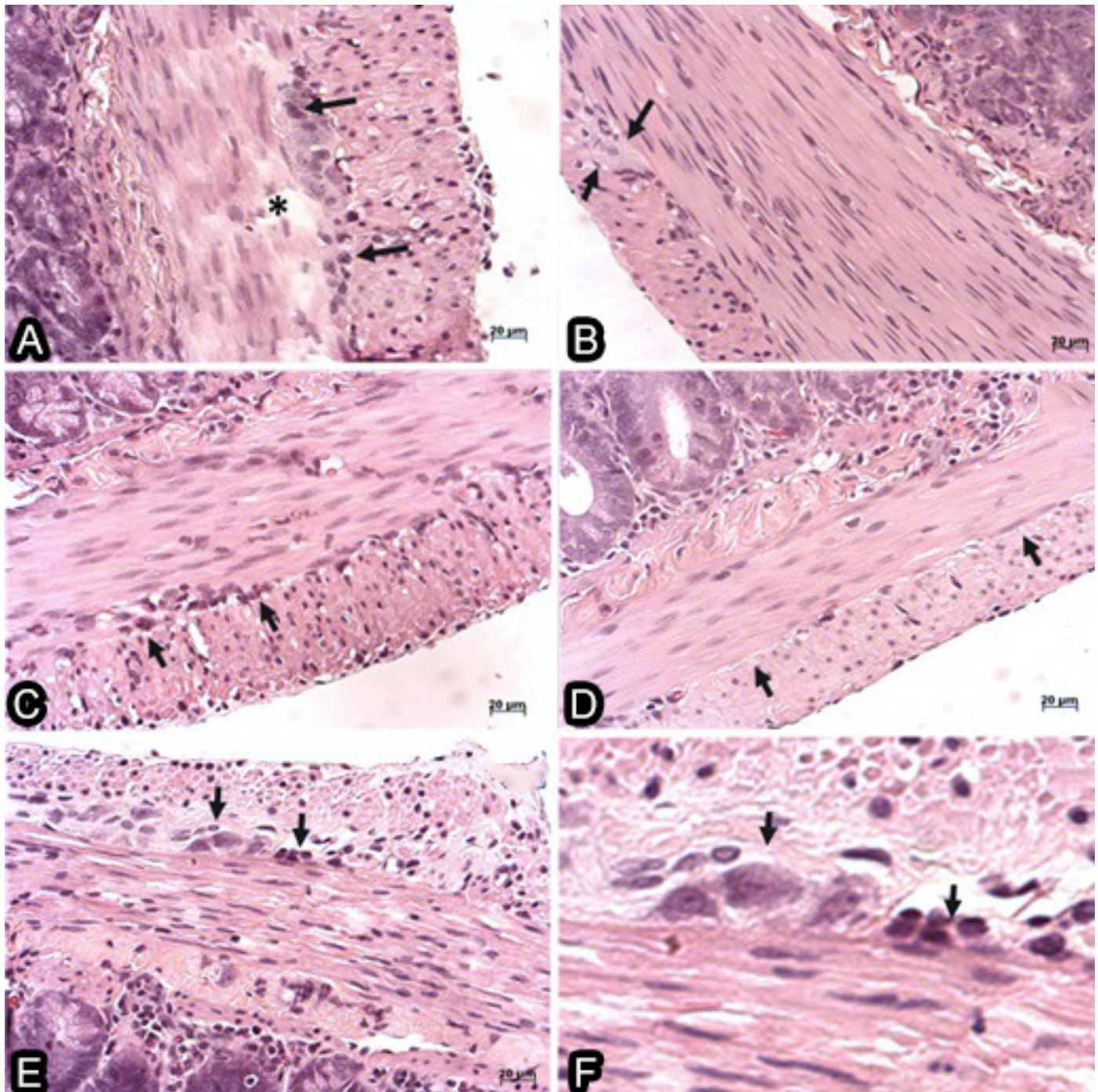


Fig.4. Ileal segment of wistar rat. (A) Control Group 24 hours, in the vicinity of traumatic lesions (asterisk), myenteric neurons with no morphometric changes (arrow). Giemsa stain, obj.40x. (B) GB1 (Benzalkonium Group), 24 hours, myenteric neurons with cytoplasmic vacuolation. Giemsa stain, obj.40x. (C) GB2, 48 hours, numerous apoptotic and inflammatory cells in the myenteric nodes (arrow). Giemsa stain, obj.40x. (D,E) GB3, 30 days, and GB4, 90 days, no cells in apoptosis are observed, important rarefaction of myenteric neurons in areas close to the traumatic injuries of the mucosa. Giemsa stain, obj.40x. (F) Hypertrophied residual myenteric neurons, can be viewed with nonspecific zoom at F (arrow). Giemsa stain, obj.40x.

this method of application is associated with complications such as peritonitis and small bowel adhesions near to the application site.

The toxicity of the CB is well known (Johnson 2018). It is known that low concentrations (0.1 to 0.5%) can cause

irritation in the conjunctiva and mucosas (Gosselin et al. 1984) membranes (Leo & Hollister 1991, Rowe et al. 2009, Papakrivou et al. 2017, Johnson 2018). Some authors (Marks & Deleo 1992) recommend caution when using this substance in body cavities. Low concentrations of CB does not damage the

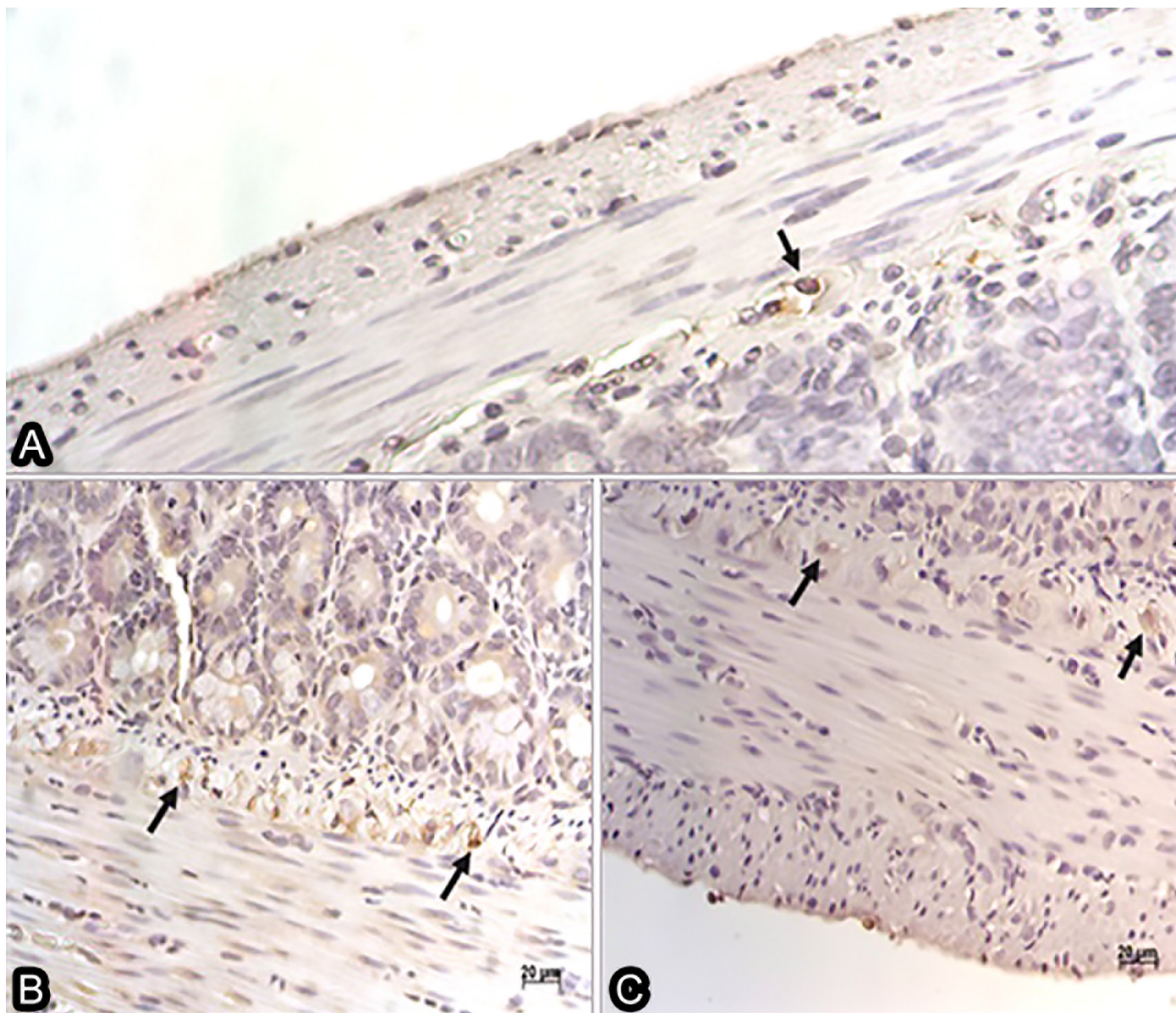


Fig.5. Immunohistochemistry of the diverticulum. (A) GB1 (Benzalkonium Group), 24 hours, and (B) GB2, 48 hours, intense nuclear H2AX expression in myenteric and submucosal neurons (arrows). (C) GB2, 48 hours, intense cytoplasmic marking of caspase-3 in myenteric and submucosal neurons (arrows). IHC, bar = 20µm.

intestinal smooth muscle layer, while higher concentrations can break through the muscle layers and cause inflammation and intestinal perforation (Fox et al. 1983, Papakrivou et al. 2017). Despite the lack of updated literature about complications associated with the use of this technique, we noted in earlier studies, the occurrence of peritonitis followed by adhesions in some animals when treated with CB 0.3% via local applications in different intestinal segments. Thus, this study brings the possibility of minimizing the rates of adverse complications because of intramural microinjections, which does not lead to direct contact of CB with adjacent organs and abdominal cavity.

The reduction in the number of myenteric neurons found in this study corroborates the findings of other studies (Sato et al. 1978, Sakata et al. 1979, Garcia et al. 1999, Carvalho et al. 2006, Fujimura et al. 2016, Yu et al. 2016,

Belkind-Gerson et al. 2017) as the application of CB in serosa of the intestinal segment provoked myenteric denervation. In our study, it was possible to promote such change with the use of the technique of intramural microinjections, being effective in the myenteric denervation with minor complications, since peritonitis or small bowel adhesions were not observed.

In this study, we observed a decrease in the number of myenteric neurons over the different times, being more evident at 30 and 90 days after denervation, corroborating previous findings (Garcia et al. 1995, Zucoloto et al. 1997, Fujimura et al. 2016, Yu et al. 2016) and other authors, which supported these results indicating that the application of 2mm solution of CB in the descending colon has reduced the number of neurons of the myenteric plexus in 10, 45 and 120 days after treatment compared to Control Groups.

After histopathological and immunohistochemical analysis in this study, it was possible to see the selective CB effect to cause apoptosis of myenteric neurons, also noticed by other authors (Yu et al. 2016). This fact might be noticed by neuronal count reduced over time and by specific H2AX immunolabeling and Caspase-3, corroborating the findings of other authors (Sato et al. 1978), who demonstrated that this fact occurs due to its positive charge. It is believed that the membrane of the nerve is more negative than the muscle membrane and thus the nerve fibers would be more susceptible to damage by cationic agent. However, according to the same authors, evidence supporting the notion that enteric neurotoxicity induced by surfactants can be attributed more to the general properties of the surfactants than the charge specifically. Another study (Sakata et al. 1979) found that CB has a detrimental effect on cell membranes causing depolarization or affecting the sodium active transport. Once the negative charge is known to be higher in nervous tissue than in other tissues such as the smooth muscles, this mechanism may explain the selective denervation occurred.

The positive assessment of H2AX detects oxidative stress, which may result in breaking the double stranded DNA and thus begin the apoptosis process, evaluated by immunolabeling Caspase-3 (Berra et al. 2006, Terzian et al. 2007, Cleaver 2011, Redon et al. 2012, Ghosal & Chen 2013, El-Aal et al. 2016). For verification of the effectiveness of denervation by CB, attesting the neuronal death and determination of the period in which this event occurred, was evaluated in this study, the presence of severe damage to genetic material, by means of immunohistochemical technique for detection of cells positively marked to H2AX, effective scorer in the presence of the double-stranded breaks of DNA (Sak & Stuschke 2010, Cleaver 2011, Ghosal & Chen 2013), characterizing the oxidative stress and the presence of neuronal apoptosis through immunolabeling of Caspase-3 in different times (24, 48 hours, 30 and 90 days). We confirm once the CB served for inducing neuronal oxidative stress lions, followed by apoptosis.

CONCLUSION

The application of benzalkonium chloride (CB) at 0.3% by means of intramural microinjections in the ileal wall is able to induce intrinsic chemical denervation of the diverticulum of wistar rats, through neuronal injury by oxidative stress beginning at 24 hours and neuronal apoptosis 48 hours after application.

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

REFERENCES

Belkind-Gerson J., Graham H.K., Reynolds J., Hotta R., Nagy N., Cheng L., Kamionek M., Shi H.N., Aherne C.M. & Goldstein A.M. 2017. Colitis promotes neuronal differentiation of Sox2+ and PLP1+ enteric cells. *Scient. Reports* 7(1):2525. <<http://dx.doi.org/10.1038/s41598-017-02890-y>> <PMid:28566702>

Berra C.M., Menck C.F.M. & Di Mascio P. 2006. Estresse oxidativo, lesões no genoma e processos de sinalização no controle do ciclo celular. *Química Nova* 29(6):1340-1344. <<http://dx.doi.org/10.1590/S0100-40422006000600032>>

Buttow N.C., Zucoloto S., Espreafico E.M., Gama P. & Alvares E.P. 2003. Substance P enhances neuronal area and epithelial cell proliferation after colon denervation in rats. *Digest. Dis. Sci.* 48(10):2069-2076. <<http://dx.doi.org/10.1023/A:1026103311800>> <PMid:14627357>

Carvalho C.E.V., Basile F.V.A., Vespúcio M.V.O., Iglesias A.C.G., Gava N.F. & Garcia S.B. 2006. Efeitos da desnervação intrínseca do jejuno após enterectomia extensa na síndrome do intestino curto em ratos. *Acta Cirúrg. Bras.* 21(1):43-46.

Cleaver J.E. 2011. γH2AX: Biomarker of damage of functional participant in DNA repair “all the glitters is not gold”. *Photochem. Photobiol.* 87(6):1230-1239. <<http://dx.doi.org/10.1111/j.1751-1097.2011.00995.x>> <PMid:21883247>

El-Aal A.A., El-Gebaly N., Al-Antably A., Hassan M. & El-Dardiry M. 2016. Post-immunization immunohistochemical expression of Caspase 3 and p53 apoptotic markers in experimental hydatidosis. *Revta Bras. Parasitol.* 25(3):333-340. <<http://dx.doi.org/10.1590/S1984-29612016058>> <PMid:27683842>

Fox D.A., Epstein M.L. & Bass P.A.U.L. 1983. Surfactants selectively ablate enteric neurons of the rat jejunum. *J. Pharmacol. Exp. Therap.* 227(2):538-544. <PMid:6195330>

Fujimura T., Shibata S., Shimojima N., Morikawa Y., Okano H. & Kuroda H. 2016. Fluorescence visualization of the enteric nervous network in a chemically induced aganglionosis model. *Plos One* 11(3):150-579. <<http://dx.doi.org/10.1371/journal.pone.0150579>> <PMid:26943905>

Garcia S.B., Pinto L.Z., Zucoloto S. & Oliveira J.S.M. 1995. Experimental megacolon. *Res. Exp. Med.* 195(4):249-253. <<http://dx.doi.org/10.1007/BF02576795>> <PMid:8525076>

Garcia S.B., Kawasaky M.C., Silva J.C., Garcia-Rodrigues A.C., Borelli-Bovo T.J., Iglesias A.C. & Zucoloto S. 1999. Intrinsic myenteric denervation: a new model to increase the intestinal absorptive surface in short-bowel syndrome. *J. Surg. Res.* 85(2):200-203. <<http://dx.doi.org/10.1006/jsre.1999.5670>> <PMid:10423319>

Ghosal G. & Chen J. 2013. DNA damage tolerance: a double-edged sword guarding the genome. *Translational Cancer Res.* 2(3):107-129. <PMid:24058901>

Gosselin R.E., Smith R.P. & Hodge H.C. 1984. *Clinical Toxicology of Commercial Products*. 5th ed. Williams and Wilkins, Philadelphia.

Hanani M., Ledder O., Yutkin V., Abu-Dalu R., Huang T., Härtig W., Vannucchi M. & Faussone-Pellegrini M. 2003. Regeneration of myenteric plexus in the mouse colon after experimental denervation with benzalkonium chloride. *J. Comp. Neurol.* 462(3):315-327. <<http://dx.doi.org/10.1002/cne.10721>> <PMid:12794735>

Johnson NF. 2018. Pulmonary toxicity of benzalkonium chloride. *J. Aerosol Med. Pulmonary Drug Delivery* 31(1):1-17. <<http://dx.doi.org/10.1089/jamp.2017.1390>> <PMid:28683210>

Laranjeira C., Sandgren K., Kessaris N., Richardson W., Potocnik A., Vanden Berghe P. & Pachnis V. 2011. Glial cells in the mouse enteric nervous system can undergo neurogenesis in response to injury. *J. Clin. Invest.* 121(9):3412-3424. <<http://dx.doi.org/10.1172/JCI58200>> <PMid:21865647>

Leo E., Hollister M.D. 1991. Drug evaluations annual 1991. *J. AMA* 266(3):424.

Marks J.G. & Deleo V.A. 1992. *Contact and Occupational Dermatology*. Mosby Year Book, Missouri, p.30-49.

Oliveira J., Llorach-Velludo M. & Sales-Neto V. 1990. Megacolon in rats. *Digestion* 45(3):166-171. <<http://dx.doi.org/10.1159/000200239>> <PMid:2373271>

Papakrivou E., Makris D., Manoulakas E., Mitroudi M., Tepetes K., Papazoglou K. & Zakyntinos E. 2017. Intra-abdominal hypertension causes bacterial growth in lungs: an animal study. *BioMed Res. Int.* 2017:1-7. <<http://dx.doi.org/10.1155/2017/4601348>> <PMid:28357400>

Redon C.E., Weyemi U., Parekh P.R., Huang D., Burrell A.S. & Bonner W.M. 2012. c-H2AX and other histone posttranslational modifications in the clinic. *Biochim. Biophys. Acta* 1819(7):743-756. <<http://dx.doi.org/10.1016/j.bbarm.2012.02.021>> <PMid:22430255>

Rowe R.C., Sheskey P.J. & Quinn M.E. 2009. *Handbook of Pharmaceutical Excipients*. 6th ed. Pharmaceutical Press, London, p.209-285.

- Sak A. & Stuschke M. 2010. Use of γ H2AX and other biomarkers of double-strand breaks during radiotherapy. *Semin. Radiation Oncol.* 20(4):223-231. <<http://dx.doi.org/10.1016/j.semradonc.2010.05.004>> <PMid:20832014>
- Sakata K., Kunieda T., Furuta T. & Sato A. 1979. Selective destruction of intestinal nervous elements by local application of benzalkonium chloride solution in the rat. *Experientia* 35(12):1611-1613. <<http://dx.doi.org/10.1007/BF01953222>> <PMid:520469>
- Sato A., Yamamoto M., Imamura K., Kashiki Y., Kunieda T. & Sakata K. 1978. Pathophysiology of aganglionic colon anal anorectum: na experimental study of aganglionosis produced by a new method in the rat. *J. Pediatr. Surg.* 13(4):399-435. <[http://dx.doi.org/10.1016/S0022-3468\(78\)80464-3](http://dx.doi.org/10.1016/S0022-3468(78)80464-3)> <PMid:682089>
- Terzian A.C., Zuccari D.A.P.C., Pereira R.S., Pavam M.V., Ruiz C.M., Sueiro F.A.R. & Coelho J. 2007. Avaliação da caspase-3 e Ki-67 como marcadores prognósticos nas neoplasias mamárias em cadelas. *Braz. J. Vet. Res. Anim. Sci.* 44(2):96-102. <<http://dx.doi.org/10.11606/issn.1678-4456.bjvras.2007.26647>>
- Yu H., Pan W., Wang H. & Gao Y. 2016. A time-limited and partially reversible model of hypoganglionosis induced by benzalkonium chloride treatment. *Neurochem. Res.* 41(5):1138-1144. <<http://dx.doi.org/10.1007/s11064-015-1806-8>> <PMid:26738989>
- Zucoloto S., Silva J.C., Oliveira J.S.M. & Muccillo G. 1991. The chronological relationship between the thickening of smooth muscle, epithelial cell proliferation and myenteric neural denervation in the rat jejunum. *Cell Proliferation* 24(1):15-20. <<http://dx.doi.org/10.1111/j.1365-2184.1991.tb01507.x>> <PMid:2009313>
- Zucoloto S., de Deus D.A., Martins A.A., Muglia V.F., Kajiwarra J.K. & Garcia S.B. 1997. The relation between myenteric neuronal denervation, smooth muscle thickening and epithelial cell proliferation in the rat colon. *Res. Exp. Med.* 197(2):117-124. <<http://dx.doi.org/10.1007/s004330050061>> <PMid:9380951>

Comparison between total intravenous anesthesia with propofol and intermittent bolus of tiletamine-zolazepam in capuchin monkey (*Sapajus apella*)¹

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ABSTRACT- Galante R., Carvalho E.R., Muniz J.A.P.C., Castro P.H.G., Gris V.N., Amora Júnior D.S. & Vilani R.G.D'O.C. 2019. **Comparison between total intravenous anesthesia with propofol or intermittent bolus of tiletamine-zolazepam in capuchin monkey (*Sapajus apella*)**. *Pesquisa Veterinária Brasileira* 39(4):271-277. Centro Nacional de Primatas, Evandro Chagas Instituto, Ministério da Saúde, Rodovia BR-316 Km 7, Ananindeua, PA 67030-000, Brazil. E-mail: beth_rcarvalho@hotmail.com

Dissociative anesthesia results in stressful and long recovery periods in monkeys and use of injectable anesthetics in medical research has to be refined. Propofol has promoted more pleasant wake up from anesthesia. The objectives of this study were to investigate the use of intravenous anesthetic propofol, establishing the required infusion rate to maintain surgical anesthetic level and comparing it to tiletamine-zolazepam anesthesia in *Sapajus apella*. Eight healthy capuchin monkeys, premedicated with midazolam and meperidine, were anesthetized with propofol (PRO) or tiletamine-zolazepam (TZ) during 60 minutes. Propofol was infused continually and rate was titrated to effect and tiletamine-zolazepam was given at 5mg/kg IV bolus initially and repeated at 2.5mg/kg IV bolus as required. Cardiopulmonary parameters, arterial blood gases, cortisol, lactate and quality and times to recovery were determined. Recovery quality was superior in PRO. Ventral recumbency (PRO = 43.0±21.4 vs TZ = 219.3±139.7 min) and normal ambulation (PRO = 93±27.1 vs TZ = 493.7±47.8 min) were faster in PRO ($p<0.05$). Cardiopulmonary effects did not have marked differences between groups. Median for induction doses of propofol was 5.9mg/kg, varying from 4.7 to 6.7mg/kg, Mean infusion rate was 0.37±0.11mg/kg/min, varying during the one-hour period. In TZ, two animals required three and five extra doses. Compared to tiletamine-zolazepam, minor post-anesthetic adverse events should be expected with propofol anesthesia due to the faster and superior anesthetic recovery.

INDEX TERMS: Intravenous anesthesia, propofol, intermittent bolus, tiletamine-zolazepam, capuchin monkey, *Sapajus apella*, recovery, refinement, primate, TIVA, clinics.

RESUMO.- [Comparação entre anestesia total intravenosa com propofol e bolus intermitente de tiletamina-zolazepam em macaco-prego (*Sapajus apella*).] A anestesia dissociativa em primatas resulta em recuperação anestésica lenta e estressante, e, portanto, o uso de anestesia injetável em

pesquisas médicas precisa ser refinado. Por outro lado, o propofol promove recuperação mais suave. Os objetivos desse estudo foram investigar o uso do anestésico intravenoso propofol, estabelecer a taxa de infusão contínua necessária para manter anestesia cirúrgica, e comparar tal técnica com a dissociativa tiletamina-zolazepam em *Sapajus apella*. Oito macacos-prego saudáveis foram pré-medicados com midazolam e meperidina, e posteriormente anestesiados com propofol (PRO) ou tiletamina-zolazepam (TZ) durante 60 minutos. O propofol foi administrado em infusão contínua, e a taxa foi titulada ao efeito, já a tiletamina-zolazepam foi administrada em 5mg/kg IV como *bolus* inicial, e repiques de 2,5mg/kg IV conforme necessário. Os parâmetros

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cardiopulmonares, hemogasometria arterial, cortisol, e lactato, além da qualidade e duração da recuperação anestésica foram determinados. A qualidade da recuperação anestésica foi superior em PRO. O tempo para atingir decúbito ventral (PRO = $43,0 \pm 21,4$ vs TZ = $219,3 \pm 139,7$ min) e ambulação normal (PRO = $93 \pm 27,1$ vs TZ = $493,7 \pm 47,8$ min) foram mais rápidos em PRO ($p < 0,05$). As variáveis cardiopulmonares não diferiram entre os grupos. A mediana para dose de indução com propofol foi de $5,9 \text{ mg/kg}$, variando de $4,7$ a $6,7 \text{ mg/kg}$. A taxa de infusão contínua média de propofol foi de $0,37 \pm 0,11 \text{ mg/kg/min}$, variando ao longo dos 60 minutos. Em TZ, dois animais necessitaram de três e cinco repiques. Comparado à tiletamina-zolazepam, menos efeitos adversos pós-anestésicos devem ser esperados com o propofol, devido à recuperação mais suave e rápida.

TERMOS DE INDEXAÇÃO: Anestesia total intravenosa, propofol, *bolus* intermitente, tiletamina-zolazepam, macaco-prego, *Sapajus apella*, recuperação, refinamento, primata, TIVA, clínica.

INTRODUCTION

Capuchin monkeys (*Sapajus apella*) are important nonhuman primates for biomedical research and the best neotropical primates at adapting to captivity (Domingues et al. 2003). Anesthesia for clinical and surgical procedures in captive primates is often required in zoos and research centers. The most common and widely established injectable protocols involve the use of dissociative agents (Olberg 2007, Vilani 2009). However, dissociative anesthesia presents long anesthetic recoveries after extended procedures, mainly when surgical anesthesia is required. Such recoveries are problematic when handling social species that require quick reintegration into the group (Galante et al. 2014) and a refinement of protocol is essential, minimizing stressful recuperation of anesthesia, once medical research is required.

We have recently described a shorter and smoother anesthetic recovery of a total intravenous anesthesia (TIVA) with continuous infusion of propofol in feline night monkeys (Galante et al. 2014). TIVA involves induction and maintenance of the anesthetic plan using only intravenous drugs (Camu et al. 2001). Propofol (2,6-diisopropylphenol) is one of the most advantageous drugs for this due to its rapid induction (Bufalari et al. 1995) and recovery (Smith et al. 1994, Ganem et al. 2002) without compromising the anesthetic plan. The rapid recovery occurs even in cases of prolonged administrations due to its rapid metabolism by the liver and also by other metabolic sites, especially the lungs, but also observed in kidney, intestine and brain (Matot et al. 1993, Court et al. 2001). The most common side effects are dose-dependent hypotension and ventilatory depression (Kotani et al. 2008, Paula et al. 2012).

The purpose of this research is to investigate the required infusion rate of propofol to maintain a surgical anesthetic level and compare this protocol to the dissociative tiletamine-zolazepam anesthesia, evaluating alterations in physiological parameters, recovery time and quality of anesthesia in capuchin monkeys.

MATERIALS AND METHODS

Capuchin monkeys (*Sapajus apella*) were captive born and belonged to the National Primate Center (CENP) breeding colony, located in the Municipality of Ananindeua, state of Pará, Brazil (latitude $1038'26''$

and longitude $48038'22''$). Eight specimens were used, four males and four females, with ages from four to 16 years. The monkeys were maintained in groups of four to six animals in indoor facilities of $3 \times 5 \times 2.5 \text{ m}$ accredited by the Governmental Institute of Environment and Natural Resources. Animal housing included a natural light and dark cycle, untreated water ad libitum via automatic valves and feeding of fresh fruits and vegetables and commercial pelleted feed (FOXY Junior Supreme, 28% protein; PROVIMI, São José dos Pinhais, Brazil). The room temperature was $24\text{--}30^\circ\text{C}$ in a humid tropical climate.

At the day of experiment, the monkeys were captured with hand nets, and immediately premedicated. Food, but not water, was withdrawn six hours before the experiment. Temperature of the experimental laboratory was controlled, ranging between 25 and 26°C . During anesthesia recovery, the specimens were housed in a $0.9 \times 0.8 \times 0.8 \text{ m}$ aluminum cage until normal ambulation, returning to original facilities at the same day. This study was conducted in accordance with the Brazilian National Council for Control of Animal Experimentation guidelines and approved by the Animal Use Ethics Committee of the Agricultural Sciences Campus of the Federal University of Paraná and by the Authorization and Information on Biodiversity System from the Brazilian Institute of Environment and Natural Resources.

Pre-anesthetic management. Preanesthetic medication consisted of 9 mg of meperidine (Dolosal® 50 mg/ml , Cristália, São Paulo/SP) and 1.2 mg of midazolam (Dormire® 5 mg/ml , Cristália, São Paulo/SP). After premedication, blood samples were collected for a complete blood count using an automated blood cell counter (CC-550, Celm, Brazil). Renal and hepatic biochemical profile included creatinine, urea, alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), total protein (TP), and electrolytes (sodium, chloride and calcium) were determined by dry chemistry (Vitros® DT60II, Johnson & Johnson Healthcare Systems, NY, USA) in serum sample.

Anesthesia. After weighing and cannulating the saphenous vein, monkeys were divided in propofol group (PRO) and tiletamine+zolazepam group (TZ). Randomization was performed by pulling a piece of paper from a bag with either group written on it. Weights, age, and other parameters were not determined for group assignment prior to capture and therefore randomized. The individuals responsible for initial capture and restraint were blinded to group divisions.

In TZ, 5 mg/kg of tiletamine+zolazepam (Zoletil® 50, Virbac, Brazil) was administered intravenously. Saline was infused at 10 ml/kg/h with a peristaltic roller pump (LF2001, Lifemed, Brazil). In PRO, anesthetic induction was performed with using a syringe infusion pump (PSK-01, Nikkiso Co., Japan) at 2 mg/kg/min of propofol (Propovan® 10 mg/ml , Cristália, São Paulo/SP) until complete loss of palpebral reflexes. Saline solution was administered at a rate of 10 ml/kg/h in contralateral saphenous vein. A laryngeal mask was placed, with oxygen flow of 1 L/min , administered through a Mapleson D Rees-Baraka non-rebreathing system, with spontaneous breathing. During anesthesia, animals were lying in dorsal recumbency.

A scale was used to determine anesthetic depth and determined the adjustment of the maintenance dose in order to provide similar depth of anesthesia. Scores from 1 to 4 were given based on the following scale: 1) poor muscle relaxation, intense palpebral reflex or voluntary movements of the tail, limbs or head; 2) no substantial changes in cardiopulmonary parameters (ranging less than 10% from the first measurement), complete relaxation, tongue can be exteriorized, no movements of the tail, limbs or head, slow palpebral reflex; 3) no substantial changes in cardiopulmonary parameters (ranging less than 10% from the first measurement), complete relaxation, tongue can

be exteriorized, complete loss of palpebral reflexes and jaw muscle tone, no movements of the tail, limbs or head; 4) anesthetic plane is too deep, no reflexes or cardiopulmonary depression (at least two parameters 10% lower than the first measurement).

In TZ, when anesthetic depth was 1, 2.5mg/kg of tiletamine+zolazepam was administered IV. In PRO, an infusion of propofol (0.4mg/kg/min) was started the induction of anesthesia. In order to maintain an anesthetic score of 3, the infusion rate was increased by 0.1mg/kg/min increments when the score was 2, or by 0.2mg/kg/min when the score was 1, and decreased by 0.1mg/kg/min when a score was 4. Anesthesia was continued for 60 minutes and changes in propofol infusion rate, when necessary, were made every 5 minutes.

Plasma cortisol concentrations were measured by means of a commercial ELISA kit (DBC, Diagnostics Biochem, Canada) in the beginning (0 minute, immediately after induction of anesthesia) and end (60 minutes) of anesthesia. Cortisol was evaluated as the percentage of increase or decrease of the concentration. Every 5 minutes the animal had the following parameters evaluated: heart rate (HR), respiratory rate (fR), non-invasive systolic (SAP), mean (MAP) and diastolic (DAP) arterial blood pressures, rectal temperature and arterial oxygen saturation of hemoglobin (SpO₂) using a veterinary portable multi-parameter monitor (MEC-1200Vet, Mindray Medical International, China). Non-invasive arterial blood pressures were measured by the oscillometric method with a number 2 sized cuff placed around the right arm. At the end of the 60 minutes' period, an arterial blood sample was collected from the femoral artery for blood gas analysis using a portable analyzer (EPOC Blood Analysis™, EPOCAL Inc., Canada). Parameters evaluated were: ionized calcium, bicarbonate (HCO₃), potassium, sodium, pH, arterial partial pressures of carbon dioxide (PaCO₂) and oxygen (PaO₂), base excess (BE), glucose and lactate concentrations.

After 60 minutes of anesthesia, infusion was stopped, the laryngeal mask was removed and the animal was put on a table in dorsal recumbency, without any physical restraint.

Recovery. Anesthesia recovery times recorded were: time to first voluntary movement, time to voluntary lateral recumbency, time to voluntary ventral recumbency and time to normal ambulation. The subjects were transferred to the recovery cage immediately after ventral recumbency was observed. A score for the quality of anesthetic recovery was given based on the following: excellent - recumbency

change with minimal ataxic movements, animal gets up in one or two attempts and it is able to ambulate with only slight ataxia; good - recumbency change with moderate ataxic movements, requiring more than two attempts to get up, ambulates with moderate ataxia and incoordination; average - makes several attempts for recumbency change with severe ataxia, tries to get up but often falls down and finally walks with notable ataxia and incoordination; poor - animal stays on the same recumbency for more than 30 minutes after the end of anesthesia, it is not responsive to stimuli and makes no effort to change recumbency, or has a very agitated recovery with severe ataxia and risk of injury.

Normality of data was verified by Kolmogorov-Smirnov test. Statistical analyses of parametric values were performed comparing groups using the t-test. The physiological parameters analyzed continuously during the anesthesia were tested using analysis of variance (ANOVA). When statistical differences were observed, mean values were compared by Tukey-Kramer post-hoc test. StatView 5.0.1 (SAS Institute Inc., NC, USA) was used. Quality of anesthetic recovery was analyzed by Fisher's exact test.

RESULTS

Preanesthetic testing and preanesthetic medication

Animals aged from four to 16 years old composed both groups. Each group was formed by two males and two females. The mean weight of PRO was 3.0±0.6 and of TZ was 3.1±0.9 kg. There were no statistical differences in ages or weights between groups. Hematologic and serum biochemistry values were similar in both groups (Table 1). Preanesthetic medication doses ranged from 2.3 to 4.6mg/kg of meperidine and 0.30 to 0.61mg/kg of midazolam. This protocol provided enough sedation to allow easy venous catheter placement with only light physical restraint.

Propofol infusion rates

The median time for induction of anesthesia in PRO was 177.5 seconds, ranging from 140 to 200 seconds, resulting in median induction dose of 5.9mg/kg, varying from 4.7 to 6.7mg/kg, during total induction period until complete loss of palpebral reflex. The mean infusion rate during anesthesia maintenance

Table 1. Hematological and biochemical values measured in capuchin monkeys (*Sapajus apella*) before anesthesia (n=8), mean ± SD

Parameter	Group		p-value
	PRO	TZ	
Ht (%)	38.6 ± 2.0	39.4 ± 7.1	0.814
Hb (g/dl)	12.5 ± 0.6	12.6 ± 2.3	0.936
TP (g/dl)	7.5 ± 0.5	7.4 ± 0.4	0.836
Ca (mmol/l)	2.1 ± 0.1	2.12 ± 0.08	0.454
Sodium (mmol/l)	147.3 ± 8.5	148 ± 3.5	0.876
Chloride (mmol/l)	115 ± 4.2	117 ± 2.5	0.451
Creatinine (mg/dl)	0.91 ± 0.15	0.96 ± 0.12	0.589
Urea (mg/dl)	26 ± 11.1	45 ± 16	0.102
AST (U/l)	25.3 ± 21.1	56.5 ± 42.8	0.239
ALT (U/l)	40.3 ± 26	78.2 ± 49.3	0.222
ALP (U/l)	60.8 ± 17.7	72.5 ± 19.1	0.401
GGT (U/l)	89 ± 66.6	61.5 ± 21.8	0.462

PRO = propofol group, TZ = tiletamine+zolazepam group, Ht = hematocrit, Hb = hemoglobina, TP = total plasma protein, Ca = calcium, AST = aspartate aminotransferase, ALT = alanine aminotransferase, ALP = alkaline phosphatase, GGT= gamma-glutamyltransferase.

was $0.37 \pm 0.11 \text{ mg/kg/min}$, varying during the one-hour period, according to the Figure 1.

Tiletamine+zolazepam volume

All animals were induced with 5 mg/kg tiletamine+zolazepam, IV. Two animals did not require any extra dose to maintain anesthetic level. The other two animals needed three and five extra doses of tiletamine+zolazepam (2.5 mg/kg), IV, to maintain the proposed anesthetic level.

Physiological parameters

Continuous physiological parameters evaluated are shown in Table 2. There were no statistically significant differences in HR between groups. In TZ, HR decreases significantly in the last 10 minutes of anesthesia when compared to the 5 minutes' assessment ($p < 0.05$). fR was higher in TZ than in PRO, presenting statistical difference at 35, 45 and 60 minutes ($p < 0.05$). No significant differences were found in arterial blood pressure and SpO_2 . The temperature decreased in both groups in relation to the beginning of anesthesia, presenting

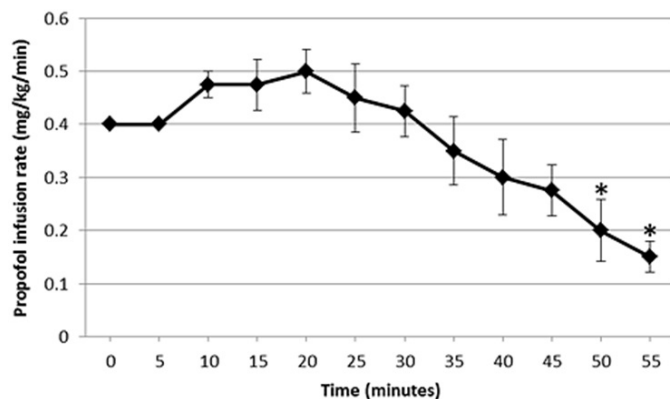


Fig.1. Propofol infusion rate variation ($n=4$) during 1-hour of anesthesia in capuchin monkeys (*Sapajus apella*) premedicated with pethidine and midazolam. Mean $\pm$ SD.

faster decreases in PRO ($p < 0.05$), but no significant difference between groups was noticed at the end of procedure.

Arterial blood gas analysis and cortisol

Both groups presented moderate acidemia at the end of anesthesia, as high base deficit, with clinically important higher values in TZ. Relatively high PCO_2 and low HCO_3^- were found. There were PaO_2 increased in both groups, however higher values are found in TZ group ($p < 0.05$). Lactate was also high in both groups. All the values are presented in Table 3.

Different levels of cortisol were observed during the day, with the lowest values at the beginning of the morning ($65.22 \mu\text{g/dL}$), and the highest values at the middle of the day ($354.58 \mu\text{g/dL}$). In both groups, it was observed increasing in the cortisol level during the anesthesia, but no statistical difference was noticed in the mean percentage of raise in cortisol levels between TZ (70.7 ± 118.8) and PRO (62.2 ± 46).

Recovering

The anesthesia recovery evaluation is shown in Table 4. There was no statistical difference in time of first voluntary movements or posture repositioning. However, the times to postural recovery and normal ambulation were significantly faster in PRO ($p < 0.05$). The anesthetic recovery quality was considered excellent in 4 out of 4 animals of PRO, and average in 2 out of 4 and poor in 2 out of 4 monkeys of TZ.

DISCUSSION

The use of animals in medical research follow guidelines to consideration and, if feasible, implementation of replacement, refinement and reduction methods. This means that, if possible, experiments have to be performed without animals (Replacement), with fewer animals (Reduction) and/or with less pain/distress for the animals (Refinement) (Russell & Burch 1959, Luijk et al. 2013). Because of the easy intramuscular administration and effective chemical restraint, dissociative drugs as ketamine and tiletamine have been preferred in worldwide primate facilities. However, abnormal behavior,

Table 2. Physiologic variables recorded in capuchin monkeys (*Sapajus apella*) during 1-hour of propofol infusion (PRO, $n=4$) or tiletamine+zolazepam (TZ, $n=4$) anesthesia, mean $\pm$ SD

Time (min)	HR (beats/minute)		fR (breaths/minute)		SAP (mmHg)		MAP (mmHg)		DAP (mmHg)		SpO_2 (%)		Temp ($^{\circ}\text{C}$)	
	PRO	TZ	PRO	TZ	PRO	TZ	PRO	TZ	PRO	TZ	PRO	TZ	PRO	TZ
5	202 $\pm$ 42	236 $\pm$ 37	37 $\pm$ 9	40 $\pm$ 5	108 $\pm$ 12	121 $\pm$ 9	79 $\pm$ 12	96 $\pm$ 9	55 $\pm$ 17	73 $\pm$ 11	97 $\pm$ 0	97 $\pm$ 1	37.9 $\pm$ 0.4	38.6 $\pm$ 0.3
10	168 $\pm$ 43	227 $\pm$ 27	30 $\pm$ 8	43 $\pm$ 14	109 $\pm$ 12	108 $\pm$ 6	78 $\pm$ 13	78 $\pm$ 6	54 $\pm$ 16	60 $\pm$ 6	98 $\pm$ 2	98 $\pm$ 2	37.4 $\pm$ 0.5	38.4 $\pm$ 0.2*
15	172 $\pm$ 36	221 $\pm$ 23	33 $\pm$ 10	35 $\pm$ 10	103 $\pm$ 11	105 $\pm$ 3	78 $\pm$ 10	77 $\pm$ 8	50 $\pm$ 15	57 $\pm$ 9	99 $\pm$ 2	97 $\pm$ 2	37.2 $\pm$ 0.3	38.1 $\pm$ 0.2*
20	162 $\pm$ 41	211 $\pm$ 19	27 $\pm$ 8	41 $\pm$ 11§	104 $\pm$ 7	107 $\pm$ 5	74 $\pm$ 11	81 $\pm$ 9	54 $\pm$ 15	58 $\pm$ 11	98 $\pm$ 2	99 $\pm$ 1	36.9 $\pm$ 0.3	37.7 $\pm$ 0.3
25	164 $\pm$ 36	207 $\pm$ 17	31 $\pm$ 5	39 $\pm$ 11	102 $\pm$ 10	110 $\pm$ 8	74 $\pm$ 11	83 $\pm$ 10	54 $\pm$ 10	61 $\pm$ 12	98 $\pm$ 2	98 $\pm$ 2	36.6 $\pm$ 0.2#	37.4 $\pm$ 0.3
30	174 $\pm$ 23	202 $\pm$ 14	27 $\pm$ 9	36 $\pm$ 17	101 $\pm$ 10	106 $\pm$ 9	79 $\pm$ 10	80 $\pm$ 10	54 $\pm$ 8	62 $\pm$ 11	98 $\pm$ 2	97 $\pm$ 2	36.3 $\pm$ 0.2#	37.1 $\pm$ 0.3
35	171 $\pm$ 26	197 $\pm$ 8	26 $\pm$ 4	36 $\pm$ 3*	103 $\pm$ 10	108 $\pm$ 10	78 $\pm$ 8	81 $\pm$ 10	50 $\pm$ 11	63 $\pm$ 10	99 $\pm$ 2	97 $\pm$ 2	36.1 $\pm$ 0.2#	36.8 $\pm$ 0.4
40	169 $\pm$ 29	196 $\pm$ 9	28 $\pm$ 5	37 $\pm$ 11	100 $\pm$ 9	107 $\pm$ 9	77 $\pm$ 8	79 $\pm$ 8	58 $\pm$ 9	64 $\pm$ 11	99 $\pm$ 2	96 $\pm$ 2	35.8 $\pm$ 0.2#	36.4 $\pm$ 0.3#
45	160 $\pm$ 28	192 $\pm$ 6	28 $\pm$ 2	45 $\pm$ 13*	101 $\pm$ 10	106 $\pm$ 7	76 $\pm$ 9	78 $\pm$ 5	52 $\pm$ 12	61 $\pm$ 7	99 $\pm$ 2	96 $\pm$ 2	35.6 $\pm$ 0.2#	36.2 $\pm$ 0.3#
50	158 $\pm$ 26	184 $\pm$ 6#	24 $\pm$ 9	41 $\pm$ 18	103 $\pm$ 8	106 $\pm$ 8	75 $\pm$ 8	79 $\pm$ 9	53 $\pm$ 6	59 $\pm$ 8	99 $\pm$ 2	97 $\pm$ 2	35.5 $\pm$ 0.2#	35.9 $\pm$ 0.4#
55	163 $\pm$ 30	182 $\pm$ 9#	28 $\pm$ 5	35 $\pm$ 17	104 $\pm$ 3	107 $\pm$ 9	76 $\pm$ 7	82 $\pm$ 10	56 $\pm$ 12	59 $\pm$ 16	99 $\pm$ 2	98 $\pm$ 1	35.2 $\pm$ 0.2#	35.8 $\pm$ 0.4#
60	152 $\pm$ 33	177 $\pm$ 11#	26 $\pm$ 3	38 $\pm$ 7*	109 $\pm$ 5	120 $\pm$ 17	81 $\pm$ 4	88 $\pm$ 14	53 $\pm$ 11	66 $\pm$ 9	99 $\pm$ 2	97 $\pm$ 2	35.1 $\pm$ 0.2#	35.5 $\pm$ 0.3#

* Difference between groups $p < 0.05$, # Difference inside the group (in relation to baseline at 5 minutes) $p < 0.05$; HR = heart rate, fR = respiratory rate, SpO_2 = hemoglobin oxygen saturation, Temp = temperature, SAP = systolic arterial pressure, MAP = mean arterial pressure, DAP = diastolic arterial pressure.

Table 3. Arterial blood gas values and serum electrolytes in capuchin monkeys (*Sapajus apella*) at the end of 1- hour of propofol (PRO, n=4) or tiletamine+zolazepam (TZ, n=4) anesthesia, mean $\pm$ SD

Parameter	Group		p-value
	PRO	TZ	
pH	7.238 $\pm$ 0.062	7.191 $\pm$ 0.020	0.195
PaCO ₂ (mmHg)	47.7 $\pm$ 9.7	48.0 $\pm$ 3.5	0.963
PaO ₂ (mmHg)	476 $\pm$ 64	598 $\pm$ 47*	0.022
HCO ₃ (mmol L ⁻¹)	19.4 $\pm$ 3.3	17.7 $\pm$ 1.1	0.361
BE (mmol L ⁻¹)	-6.9 $\pm$ 3.2	-9.1 $\pm$ 1.0	0.227
SaO ₂ (%)	99.9 $\pm$ 0.1	99.8 $\pm$ 0.1	0.278
Sodium (mmol L ⁻¹)	147 $\pm$ 6	151 $\pm$ 3	0.339
Potassium (mmol L ⁻¹)	4.6 $\pm$ 1.2	5.2 $\pm$ 0.6	0.351
Ionized Calcium (mmol L ⁻¹)	1.8 $\pm$ 0.2	2.0 $\pm$ 0.1	0.235
Total Serum Calcium (mmol L ⁻¹)	2.1 $\pm$ 0.1	2.1 $\pm$ 0.1	0.550
Glucose (mg dL ⁻¹)	85 $\pm$ 17	81 $\pm$ 16	0.786
Lactate (mg dL ⁻¹)	5.4 $\pm$ 0.1	6.9 $\pm$ 2.5	0.252

PaCO₂ = arterial partial pressure of carbon dioxide, PaO₂ = arterial partial pressure of oxygen, HCO₃ = bicarbonate, BE = base excess, SaO₂ = oxygen saturation; * Difference between groups $p < 0.05$.

Table 4. Times (minutes) to stages of recovery from 1-hour of propofol (PRO, n=4) or tiletamine+zolazepam (TZ, n=4) anesthesia in capuchin monkeys (*Sapajus apella*), mean $\pm$ SD

Recovery times (min)	Group		p-value
	PRO	TZ	
Time to first voluntary movement	9.2 $\pm$ 5.6	18.5 $\pm$ 9.2	0.277
Time to lateral recumbency	19.2 $\pm$ 16.5	50.7 $\pm$ 32.9	0.153
Time to ventral recumbency	43 $\pm$ 21.4	244.0 $\pm$ 97.0*	0.009
Time to normal ambulation	93 $\pm$ 27.1	493.7 $\pm$ 47.8*	<0.001

* Difference between groups $p < 0.05$.

which may progress to delirium, may occur during emergence from dissociative anesthesia (Lin 2007). Depression of the inferior colliculus and medial geniculate nucleus leading to misperception of auditory and visual stimuli may be responsible for this reaction (White et al. 1982). Emergence reactions are characterized by ataxia, increased motor activity, hyperreflexia, sensitivity to touch, and sometimes violent recovery (Wright 1982). These reactions usually disappear within several hours without recurrence (Amend et al. 1972). Because of this, refinement of anesthesia, excluding use of dissociative anesthetics to promote unconsciousness, needs to be discussed.

On the other hand, the greatest advantage in the use of propofol is the quality of anesthesia recovery. It propitiates smooth return to consciousness, besides that, it presents high clearance rate, with fast metabolism by the hepatic and extra hepatic routes, with no active metabolites described, properties that favor a rapid recovery from anesthesia, depending on the duration of infusion (Aguiar 2010, Jimenez et al. 2012). Recovery was remarkably faster and more pleasant with continuous infusion of propofol in these capuchin monkeys. All specimens anesthetized with propofol were able to get up in first attempt with only slight ataxia during early ambulation.

It was considered complete recovery when the animal returns to normal ambulation, which is the moment when it is possible to the monkey to go back to its group without any social problems. Monkeys that received dissociative anesthesia spent at least seven hours to return to original facilities after anesthesia, and their recovery was characterized by very agitated awakening with severe ataxia and risk of injury in two animals and by several attempts to get up, but often falling and notable ataxia and incoordination in other two. Compared to dissociative anesthesia, it is expected less abnormal events in recovery with propofol, after anesthetic procedures and faster return of animals to their cages.

Cortisol, the principal glucocorticoid in primates, can be an important indicator of pathophysiological processes, and the cortisol response to an event is often considered a useful indicator of stress (Bentson et al. 2003). High absolute basal levels of cortisol characterize New World primate species. Moreover, capuchin monkeys, as other primates, have shown to present increased cortisol levels in response to handling (Dettmer et al. 1996). Cortisol is capable of increasing in response to capture and chair restraint and turnover rate of cortisol is extremely high (Brown et al. 1970). It may be secreted as a direct response to an environmental or social stress and it may also be considered an enhancer for rapid physical response, as it mobilizes circulating glucose to provide readily available energy for muscular activity (Sapolsky 1992, Lynch et al. 2002).

Since it is known that cortisol levels change during the day (Lynch et al. 2002), it is erroneous to compare serum levels among animals that were anesthetized in different periods. We only used the variation between the start and the end of anesthesia. However, blood sample was collected before recovery and this method did not permit we observe the impact of that in cortisol response.

Both experimental groups presented similar conditions of weight, health and sex. Pre-anesthetic testing was performed just to assure health status, and all the results are under normal reference range for the species (Wirz & Riviello 2008). Premedication with meperidine and midazolam permitted

satisfactory manipulation and instrumentation, making the venous cannulation easier.

The 2mg/kg/min rate of propofol for induction was able to abolish palpebral reflex between 140 and 200 seconds. Administration of induction doses of propofol over 2 minutes provides a reasonable rate and improved titration to effect, yet avoids excessively high arterial concentrations and cardiovascular and ventilatory depressive effects (Ludbrook et al. 1998). The maintenance infusion rate of propofol had to be lightly increased until 20 minutes of anesthesia, probably the moment of compartmental equilibrium, and then reduced, reaching statistically significant difference in 50 and 55 minutes. This behavior was different from the observed in feline night monkeys, where infusion rates above 0.4mg/kg/min of propofol were necessary until the end of anesthesia (Galante et al. 2014). A variable-rate infusion, in which the rate is altered according to the current or anticipated stimulus, is more useful clinically than a constant rate infusion (Shafer et al. 1988). Nevertheless, in case of total intravenous anesthesia to be performed using a constant rate infusion, rates lower than 0.4 mg/kg/min will be enough after 20 minutes to capuchin monkeys.

Tiletamine-zolazepam is known to cause cardiovascular stimulation (Hall et al. 2001), as long as the adrenergic system is intact (Paddleford 1999). The clinically, but not statistically, high HR in TZ group, observed in beginning of anesthesia, is attributed to enhanced sympathetic tone and perhaps decreased vagal tone (Thurmon et al. 1996, Saha et al. 2007). HR decreased progressively due to the consumption of catecholamines. Otherwise, even presenting the highest HR in the first evaluation in PRO, probably because of the stress caused by initial manipulation, HR in PRO remained around a constant and physiological rate.

Parasympathetic activity promoted by propofol can decrease arterial blood pressure. That has been attributed to a decrease in systemic vascular resistance or in cardiac output caused by a combination of venous and arterial vasodilation, impaired baroreflex mechanisms, and depression of myocardial contractility (Kanaya et al. 2003, Win et al. 2005). However, all these effects seem to be dose-dependent (Zausig et al. 2009), and using titrated continuous rate infusion we did not observed any statistical difference in arterial blood pressure.

Minor respiratory alteration occurred either with propofol or tiletamine-zolazepam anesthesia. In the present study, arterial blood gas analysis was not performed in the beginning of anesthesia, but slight high values in PCO₂ in the end of the procedure were detected. This is an expected alteration in a long-term anesthesia with spontaneous breathing and stronger alterations are reported with propofol and dissociative anesthesia with this condition (Booker Junior et al. 1982, Hou et al. 1992). As anesthesia with propofol is likely to be accompanied by respiratory depression and hypotension, further studies with a larger number of animals could show alterations that were not reported in this research.

Capuchin monkeys have social behavior and are caged in groups but, even being a recurrent procedure, capture of the animals for drug administration was slow and slightly stressful. High lactic acid production caused by intense muscular activity during capture, indicated by elevated lactate levels, induced metabolic acidosis. Low pH and base excess illustrate metabolic acidosis, which are common findings in physically

restrained primates (Bush et al. 1977, Galante et al. 2014). As other physiological parameters did not show important modifications during anesthesia to justify the observed blood gas alterations, we are assuming that this metabolic acidosis had happened due to stressful capture.

CONCLUSIONS

Propofol induced complete unconsciousness in capuchin monkey after 173.7±26.8 seconds at 2mg/kg/min infusion rate and the mean rate to maintain a 60 minutes anesthesia was 0.37±0.11mg/kg/min, despite the necessity of titrate this rate during the procedure.

Compared to the well-known tiletamine-zolazepam protocol, closed physiological alterations were observed, but faster and smoother anesthetic recovery and minor post-anesthetic unpleasant events are expected with propofol anesthesia.

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REFERENCES

- Aguiar A.A. 2010. Anestesia intravenosa total, p.275-297. In: Fantoni D.T & Cortopassi S.R.G (Eds), Anestesia em Cães e Gatos. 2ª ed. Roca, São Paulo.
- Amend J.F., Klavano P.A. & Stone E.C. 1972. Premedication with xylazine to eliminate muscular hypertonicity in cats during ketamine anesthesia. Vet. Med. Small Anim. Clin. 67(12):1305-1307. <PMid:4486539>
- Bentson K.L., Capitano J.P. & Mendoza S.P. 2003. Cortisol responses to immobilization with Telazol or ketamine in baboons (*Papio cynocephalus/anubis*) and rhesus macaques (*Macaca mulatta*). J. Med. Primatol. 32(3):148-160. <http://dx.doi.org/10.1034/j.1600-0684.2003.00018.x> <PMid:12823625>
- Booker Junior J.L., Erickson H.H. & Fitzpatrick E.L. 1982. Cardiodynamics in the rhesus macaque during dissociative anesthesia. Am. J. Vet. Res. 43(4):671-675. <PMid:7073088>
- Brown G.M., Grota L.J., Penney D.P. & Reichlin S. 1970. Pituitary-adrenal function in the squirrel monkey. Endocrinology 86(3):519-529. <http://dx.doi.org/10.1210/endo-86-3-519> <PMid:4312110>
- Bufalari A., Nilsson L.E., Short C.E. & Giannoni C. 1995. A comparative study of neurologically-equivalent propofol anaesthetic combinations in the dog. J. Vet. Anaesth. 22(1):19-24. <http://dx.doi.org/10.1111/j.1467-2995.1995.tb00137.x>
- Bush M., Custer R., Smeller J. & Bush L.M. 1977. Physiologic measures of nonhuman primates during physical restraint and chemical immobilization. J. Am. Vet. Med. Assoc. 171(9):866-869. <PMid:21868>
- Camu F., Lauwers M. & Vanlersberghe C. 2001. Anestesia venosa total, p.370-386. In: White P.F (Ed), Tratado de Anestesia Venosa. Artmed, Porto Alegre.
- Court M.H., Duan S.X., Hesse L.M., Venkatakrishnan K. & Greenblatt D.J. 2001. Cytochrome P-450 2B6 is responsible for interindividual variability of propofol hydroxylation by human liver microsomes. Anesthesiology 94(1):110-119.
- Dettmer E.L., Phillips K.A., Rager D.R., Bernstein I.S. & Fragaszy D.M. 1996. Behavioral and cortisol responses to repeated capture and venipuncture in *Cebus apella*. Am. J. Primatol. 38(4):357-362. <http://dx.doi.org/10.1002/(SICI)1098-2345(1996)38:4<357::AID-AJP6>3.0.CO;2-Y>
- Domingues S.F.S., Ferreira H.S., Muniz J.A.P.C., Lima A.K.F., Ohashi O.M., Figueiredo J.R. & Silva L.D.M. 2003. Mechanical isolation of capuchin monkey (*Cebus*

- apella*) preantral ovarian follicles. Arq. Bras. Med. Vet. Zootec. 55(3):301-308. <<http://dx.doi.org/10.1590/S0102-09352003000300009>>
- Galante R., Muniz J.A.P.C., Castro P.H.G., Amora Jr. D.S., Gris V.N., Carvalho E.R. & Vilani R.G.D'O.C. 2014. Continuous infusion of propofol or intermittent bolus of tiletamine-zolazepam in feline night monkeys (*Aotus infulatus*). J. Med. Primatol. 43(2014):22-30.
- Ganem E.M., Fukushima F.B., Silva D.S., Nakamura G., Castiglia Y.M. & Vianna P.T. 2002. Efficacy of propofol and propofol plus dexamethasone in controlling postoperative nausea and vomiting of gynecologic laparoscopy. Revta Bras. Anesthesiol. 52(4):394-401. <PMid:19479103>
- Hall L.W., Clarke K.W. & Trim C.M. 2001. Veterinary Anaesthesia. 10th ed. W.B. Saunders Company, London. 576p.
- Hou W.Y., Huang F.Y., Sun W.Z., Susetio L., Chen C.L., Liang H.C. & Huang C.H. 1992. The effect of total intravenous propofol on spontaneous respiration during anesthesia for minor surgery. Ma Zui Xue Za Zhi 30(1):7-11. <PMid:1608323>
- Jiménez C.P., Mathis A., Mora S.S., Brodbelt D. & Alibhai H. 2012. Evaluation of the quality of the recovery after administration of propofol or alfaxalone for induction of anaesthesia in dogs anaesthetized for magnetic resonance imaging. Vet. Anaesth. Analg. 39(2):151-159. <<http://dx.doi.org/10.1111/j.1467-2995.2011.00678.x>> <PMid:22117934>
- Kanaya N., Hirata N., Kurosawa S., Nakayama M. & Namiki A. 2003. Differential effects of propofol and sevoflurane on heart rate variability. Anesthesiology 98(1):34-40. <<http://dx.doi.org/10.1097/00000542-200301000-00009>> <PMid:12502976>
- Kotani Y., Shimazawa M., Yoshimura S., Iwama T. & Hara H. 2008. The experimental and clinical pharmacology of propofol, an anesthetic agent with neuroprotective properties. CNS Neurosci. Therap. 14(2):95-106. <<http://dx.doi.org/10.1111/j.1527-3458.2008.00043.x>> <PMid:18482023>
- Lin H.C. 2007. Dissociative anesthetics, p.301-354. In: Tranquilli W.J., Thurmon J.C. & Grimm K.A. (Eds), Lumb & Jones' Veterinary Anesthesia and Analgesia. 3rd ed. Blackwell Publishing, Ames.
- Ludbrook G.L., Upton R.N., Grant C. & Martinez A. 1998. The effect of rate of administration on brain concentrations of propofol in sheep. Anesth. Analg. 86(6):1301-1306. <PMid:9620524>
- Luijk J., Cuijpers Y., van der Vaart L., De Roo T.C., Leenaars M. & Ritskes-Hoitinga M. 2013. Assessing the application of the 3Rs: a survey among animal welfare officers in The Netherlands. Lab. Anim. 47(3):210-219.
- Lynch J.W., Ziegler T.E. & Strier K.B. 2002. Individual and seasonal variation in fecal testosterone and cortisol levels of wild male tufted capuchin monkeys, *Cebus apella nigratus*. Hormones Behavior 41(3):275-287. <<http://dx.doi.org/10.1006/hbeh.2002.1772>> <PMid:11971661>
- Matot I., Neely C.F., Katz R.Y. & Neufeld G.R. 1993. Pulmonary uptake of propofol in cats: effect of fentanyl and halothane. Anesthesiology 78(6):1157-1165.
- Olberg R.A. 2007. Monkeys and gibbons, p.375-386. In: West G., Heard D. & Caulkett N. (Eds), Zoo Animal & Wildlife Immobilization and Anesthesia. Blackwell Publishing, Ames. <<http://dx.doi.org/10.1002/9780470376478.ch32>>.
- Paddleford R.R. 1999. Manual of Small Animal Anesthesia. 2nd ed. W.B. Saunders, Philadelphia.
- Paula D.P., Nunes N., Nishimori C.T.D., Lopes P.C.F., Carareto R. & Santos P.S.P. 2012. Efeitos da infusão contínua de propofol ou etomidato sobre variáveis intracranianas em cães. Arq. Bras. Med. Vet. Zootec. 62(2):302-308. <<http://dx.doi.org/10.1590/S0102-09352010000200009>>
- Russell W.M.S. & Burch R.L. 1959. The Principles of Humane Experimental Technique. Methuen, London. 238p.
- Saha D.C., Saha A.C., Malik G., Astiz M.E. & Rackow E.C. 2007. Comparison of cardiovascular effects of tiletamine-zolazepam, pentobarbital, and ketamine-xylazine in male rats. J. Am. Assoc. Lab. Anim. Sci. 46(2):74-80. <PMid:17343357>
- Sapolsky R.M. 1992. Cortisol concentrations and the social significance of rank instability among wild baboons. Psychoneuroendocrinology 17(6):701-709. <[http://dx.doi.org/10.1016/0306-4530\(92\)90029-7](http://dx.doi.org/10.1016/0306-4530(92)90029-7)> <PMid:1287688>
- Shafer A., Doze V.A., Shafer S.L. & White P.F. 1988. Pharmacokinetics and pharmacodynamics of propofol infusions during general anesthesia. Anesthesiology 69(3):348-356. <<http://dx.doi.org/10.1097/00000542-198809000-00011>> <PMid:3261954>
- Smith I., White P.F., Nathanson M. & Gouldson R. 1994. Propofol: an update on its clinical use. Anesthesiology 81(4):1005-1043. <<http://dx.doi.org/10.1097/00000542-199410000-00028>> <PMid:7943815>
- Thurmon J.C., Tranquilli W.J. & Benson G.J. 1996. Preanesthetics and anesthetic adjuncts, p.183-209. In: Tranquilli W.J., Thurmon J.C. & Grimm K.A. (Eds), Lumb and Jones' Veterinary Anesthesia. 3rd ed. Lea and Feabiger, Philadelphia.
- Vilani R.G.D.C. 2009. Anestesia em primatas não-humanos, p.297-310. In: Kindlovits A. & Kindlovits L.M. (Eds), Clínica e Terapêutica em Primatas Neotropicals. 2ª ed. L.F. Livros, Rio de Janeiro.
- White P.F., Way W.L. & Trevor A.J. 1982. Ketamine-its pharmacology and therapeutic uses. Anesthesiology 56(2):119-136. <<http://dx.doi.org/10.1097/00000542-198202000-00007>> <PMid:6892475>
- Win N.N., Fukayama H., Kohase H. & Umino M. 2005. The different effects of intravenous propofol and midazolam sedation on hemodynamic and heart rate variability. Anesth. Analg. 101(1):97-102. <<http://dx.doi.org/10.1213/01.ANE.0000156204.89879.5C>> <PMid:15976213>
- Wirz A. & Riviello M.C. 2008. Reproductive parameters of a captive colony of capuchin monkeys (*Cebus apella*). Primates 49(4):265-270.
- Wright M. 1982. Pharmacologic effects of ketamine and its use in veterinary medicine. J. Am. Vet. Med. Assoc. 180(12):1462-1471. <PMid:6124527>
- Zausig Y.A., Busse H., Lunz D., Sinner B., Zink W. & Graf B.M. 2009. Cardiac effects of induction agents in the septic rat heart. Critical Care 13(5):1-8. <<http://dx.doi.org/10.1186/cc8038>> <PMid:19737388>

Morphometry of the mandibular foramen applied to local anesthesia in hoary fox (*Lycalopex vetulus*)¹

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ABSTRACT.- Magalhães H.I.R., Romão F.B., Paula Y.H., Luz M.M., Barcelos J.B., Silva Z., Carvalho-Barros R.A. & Ribeiro L.A. 2019. **Morphometry of the mandibular foramen applied to local anesthesia in hoary fox (*Lycalopex vetulus*)**. *Pesquisa Veterinária Brasileira* 39(4):278-285. Laboratório de Anatomia Animal, Faculdade de Medicina Veterinária, Centro Universitário de Patos de Minas, Rua Major Gote 808, Caiçaras, Patos de Minas, MG 38702-054, Brazil. E-mail: henrique123magalhaes@yahoo.com.br

Great part of the veterinary care in wild carnivores is intended to treat the dental disorders, and oral cavity disorders may generally affect the animal health as a whole. It is factual that knowing the location of the mandibular foramen is vital for local anesthetic block of the inferior alveolar nerve, however, there is still no data on the morphometry of the hoary fox mandibular foramen. The aim was describing morphometry of the mandibular foramen of this species and associating its position with anatomic reference points in the mandible, thus providing support for more effective local anesthetic block of the inferior alveolar nerve in such species. Four adult jaws of *Lycalopex vetulus* were used. Radiography and biometrics of the hemimandibulas were performed. The rostral third of the jaw body in a lateral view presented three mental foramina, being a rostral, a medium and a caudal. Each hemimandibula presented I3/C1/PM4/M3. The angle of the mandible was marked by the masseteric fossa, the angular incisure, the angular process and the mandibular foramen, and this last one located perpendicularly to the dorsal edge of the angular incisures in medial view. In this view, the crevice to the milohyoid nerve, projected in the caudodorsal rostral direction, was also evidenced. The ramus of the mandible was characterized by the presence of the condylar and coronoid processes, and by the dorsal and ventral mandibular incisions. Statistical analyzes did not present significant differences between the antimeres of the studied animals, and the penetration of the needle perpendicularly to the dorsal end of the angular incision on average 8.79mm, overlapped to the medial face of the angle of the mandible could be indicated. Alternatively, the access may also be achieved by inserting the needle in an average of 17.69mm perpendicular to the dorsal end of the angular process, in contact with the medial aspect of the angle of the mandible, and in caudo-rostral projection, also allowing a better anesthetic blockade of the inferior alveolar nerve in *L. vetulus*. It can also be concluded that the masseteric fossa, the dorsal and ventral mandibular angles, the crevice to the milohyoid nerve, the ramus of the mandible and the mandibular foramen presented differences in their topographic descriptions when compared to the other canids.

INDEX TERMS: Morphometry, anesthesia, mandibular foramen, hoary fox, *Lycalopex vetulus*, jaw, morphology.

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RESUMO.- [Morfometria do forame mandibular aplicada à anestesia local em raposa-do-campo (*Lycalopex vetulus*).]

A maior parte dos atendimentos veterinários em carnívoros silvestres destina-se ao tratamento das afecções dentárias, sendo que doenças de cavidade oral podem afetar a saúde do animal como um todo. É factível que o conhecimento da localização do forame mandibular é essencial para o bloqueio anestésico local do nervo alveolar inferior; entretanto, ainda são inexistentes dados sobre a morfometria do forame mandibular de raposa-do-campo. Objetivou-se descrever a morfometria do forame mandibular desta espécie, e correlacionar sua posição com pontos de referência anatômica na mandíbula, oferecendo subsídio para um bloqueio anestésico local mais efetivo do nervo alveolar inferior nesta espécie. Foram utilizadas quatro mandíbulas de cadáveres adultos de *Lycalopex vetulus*. Realizou-se a radiografia e as biometrias das hemimandíbulas. O terço rostral do corpo da mandíbula em uma vista lateral apresentou três forames mentuais, sendo um rostral, um médio e um caudal. Cada hemimandíbula apresentou I3/C1/PM4/M3. O ângulo da mandíbula foi marcado pela fossa massetérica, pela incisura angular, pelo processo angular e pelo forame mandibular; e este último localizado perpendicularmente ao extremo dorsal da incisura angular em vista medial. Nesta vista, também se evidenciou o sulco para o nervo milohióideo, projetado em sentido caudodorso-rostral. O ramo da mandíbula foi caracterizado pela presença dos processos condilar e coronóide, e pelas incisuras mandibulares dorsal e ventral. As análises estatísticas não apresentaram diferenças significantes entre os antímeros dos animais estudados, podendo-se indicar a penetração da agulha perpendicularmente ao extremo dorsal da incisura angular em média 8,79mm, justaposto à face medial do ângulo da mandíbula. Alternativamente, o acesso também poderá ser realizado introduzindo a agulha em média 17,69mm de forma perpendicular ao extremo dorsal do processo angular, em contato com a face medial do ângulo da mandíbula, e em projeção caudorostral, permitindo também um melhor bloqueio anestésico do nervo alveolar inferior na *L. vetulus*. Também se pode concluir que a fossa massetérica, as incisuras angular, mandibulares dorsal e ventral, o sulco para o nervo milohióideo, o ramo da mandíbula e o forame mandibular apresentaram diferenças em suas descrições topográficas quando comparados aos demais canídeos.

TERMOS DE INDEXAÇÃO: Morfometria, anestesia, forame mandibular, raposa-do-campo, *Lycalopex vetulus*, mandíbula, morfologia.

INTRODUCTION

The order Carnivora is comprised of 15 families, 287 species and divided into two suborders: Feliformia and Caniformia (Wozencraft 2005). It is known that approximately 195 species within such suborders inhabit the Brazilian biome Cerrado, out of which 18 are endemic (Brasil 2002). Among the canidae found in such biome, the hoary fox (*Lycalopex vetulus* Lund, 1842) is considered as the smallest among them with its occurrence confirmed only in Brazil (Dalponte & Courtenay 2004).

The geographic span of this animal species, at first, seems to be associated with the extent thresholds of the Cerrado (Dalponte 2009), but they may also be found in transition zones, including open areas of Pantanal (Lemos et al. 2013). Considering the

new information regarding this species occurrence area, we know their living area ranges from Northeastern and Western regions of the State of São Paulo (Dalponte 2009), Northern region in Piauí (Costa & Courtenay 2003) and middle-eastern region of Maranhão (Lemos et al. 2013).

The *Lycalopex vetulus* gets to the weight of up to 4.0 kg, and their body ranges from 58.5 to 64.0cm and their tail from 28.0 to 32.0cm (Nowak 1999). Their head has a red-brownish colored fur and, on the back, their fur has a grey-brownish color, with a dark-colored strip extending from the scruff to the tail edge, being more evident in the adult males (Vieira 1946). Their limbs are whitish, their tail is thick and has a dense fur, which may often have a dark spot in their base (Nowak 1999, Dalponte & Courtenay 2004).

They have their dental formula by quadrants equivalent to: I 3/3; C 1/1; PM 4/4; M 2/3 = 42 (Cheida et al. 2011), being considered insectivorous-omnivorous carnivores which use as their food basis the termites, also consuming in smaller quantities locusts and beetles. According to their availability in the environment and season of the year, they also feed of wild, exotic fruits, small-sized mammals, lizards and snakes, Anura and birds (Dalponte 1997, Juarez & Marinho-Filho 2002, Jácomo et al. 2004, Ferreira-Silva & Lima 2006, Trovati et al. 2006, Dalponte 2009, Lemos et al. 2011).

In the Brazilian States of São Paulo and Minas Gerais, Chiarello et al. (2008) mention the hoary fox as an endangered species (EN), however, this dog is found as a species of Least Concern (LC) as to the endangered scope, under the International Union for Conservation of Nature (IUCN 2017).

Great part of the veterinary care in wild carnivores is intended to treat the dental disorders, and oral cavity disorders may generally affect the animal health as a whole (Pachaly & Gioso 2001), and cause variable impact upon the wild populations, even suggested as limiting to the life expectancy (Hungerford et al. 1999, Stimmelmayer et al. 2006). For Pachaly & Gioso (2001), the oral cavity health is directly associated with maintenance of a good nutritional condition, increased reproductive capacity, longevity and life quality of the wild carnivores in captivity.

It is factual that knowing the location of the mandibular foramen is vital for local anesthetic block of the inferior mandibular and alveolar nerves (Nickel et al. 1981, Nicholson 1985, Blanton & Roda 1995). The inferior alveolar nerve is a sensitive nerve, derived from the mandibular nerve, which penetrates the mandibular foramen and spreads as it extends along the mandibular canal, in caudal, mean and rostral branches, responsible for both the innervation of the molar teeth, premolar, canine and incisors, and the corresponding gums and the skin of the mental region (Godinho & Getty 1986).

Local anesthesia provides some benefits, such as decreased central sensitivity to pain, mitigated inflammatory tissue reaction, reduced volume of general anesthetic required during the surgical procedure, and decreased dose and frequency of analgesics used in post-op (Gross et al. 1997, Hellyer & Gaynor 1998, Holmstrom et al. 2004). For occurrence of the anesthetic stunning of the inferior alveolar nerve, a local anesthetic injection must be administered near the ventral limit of such nerve (Lumb & Jones 2014).

Thus, knowledge of the topographic anatomy of the mandibular foramen and inferior alveolar nerve is extremely important for clinical practice, since, according to Milken et al. (2006)

the procedure for anesthesia of the inferior alveolar nerve may cause damages to the peripheral nerves, and the accurate mechanism for it is unknown, as well as its prevention and treatment. Still according to the same authors, the conventional regional block techniques are associated with risks and complications, such as neurological and vascular disorders, intravascular injections and muscular disorders.

Studies approaching morphometry of the mandibular foramen in domestic felines with no defined breed (Barroso et al. 2009), mandibular foramen of crab-eating fox (Souza Junior et al. 2013) and the mandibular, mental and infraorbital foramina of maned wolf (Moraes 2016) have been previously conducted, however, there is still no data on the morphometry of the hoary fox mandibular foramen.

Therefore, the aim was describing morphometry of the hoary fox mandibular foramen, and associating its position with anatomic reference points in the mandible, thus providing support for more effective local anesthetic block of the inferior alveolar nerve in such species.

MATERIALS AND METHODS

Eight hemimandibles taken from four adult corpses (two males and two females) of *Lycalopex vetulus* (Lund, 1842), components of the research property of the Anatomy Laboratory of the Biological Science College of the Federal University of Goiás (UFG), Campus Catalão, were used. The mandibles were prepared by, firstly, removing the skin and soft tissues of the head region, with subsequent temporal mandibular disjuncting. Then, the mandibles were subjected to chemical maceration with sodium hydroxide (NaOH, Lavitex®, concentration 98%-99%) during 30 minutes for cleaning, and later bleached through immersion into water solution of hydrogen peroxide (H₂O₂, Dinâmica®, concentration 30%-36%), with 1:10 dilution, during 30 minutes.

For topographic record, the radiography of a hemimandible was taken, displaying the mandibular foramen and the mandibular canal position (Fig.1).

After this stage, the biometric information was taken in each hemimandible by a single analyzer and in duplicate, using the digital electronic caliper Starrett® (capacity 0-150mm, resolution 0.01mm, accuracy ± 0.02 mm). Based on the studies conducted by Barroso et al. (2009), Souza Junior et al. (2013) and Moraes (2016), measurements were adapted and taken in each hemimandible as follows: LAM = distance from the side border of the condylar process to the root of the mandibular medial incisor; SAM = distance from the ventral and alveolar borders of the mandible, taken between the fourth mandibular premolar and the first mandibular molar; MFVB = distance from the caudal edge of the mandibular foramen ventral border to the ventral border of the mandible in such level; MFANG = distance from the caudal edge of the mandibular foramen ventral border to the dorsal edge of the angular process; MFCOND = distance from the caudal edge of the mandibular foramen ventral border to the medial end of the condylar process; MFCOR = distance from the caudal edge of the mandibular foramen ventral border to the dorsal edge of the caudal border of the coronoid process (Fig.2).

The morphometric data obtained was submitted to descriptive statistical analysis (arithmetic mean, standard deviation, and coefficient of variation) and to student t-test ($p=0.05$) with 95% confidence interval, using the software BioEstat® 5.3. The anatomic nomenclature used to designate the identified structures was in accordance with the International Committee on Veterinary Gross Anatomical Nomenclature (I.C.V.G.A.N. 2017). The study was approved

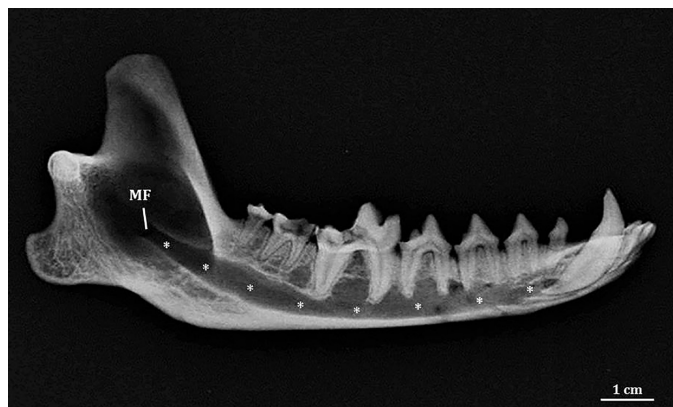


Fig.1. Lateromedial radiographic image of right hemimandible of an adult female of *Lycalopex vetulus*, highlighting the mandibular foramen (MF) and the mandibular canal (*).

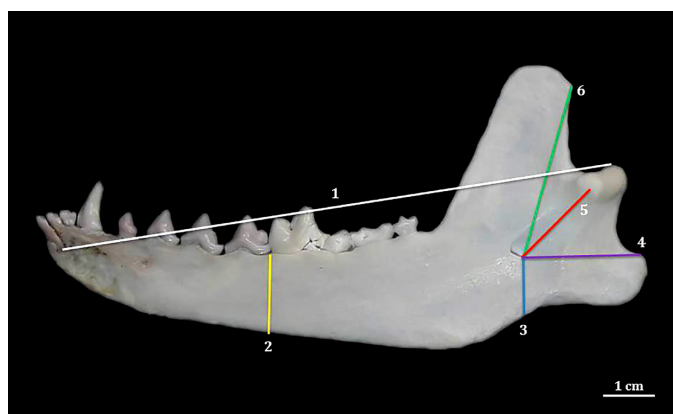


Fig.2. Medial face of right hemimandible of an adult female of *Lycalopex vetulus*, showing the LAM (1), SAM (2), MFVB (3), MFANG (4), MFCOND (5) and MFCOR (6) measurements.

by the Board of Ethics in Animal Use of the Federal University of Uberlândia, protocol nº081/14, and the Board of Ethics in Animal Use of the University Center of Patos de Minas - Unipam, protocol no. 14/17.

RESULTS

Each hemimandible was comprised of body, angle and ramus. The mandible body in side view showed in its rostral third three mental foramina, being one rostral, lower and ventral to the mandibular medial incisor; one caudal, with intermediate size and ventrally located to the third mandibular premolar; and one medium, upper and immediately ventral to the first mandibular premolar. Along the mandible body, in its alveolar border, were identified in each hemimandible I3/C1/PM4/M3. Laterally, the mandible angle was marked by a deep masseteric fossa dorsally extending to the mandible ramus. At the mandible angle ventral border, a marked angular incisure delimited the transition from the mandible body to the angle. Such incisure was caudally extended and ended in the angular process of the mandible. The dorsal projection of the mandible ramus was characterized by the presence of two processes, being the most caudal one named condylar

process of the mandible, rostrally joined by a non-articular depression, dorsal mandibular incisure, to the mandible coronoid process. One ventral mandibular incisure was noticed between the angular and condylar processes of the mandible in a caudal border.

The hemimandibles were joined in the rostral third of the mandible body through a joint of the symphysis type, it being evident in a medial view. Yet in such view, at the level of the transition point between the mandible body and angle, near the coronoid process projection, the crevice to the mylohyoid nerve was evidenced branching in the caudodorsal rostral direction. The mandibular foramen was located in the mandible angle, ventrally to the insertion area of the temporal muscle and perpendicularly to the dorsal edge of the angular incisure, representing the caudal opening of the mandibular canal, which extended rostrally to the mental foramen. Caudoventrally to such foramen, at the median surface of the angular process, the fossa to the median pterygoid muscle was viewed (Fig.3A,B).

Table 1 and 2 show the arithmetic means and standard deviations related to each measure, the coefficients of variation and the values obtained in the t-test for comparison between the mean values of the hemimandibles of each antimer.

The mean biometry of the longitudinal axis of the hemimandibles was 99.67 ± 1.14 mm (LAM), there being no statistically significant difference ($p=0.186$) between the left (100.15 ± 1.43 mm) and right (99.19 ± 0.73 mm) antimeres. Measurements concerning the sagittal axis showed a mean value of 13.46 ± 0.16 mm (SAM), and the left hemimandibles have a mean value of 13.37 ± 0.18 mm, and the right ones, 13.55 ± 0.08 mm, there being no difference between such antimeres ($p=0.113$).

The caudal edge of the mandibular foramen ventral border was, in average, 8.79 ± 0.50 mm distant from the mandible ventral border (MFVB), there being no difference ($p=0.066$) between the mean values of the left (8.45 ± 0.18 mm) and right (9.14 ± 0.48 mm) hemimandibles. Such point was still in a mean manner distant from the angular process dorsal edge by 17.69 ± 1.07 mm (MFANG), there being no statistical

difference ($p=0.073$) between the mean measures of the left (16.82 ± 0.71 mm) and right (18.56 ± 0.28 mm) antimeres.

The caudal edge of the mandibular foramen ventral border was distant from the condylar process medial end in average by 15.61 ± 0.47 mm (MFCND), there being no statistical relevance ($p=0.097$) between the median of the left (15.89 ± 0.22 mm) and right (15.34 ± 0.54 mm) antimeres. The mentioned point also did not have statistically significant variance ($p=0.062$) between the median of the left (29.66 ± 0.22 mm) and right (30.27 ± 0.49 mm) antimeres, when conducting the measurements up to the dorsal edge of the coronoid process caudal border (MFCOR), being the mean value of such measurement 29.96 ± 0.48 mm.

Table 1. Values of the morphometric measurements of the left and right hemimandibles of a *Lycalopex vetulus* (arithmetic mean $\pm$ standard deviation)

Measurements	Left hemimandibles (mm)	Right hemimandibles (mm)
LAM	100.15 ± 1.43	99.19 ± 0.73
SAM	13.37 ± 0.18	13.55 ± 0.08
MFVB	8.45 ± 0.18	9.14 ± 0.48
MFANG	16.82 ± 0.71	18.56 ± 0.28
MFCND	15.89 ± 0.22	15.34 ± 0.54
MFCOR	29.66 ± 0.22	30.27 ± 0.49

Table 2. Values of the morphometric measurements of the hemimandibles (n=8) of a *Lycalopex vetulus*

Measurements	Arithmetic mean and Standard deviation (mm)	Coefficient of variation (%)	p-test
LAM	99.67 ± 1.14	1.15	0.186
SAM	13.46 ± 0.16	1.20	0.113
MFVB	8.79 ± 0.50	5.68	0.066
MFANG	17.69 ± 1.07	6.04	0.073
MFCND	15.61 ± 0.47	3.04	0.097
MFCOR	29.96 ± 0.48	1.59	0.062

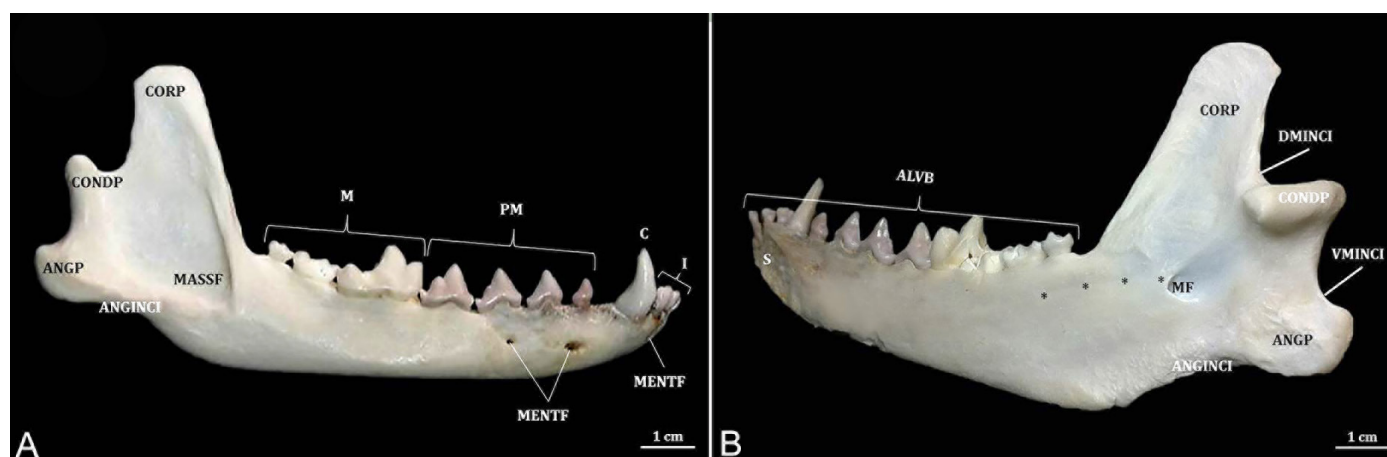


Fig.3. (A) Lateral and (B) mediocaudal faces of right hemimandible of an adult female of *Lycalopex vetulus*, evidencing the coronoid process (CORP), condylar process (CONDP), angular process (ANGP), masseteric fossa (MASSF), mental foramen (MENTF), angular incisure (ANGINCI), dorsal mandibular incisure (DMINCI), ventral mandibular incisure (VMINCI), mandibular foramen (MF), mandibular symphysis (S), alveolar border (ALVB), crevice to the mylohyoid nerve (*), mandibular incisors (I), canine (C), premolars (PM) and molar (M).

DISCUSSION

As to description of the left and right hemimandibles of a hoary fox, these may be divided into body, angle and ramus, as described by Sisson (1986) for carnivores.

Three mental foramens were viewed at the rostral end of the mandible body side face, corroborating in number with the studies disclosed by Evans & DeLahunta (1994) for domestic dogs. Moraes (2016) in turn reports a great oscillation in the number and location of such foramens in the *Chrysocyon brachyurus*, and such variations were more frequent in relation to the caudal foramens. The inferior teeth of a *Lycalopex vetulus* by hemimandible (I3/C1/PM4/M3) was similar to the studies mentioned for other canidae, such as *Canis lupus familiaris* (Evans & DeLahunta 1994), *Atelocynus microtis*, *Cerdocyon thous*, *Chrysocyon brachyurus* and *Lycalopex gymnocercus* (Cheida et al. 2011). Evans & DeLahunta (1994) also mention that the incisors, canine, first premolars and the last molars, have only one root each. The last three premolars and the first two molars, in turn, have two roots each. Such morphology was also present in the hoary fox, and could be confirmed through radiographic examination, not being required to proceed to dental extraction for such certification.

Sisson (1986), Evans & DeLahunta (1994) and König & Liebich (2016) mention the presence of the masseteric fossa at the side surface of the mandible ramus, however, in the hoary fox, such bone accident began in the mandible angle, and it extends dorsally towards the mandible ramus. The mandible angle ventral border showed a non-articular depression named angular incisure. It extends caudally, delimiting the transition between body and angle, ending in the mandible angular process.

In the hoary fox, the mandibular foramen was located at the mandible angle medial face, while Sisson (1986) in carnivores, Souza Junior et al. (2013) in the crab-eating fox, and Moraes (2016) in the maned wolf, report the presence of this caudal opening in the medial face of the mandible ramus. Evans & DeLahunta (1994), in turn, for domestic dogs, and König & Liebich (2016) for domestic animals, report only the presence of such foramen in the mandible medial face. This study showed that the mandibular foramen was positioned ventrally to the insertion area of the temporal muscle, corroborating with the information described by Godinho & Getty (1986) and Evans & DeLahunta (1994) for carnivores and Souza Junior et al. (2013) for the crab-eating fox.

Such foramen was also located perpendicularly to the dorsal edge of the angular incisure in the *L. vetulus*, being similar to the studies stressed by Moraes (2016) for the maned wolf. However, it is different from the study of Souza Junior et al. (2013), which highlights a position relatively more rostral of the mandibular foramen in relation to such incisure for the crab-eating fox.

Still assessing the hoary fox mandible medial face, there was evidence of the crevice to the mylohyoid nerve, projected in the caudodorsal rostral direction, to the transition point between the mandible body and angle, near the initial coronoid process. Godinho & Getty (1986) describe the mylohyoid nerve position for domestic carnivores, as being arranged ventrally and laterally to their paternal stem. In accordance with the above-mentioned authors, it is construed that such nerve is lodged over the crevice, thus, such characteristic may

be assumed as a variant to projection of such bone accident for the *L. vetulus*.

König & Liebich (2016) describe for domestic animals, the mandible ramus as the dorsal extension of the mandible body, it being comprised of the condylar process caudally, and by the coronoid process rostrally. Assessment of such mandible region of a *L. vetulus* enables to assume certain similarity to the studies of such authors, but in the concerned specimens, the mandible ramus extends from the angle region of it. Presence of two non-articular depressions between the mandible processes of a hoary fox allows for naming the separations between the angular and condylar processes from ventral mandibular incisure, and between the condylar and coronoid processes from dorsal mandibular incisure.

Among the main blocks used to provide appropriate analgesia of different regions of the oral cavity, there is the inferior alveolar block (Holmstrom et al. 2004), having a key procedure for efficient technique, determination of anatomic landmarks (Pachaly & Gioso 2001, Pessutti et al. 2001), being vital to know the mandibular foramen location (Nicholson 1985). Such regional block is efficient in ipsilateral stunning of the mandibular teeth and soft tissues, being possible to be conducted using the intra-oral or extra-oral techniques in domestic dogs (Gross et al. 1997, Holmstrom et al. 2004, Hale 2007, Egger & Love 2009). Indications for this anesthetic procedure include extractions of ipsilateral mandibular teeth, periodontal surgery, endodontic treatment, biopsies and mandibulectomy (Rochette 2005, Woodward 2008, Pascoe 2012).

LAM and SAM measurements were also conducted by Barroso et al. (2009), Souza Junior et al. (2013) and Moraes (2016), studying domestic felines with no defined breed, crab-eating fox and maned wolf respectively, and although not having direct relationship with the mandibular foramen position, it provides the idea of the longitudinal and sagittal measurements of the mandible. It should also be stressed that, for *L. vetulus*, statistically significant differences were not noticed in any of the measurements made, when comparing the left and right hemimandibles.

Regarding the anesthetic block of the inferior alveolar nerve to the level of its penetration into the mandibular foramen of maned wolf, Moraes (2016) indicates that the needle should be introduced perpendicularly and dorsally from the depression present in the mandible body caudal third, and near the lingual face of the bone by 11.40mm. Barroso et al. (2009) suggest for domestic cats with no defined breed, introduction of the needle between 4.10 and 4.40mm, from the mandible ventral border.

For domestic dogs, in turn, Gross et al. (1997) recommend dorsal penetration of the needle by 1 to 2cm along the mandible median surface, from 0.50cm rostral to the angular process. Higher values were published by Souza Junior et al. (2013) studying the crab-eating fox. Such authors recommend penetration of the needle by 12.10mm for females, and 13.60mm for males, perpendicularly to the ventral border of the mandible of such specimens.

For the hoary fox, it may be indicated that the needle should be introduced perpendicularly to the dorsal edge of the angular incisure in average 8.79mm, overlapped to the medial face of the mandible angle, and the technique mentioned herein corroborates with the recommendations

by Beckman & Legendre (2002), Holmstrom et al. (2004), Hale (2007) and Egger & Love (2009), which indicate that the depression present in the ventral border of the mandible should be used as anatomic reference when introducing the needle for conducting the extra-oral technique of anesthetic block of the inferior alveolar nerve.

Egger & Love (2009) also mention the angular process as a landmark for advancing the needle in the temporary stunning process of the inferior alveolar nerve in domestic dogs. Souza Junior et al. (2013) recommend that for this type of access in the *C. thous*, the needle should be introduced 20.40mm, and in diagonal from the angular process, however, such angle before the angular process was not stressed. In respect of the *C. brachyurus*, Moraes (2016) proposes penetrating the needle by 30mm from the angular process, near the lingual face and angled at 20-25° with the mandible body ventral border. Lower values were determined by Barroso et al. (2009) for *Felis catus*, requiring introduction of the needle about 12.37 and 12.57mm, also from the angular process. For the *L. vetulus*, the needle shall be introduced in average 17.69mm, perpendicularly, in contact with the mandible angle medial face, and in caudal-rostral projection from the dorsal edge of the angular process, for the anesthetic to be deposited nearer the inferior alveolar nerve.

The values provided for the hoary fox are lower than those reported by the above-mentioned authors by studying other wild canidae, such as crab-eating fox (Souza Junior et al. 2013) and maned wolf (Moraes 2016). However, such fact may have occurred due to such researchers take their measurements starting from the rostral border of the mandibular foramen, which is a reference not used in this study. The methodology adopted for the *Lycalopex vetulus* aims to preserve the neural structures of injuries possibly caused by penetrating the needle during the anesthetic procedure.

Silva et al. (2006) also indicated anesthetic application points for blocking the inferior alveolar nerve in cats. By dissecting the facial, retropharyngeal and lateral and medial mandibular sections of 10 male felines, the accurate point for the anesthetic procedure to be conducted was defined. Application of 1.0ml of Lidocaine 2% in each animal, with introduction of the needle by approximately 1.0cm, dorsally angled at 30°, medially or laterally to the palatoglossal arch, enabled the successful block in the five animals used (100%), with no side effects and adverse reactions.

Milken et al. (2006) showed in a similar manner the efficiency of such form of local block, using 1.0mg/kg of ropivacaine in 20 adult cats (100%), with application site 1.0cm rostral to the angular process and 0.5cm dorsal to the ventral border of the right mandible ramus. The application site was determined by dissecting the mandibles of three Feline corpses. All studied animals showed anesthesia of the right lateral and ventral region of the mandibles.

Findings such as these show the wide range of possibilities for varying the relative position of the mandibular foramen, even among species from the same order, which justifies obtainment of accurate data for each species (Moraes 2016). Although such anesthetic procedures are considered frequent, easy to conduct and require using anatomic references to be conducted (Gross et al. 1997, Holmstrom et al. 2004, Hale 2007, Egger & Love 2009), a lack of morphometric studies of the mandibular foramen in animals (Moraes 2016), especially wild ones, is noted.

According to Moraes (2016), the anesthetic procedures in wild species are required to be developed and enhanced, because most of such techniques were designed for domestic animals, with researches which would evidence procedures with support of the anatomy of each wild animal being indispensable. Thus, we can assume that extrapolation of the anatomic references used in domestic dogs may lead to greater index of failures in practice with wild canidae (Souza Junior et al. 2013).

The anesthetic technique of the inferior alveolar nerve is the most important regional block and used in the human dental care, and even so it has high failure percentage (15 to 20%) (Rizzolo & Madeira 2006, Strini et al. 2006). Among the several factors which infer a successful procedure, one may highlight the inaccuracy in locating the mandibular foramen (Strini et al. 2006, Lima et al. 2011), highly due to the variable location in individuals (Nicholson 1985, Blanton & Roda 1995, Afsar et al. 1998, Potocnik & Bajrovic 1999, Rizzolo & Madeira 2006).

Lack of instruction in relation to the anatomy, associated with the technical deficiencies, hinder the obtainment of anesthesia and endanger other anatomic structures (Gregori & Santos 1996). Block allows for analgesia for several hours after the procedure, minimizes the general anesthesia plan (Beckman & Legendre 2002, Hale 2007), the inflammatory tissue reaction and central sensitivity to pain (Cediel et al. 1999). Such benefits are increasingly desirable in medicine of wild canidae due to the high rate of occurrence of dental and mandibular fractures, complex drug administration, as well as frequent weak condition of the patients upon the surgical procedure (Pachaly & Gioso 2001, Pessutti et al. 2001). However, if these are incorrectly conducted, they may generate complications deriving from intramuscular injections (lockjaw, muscular pain or limited mouth movement) and anesthesia of the facial nerve (transitory facial paralysis) (Evers & Haegerstam 1991).

CONCLUSIONS

Mandibles of *Lycalopex vetulus* had morphology similar to the previously described for other canidae species. Variations were noted in relation to the topographic description of the mandibular foramen, crevice to the mylohyoid nerve, mandible ramus, masseteric fossa, angular, dorsal mandibular and ventral mandibular incisures.

Enhanced anesthetic block of the inferior alveolar nerve of adult specimens of hoary fox may be indicated, by penetrating the needle perpendicularly to the dorsal edge of the angular incisure in average 8.79mm, overlapped to the medial face of the mandible angle. Alternatively, the access may also be made by introducing the needle in average 17.69mm perpendicularly, in contact with the medial face of the mandible angle, and in caudal-rostral projection from the dorsal edge of the angular process.

Conflict of interest statement.- The authors declare that they have no conflict of interest.

REFERENCES

- Afsar A., Haas D.A., Rossouw P.E. & Wood R.E. 1998. Radiographic localization of mandibular anesthesia landmarks. *Oral Surg., Oral Med., Oral Pathol., Oral Radiol.* 86(2):234-241. <[http://dx.doi.org/10.1016/S1079-2104\(98\)90131-7](http://dx.doi.org/10.1016/S1079-2104(98)90131-7)> <PMid:9720101>

- Barroso R.M.V., Ferreira F.A., Silva R.M. & Lima E.M.M. 2009. Morphometric analysis of the mandibular foramen of cats (*Felis catus* Linnaeus, 1758) with no defined breed. *Bioscience J.* 25(4):135-142.
- Beckman B. & Legendre L. 2002. Regional nerve blocks for oral surgery in companion animals. *Compend. Cont. Educ. Pract. Vet.* 24(6):439-444.
- Blanton P.L. & Roda R.S. 1995. The anatomy of local anesthesia. *J. California Dental Assoc.* 23(4):55-69. <Pmid:7643190>
- Brasil 2002. Biodiversidade brasileira: avaliação e identificação de áreas e ações prioritárias para conservação, utilização sustentável e repartição dos benefícios da biodiversidade nos biomas brasileiros. Ministério Do Meio Ambiente, Secretaria de Biodiversidade e Florestas, Brasília, p.193-195.
- Cediel R., Sánchez M., López-San Roman J., Tendillo F. & Vilorio A. 1999. Anestesia em odontologia, p.97-110. In: Acaso F.S.R., Orozco A.W. & Muniz I.T. (Eds), *Atlas de Odontologia de Pequenos Animais*. Manole, São Paulo.
- Cheida C.C., Nakano-Oliveira E., Fusco-Costa R., Rocha-Mendes F. & Quadros J. 2011. Ordem Carnívora, p.235-288. In: Reis N.R., Peracchi A.L., Pedro W.A. & Lima I.P. (Eds), *Mamíferos do Brasil*. 2ª ed. Nelio R. dos Reis, Londrina.
- Chiarello A.G., Aguiar L.M.S., Cerqueira R., Melo F.R., Rodrigues F.H.G. & Silva V.M.F. 2008. Mamíferos ameaçados de extinção no Brasil, p.681-885. In: Machado A.B.M., Drummond G.M. & Paglia A.P. (Eds), *Livro Vermelho da Fauna Brasileira Ameaçada de Extinção*. MMA, Fundação Biodiversitas, Brasília.
- Costa C.H.N. & Courtenay O. 2003. A new record of the hoary fox *Pseudalopex vetulus* in north Brazil. *Mammalia* 67(4):593-594. <http://dx.doi.org/10.1515/mamm-2003-0416>
- Dalponete J.C. 1997. Diet of hoary fox, *Lycalopex vetulus*, in Mato Grosso, Brazil. *Mammalia* 61(4):537-546.
- Dalponete J.C. 2009. *Lycalopex vetulus* (Carnívora: Canidae). *Mammalian Species* 847(Spec.):1-7.
- Dalponete J.C. & Courtenay O. 2004. Hoary fox *Pseudalopex vetulus* (Lund, 1842), p.72-76. In: Sillero-Zubiri C., Hoffmann M. & Macdonald D.W. (Eds), *Canids: foxes, wolves, jackals and dogs. Status Survey and Conservation Action Plan*, IUCN/SSC Canid Specialist Group. Gland, Switzerland and Cambridge, UK.
- Egger C. & Love L. 2009. Local and regional anesthesia techniques. Part 3. Blocking the maxillary and mandibular nerves. *Vet. Med.* 104(6):12-15.
- Evans H.E. & DeLahunta A. 1994. Miller, Guia para a Dissecção do Cão. 3ª ed. Guanabara Koogan, Rio de Janeiro, p.156-159.
- Evers H. & Haegerstam H. 1991. Introdução à Anestesia Local Odontológica. Manole, São Paulo. 96p.
- Ferreira-Silva E. & Lima E.S. 2006. Termite predation by the hoary fox, *Pseudalopex vetulus* (Lund) (Carnívora, Canidae), in a pasture in Mato Grosso, Central Brazil. *Mammalia* 70(3/4):255-260. <http://dx.doi.org/10.1515/MAMM.2006.043>
- Godinho H.P. & Getty R. 1986. Sistema nervoso periférico, p.1583-1595. In: Getty R. (Eds), *Sisson & Grossman anatomia dos animais domésticos*. 5ª ed. Guanabara Koogan, Rio de Janeiro.
- Gregori C. & Santos W. 1996. Anestésias Intrabucais em Odontologia. Sarvier, São Paulo. 272p.
- Gross M.E., Pope E.R., O'Brien D., Dodam J.R. & Polkow-Haight J. 1997. Regional anesthesia of the infraorbital and inferior alveolar nerves during noninvasive tooth pulp stimulation in halothane-anesthetized dogs. *J. Am. Vet. Med. Assoc.* 211(11):1403-1405. <Pmid:9394889>
- Hale F. 2007. Local anesthesia in veterinary dentistry. *CUSP* 7:1-5.
- Hellyer P.W. & Gaynor J.S. 1998. Acute postsurgical pain in dogs and cats. *Compend. Cont. Educ. Pract. Vet.* 20:140-153.
- Holmstrom S.E., Frost P.F. & Eisner E.R. 2004. *Veterinary Dental Techniques for the Small Animal Practitioner*. 3rd ed. W.B. Saunders, Philadelphia. 689p.
- Hungerford L.L., Mitchell M.A., Nixon C.M., Esker T.E., Sullivan J.B., Koerkenmeier R. & Marretta M. 1999. Periodontal and dental lesions in raccoons from a farming and a recreational area in Illinois. *J. Wildl. Dis.* 35(4):728-734. <http://dx.doi.org/10.7589/0090-3558-35.4.728> <Pmid:10574532>
- I.C.V.G.A.N. 2017. *Nomina Anatomica Veterinaria*. 6th ed. International Committee on Veterinary Gross Anatomical Nomenclature, Editorial Committee, Hannover, Ghent, Columbia, Rio de Janeiro. 178p.
- IUCN 2017. Red List of Threatened Species. Available at <www.iucnredlist.org> Accessed on Feb. 15, 2017.
- Jácomo A.T.A., Silveira L. & Diniz-Filho J.A.F. 2004. Niche separation between the maned wolf (*Chrysocyon brachyurus*), the crab-eating fox (*Dusicyon thous*) and the hoary fox (*Dusicyon vetulus*) in central Brazil. *J. Zoo. Wildl. Med.* 262(1):99-106.
- Juarez K.M. & Marinho-Filho J. 2002. Diet, habitat use and home ranges of sympatric canids in central Brazil. *J. Mamm. Evol.* 83(4):925-933. <http://dx.doi.org/10.1644/1545-1542(2002)083<0925:DHUAHR>2.0.CO;2>
- König H.E. & Liebich H.G. 2016. *Anatomia dos Animais Domésticos: texto e atlas colorido*. 6ª ed. Artmed, Porto Alegre, p.71-73.
- Lemos F.G., Facure K.G. & Azevedo F.C. 2011. Comparative ecology of the hoary fox and the crab-eating fox in a fragmented landscape in the Cerrado biome at central Brazil, p.143-160. In: Rosalino L.M. & Gheler-Costa C. (Eds), *Middle-Sized Carnívores in Agricultural landscapes*. Nova Science Publishers Inc, New York.
- Lemos F.G., Azevedo F.C., Beisiegel B.M., Jorge R.P., Paula R.C., Rodrigues F.H.G. & Rodrigues L.A. 2013. Avaliação do risco de extinção da Raposa-do-campo *Lycalopex vetulus* (Lund, 1842) no Brasil. *Biod. Bras.* 3(1):160-171.
- Lima D.S.C., Figuerêdo A.A., Rocha E.A., Costa V.H.M.V., Castro M.P., Silva R.C.P., Chagas G.L., Araújo L.P., Mendonça V.R.R., Gravina P.R. & Meneses J.V.L. 2011. Estudo anatômico do forame mandibular e suas relações com pontos de referência do ramo da mandíbula. *Revta Bras. Cirurg. Craniomaxilofacial* 14(2):91-96.
- Lumb W.V. & Jones E.W. 2014. *Anestesiologia e Analgesia Veterinária*. 4ª ed. Roca, São Paulo. 1192p.
- Milken V.M.F., Freitas P.M.C., Eurides D., Silva F.O.C., Mota F.C.D., Naves E.A., Rezende R.J., Prieto L.A., Melo M.S. & Goulart T.M.R. 2006. Bloqueio do nervo alveolar mandibular com ropivacaína a 0,5% em gatos. *Ciência Rural* 36(2):550-554. <http://dx.doi.org/10.1590/S0103-84782006000200029>
- Moraes F.M. 2016. Morfometria dos forames mandibular, mental e infraorbital de lobo-guará *Chrysocyon brachyurus*, Illiger, 1815 aplicada a bloqueio anestésicos. Doctoral Dissertation, Universidade Federal de Uberlândia, Uberlândia. 55p. Available at <https://repositorio.ufu.br/bitstream/123456789/17588/1/MorfometriaForamesMandibular.pdf> Accessed on 15 Jan. 2017.
- Nicholson M.L. 1985. A study of the position of the mandibular foramen in the adult human mandible. *Anat. Rec.* 212(1):110-112. <http://dx.doi.org/10.1002/ar.1092120116> <Pmid:4073538>
- Nickel R., Schummer A. & Seiferle E. 1981. *The Anatomy of the Domestic Animals*. Verlag Paul Parey, Berlin. 141p.
- Nowak R.M. 1999. *Walker's Mammals of the World*. 6th ed. The John Hopkins University Press, Baltimore.
- Pachaly J.R. & Gioso M.A. 2001. The oral cavity, p.457-463. In: Fowler M.E. & Cubas Z.S. (Eds), *Biology, Medicine and Surgery of South American Wild Animals*. Iowa State University Press, Ames.
- Pascoe P.J. 2012. Anesthesia and pain management, p.23-42. In: Verstraete F.J.M. & Lommer M.J. (Eds), *Oral and Maxillofacial Surgery in Dogs and Cats*. Saunders Elsevier, Edinburgh. <http://dx.doi.org/10.1016/B978-0-7020-4618-6.00004-X>.
- Pessutti C., Santiago M.E.B. & Oliveira L.T.F. 2001. Order Carnívora, p.279-290. In: Fowler M.E. & Cubas Z.S. (Eds), *Family Canidae (dogs, foxes and maned wolves)*. Iowa State University Press, Ames.

- Potocnik I. & Bajrovic F. 1999. Failure of inferior alveolar nerve block in endodontics. Dent. Traum. 15(6):247-251. <<http://dx.doi.org/10.1111/j.1600-9657.1999.tb00782.x>> <PMid:10825834>
- Rizzolo R.J.C. & Madeira M.C. 2006. Anatomia Facial com Fundamentos de Anatomia Sistêmica Geral. 2ª ed. Sarvier, São Paulo. 343p.
- Rochette J. 2005. Regional anesthesia and analgesia for oral and dental procedures. Vet. Clin. N. Am., Small Anim. Pract. 35(4):1041-1058, viii-ix. <<http://dx.doi.org/10.1016/j.cvsm.2005.02.004>> <PMid:15979524>
- Silva M.L., Santana M.I., Araújo L.V. & Elston F. 2006. Topografia e bloqueio anestésico do nervo mandibular em gatos. Revta Port. Ciênc. Vet. 101:187-192.
- Sisson S. 1986. Osteologia do carnívoro, p.1337-1412. In: Getty R. (Ed.), Sisson/Grossman Anatomia dos Animais Domésticos. 5ª ed. Guanabara Koogan, Rio de Janeiro.
- Souza Junior P., Pinto R.J.F., Freitas A.B. & Carvalho N.C. 2013. Morfometria do forame mandibular de *Cerdocyon thous* (Linnaeus, 1766) (cachorro-do-mato). Biotemas 26(2):175-183.
- Stimmelmayer R., Maier J.A.K., Persons K. & Battig J. 2006. Incisor tooth breakage, enamel defects, and periodontitis in a declining Alaskan moose population. Alces 42:65-74.
- Strini P.J.S.A., Júnior W.S., Rodrigues D.A., Strini P.J.S.A., Guimarães E.C. & Júnior R.B. 2006. Avaliação topográfica do forame mandibular em peças anatômicas maceradas parcialmente dentadas e edêntulas. Revta Facul. Odont. Univ. Passo Fundo 11(2):111-115.
- Trovati R.G., Campos C.B. & Brito B.A. 2006. Nota sobre convergência e divergência alimentar de canídeos e felídeos (Mamalia: Carnivora) simpátricos no cerrado brasileiro. Neotrop. Biol. Conserv. 3(2):95-100.
- Vieira C.C. 1946. Carnívoros do estado de São Paulo. Arq. Zool. 5(3):135-175.
- Woodward T.M. 2008. Pain management and regional anesthesia for the dental patient. Topics Companion Anim. Med. 23(2):106-114. <<http://dx.doi.org/10.1053/j.tcam.2008.02.007>> <PMid:18482711>
- Wozencraft W.C. 2005. Order Carnivora, p.532-628. In: Wilson D.E. & Reeder D.M. (Eds), Mammal Species of the World: a taxonomic and geographic reference. 3rd ed. The Johns Hopkins University Press, Baltimore.

Maternal, fetal and neonatal heart rate and heart rate variability in Holstein cattle¹

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ABSTRACT. Quevedo D.A.C., Lourenço M.L.G., Bolaños C.D., Alfonso A., Ulian C.M.V. & Chiacchio S.B. 2019. **Maternal, fetal and neonatal heart rate and heart rate variability in Holstein cattle.** *Pesquisa Veterinária Brasileira* 39(4):286-291. Faculdade de Medicina Veterinária e Zootecnia, Universidade Estadual Paulista "Julio de Mesquita Filho", Distrito Rubião Júnior s/n, Botucatu, SP 18618-970, Brazil. E-mail: maria-lucia.lourenco@unesp.br

The aim of this study was to describe the normal values for maternal, fetal and neonatal heart rate (HR) and heart rate variability (HRV) indexes in the time domain (standard deviation of beat-to-beat interval - SDNN; root mean square of successive beat-to-beat differences - RMSSD) and the frequency domain (low frequency - LF; high frequency - HF; relationship between low and high frequency - LF/HF) in 23 Holstein cows, 23 fetuses and 18 neonates during the perinatal period. HR and HRV were calculated by fetomaternal electrocardiography (ECG). Fetomaternal measurements were taken six times prepartum (between days 234 and 279 of pregnancy) and measurements were taken in neonates six times after calving (after birth and five times weekly). HR, time and frequency domain were analyzed. No significant changes in maternal, fetal beat-to-beat interval (RR interval) or HR were found. In maternal variables, SDNN decreased significantly from 38.08 ± 2.6 ms (day 14 before calving) to 23.7 ± 2.5 ms (day 1 after calving) ($p < 0.05$), but the RMSSD did not change significantly. HR and RR interval of calf differed statistically from the day before delivery (163 ± 7.5 bpm; 381 ± 24.2 ms) to the day after calving (131 ± 5 bpm; 472 ± 16.2 ms). Time variables (SDNN and RMSSD) and the frequency-domain variables (LF and HF) were significantly different ($p < 0.05$) between fetal and neonatal stages. Reductions in the values of SDNN and RMSSD can reflect a sympathetic dominance. After calving, the increase in HF and decrease in LF variables can indicate activation of the vagal nerve followed by heart and respiratory modulation.

INDEX TERMS: Maternal heart rate, fetal heart rate, neonatal, Holstein cattle, calf, electrocardiogram, cattle, morphology.

RESUMO.- [Frequência cardíaca e variabilidade da frequência cardíaca materna, fetal e neonatal em bovinos da raça Holstein.] O objetivo deste estudo foi descrever os valores normais para os índices de frequência cardíaca (FC) materna, fetal e neonatal e de variabilidade da frequência

cardíaca (VFC) no domínio do tempo (desvio padrão do intervalo batimento a batimento, SDNN; raiz quadrada média de sucessivas diferenças de batimento a batimento, RMSSD) e do domínio da frequência (baixa frequência, LF; alta frequência, HF; relação entre baixa e alta frequência, LF/HF) em 23 vacas Holandesas, 23 fetos e 18 neonatos durante o período perinatal. A FC e a VFC foram calculadas por eletrocardiografia materno-fetal. As medidas materno-fetais foram realizadas seis vezes antes do parto (entre os dias 234 e 279 de gestação) as medidas neonatais foram realizadas seis vezes após o parto (um dia após nascimento e semanalmente, durante cinco semanas). FC, e variáveis no domínio do tempo e de frequência foram analisadas. Não foram encontradas alterações significativas na FC e no intervalo de batimento para batimento (intervalo RR) materno e fetal. Nas variáveis maternas, o SDNN diminuiu

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significativamente de $38,08 \pm 2,6$ ms (dia 14 antes do parto) para $23,7 \pm 2,5$ ms (dia 1 após o parto) ($p < 0,05$), mas o RMSSD não alterou significativamente. A FC e o intervalo e RR do bezerro diferiram estatisticamente a partir de um dia antes do parto ($163 \pm 7,5$ bpm, $381 \pm 24,2$ ms) até o dia seguinte ao parto (131 ± 5 bpm, $472 \pm 16,2$ ms). As variáveis de tempo (SDNN e RMSSD) e as variáveis de domínio de frequência (LF e HF) foram significativamente diferentes ($p < 0,05$) entre os momentos fetal e neonatal. As reduções nos valores de SDNN e RMSSD podem refletir domínio simpático. Após o parto, o aumento da HF e a diminuição das variáveis LF podem indicar a ativação do nervo vagal seguido de modulação cardíaca e respiratória.

TERMOS DE INDEXAÇÃO: Frequência cardíaca materna, feto, neonatal, bovinos da raça Holstein, bezerro, eletrocardiograma materno-fetal, morfologia.

INTRODUCTION

Electrocardiography (ECG) has been proposed as a useful noninvasive technique for assessing well-being of the bovine fetus (Reef et al. 1996). Fetomaternal ECG allows continuous analysis of fetal and maternal heart rate (HR) and heart rate variability (HRV) which reflects sympathoadrenal activity in the late stages of pregnancy in cows and newborn calves. To improve perinatal care of cattle and reduce the mortality of newborns, especially for animals with high genetic value, ECG can be performed, and HR and HRV parameters can be measured. To date, there have been few studies of HR and HRV in cows and fetuses. Fetal heart rate (FHR) is the most frequently reported parameter used to assess fetal well-being in the final stages of pregnancy in humans, horses and sheep (Kovács et al. 2012, 2014a, 2014b).

Short-term fluctuations in HR are caused by modulation of cardiac action via the autonomous nervous system (Moberg 2000). HR represents the net interactions between vagal (which reduces HR) and sympathetic (which increases HR) regulation (Kleiger et al. 1995). A rise in HR is mainly caused by an increase in sympathetic activity, but it may also result from a decrease in vagal regulation or from simultaneous changes in both regulatory systems (Guyton & Hall 2000). HRV analysis allows a much more accurate and detailed determination of the functional regulatory characteristics of the autonomic nervous system (ANS). HRV is a particularly good indicator for the noninvasive assessment of ANS activity in response to stress in farm animals (Von Borell et al. 2007). In human fetuses, short-term variability can be useful as an indirect indicator central nervous system function. It is based on the antagonistic oscillatory influences of the sympathetic and parasympathetic nervous system on the sinoatrial node (Van Leeuwen et al. 2007). HRV thus reflects the balance of sympathetic and parasympathetic tone in humans (Schneider et al. 2008). Reductions in the values of the HRV variables, standard deviation of beat-to-beat interval (SDNN) and root mean square of successive beat-to-beat differences (RMSSD) reflect a shift toward more sympathetic dominance, whereas increases indicate a shift toward parasympathetic dominance (Levy & Martin 1978).

Power spectrum analysis has been employed to introduce a quantitative evaluation of the autonomic nervous system in the control of heart rate variability into clinical examinations in human adults and neonates (Malik et al. 1996). Spectral

analysis of heart rate variability has potential as a noninvasive means to assess fetal autonomic nerve system activity and dynamic control in real time. Processes associated with autonomic nerve disorder can be detected through HR or blood pressure (Von Borell et al. 2007). The spectral analysis technique may provide us with a tool for the early detection of mutual imbalance between the two regulatory systems (Abboud & Sadeh 1990). Low-frequency (LF) peaks in the spectral analysis of fetal heartbeat rate variability are associated with fetal sympathetic activity and partly with parasympathetic activity (Akselrod et al. 1981).

Studies have demonstrated the usefulness of the low frequency (LF)/high-frequency (HF) ratio of the power spectrum as an indicator of sympathetic activity (Abboud & Sadeh 1990, Akselrod et al. 1981) with an increase in the LF/HF ratio being interpreted as a regulatory shift toward sympathetic dominance. High vagal tone has been linked to efficient autonomic regulatory activity, which enables an organism to increase its sensitivity and response to physiological and environmental challenges.

Interpretation of FHR is complicated due to the multiple factors involved in its regulation: chemoreceptors, the sympathetic and parasympathetic nervous system, and fetal activity (Guyton & Hall 2000). FHR not only enables verification of human fetal health and viability but also provides information about the stage of development of the autonomic nervous system (Camm et al. 1996). There is a need for clinical studies using fetal HRV measures under realistic conditions, and it is important to describe the normal ranges for such measurements. Thus, the aim of this study was to describe the normal values for maternal, fetal and neonatal HR and HRV indexes in the time domain (standard deviation of beat-to-beat interval, SDNN; root mean square of successive beat-to-beat differences, RMSSD) and the frequency domain (low frequency, LF; high frequency, HF; relationship between low and high frequency, LF/HF).

MATERIALS AND METHODS

Animals. The farm is located at an altitude of 804 meters above sea level, latitude $22^{\circ}53'09''$ S and longitude $48^{\circ}26'42''$ W. The region has a humid subtropical climate. This project had the approval of the Ethics Committee on Animal Use - CEUA (No. 89-2014), and informed consent was given by the owner of the farm.

The 23 cows were selected from among the healthy, trouble-free specimens in the dry period stage. The exact day of pregnancy was calculated from reproductive records (artificial insemination or natural service) with pregnancy confirmation. From the beginning of the dry period, the animals were kept in a maternity paddock where they received corn silage three times a day. All of the cows were brought into an enclosed barn for sampling once a week. Two weeks prior to the start of the trial, the cows were acclimated to the facility and to the handling and electrocardiogram procedures.

Following birth, 17 clinically healthy and full-term-born Holstein calves (9 males and 8 females), were monitored. Heifer calves were removed from their mothers and transferred to individual pens. They received colostrum for the first 2 days and were then fed according to a regular schedule for rearing calves based on the provision of whole milk (milk offered twice a day, and liters calculated according to the weight of each animal), and gradual supplementation with concentrates. Bull calves were removed from their mothers

and housed together, and they were fed with the same quantity of colostrum and ad libitum roughage (maize silage).

Experimental design. Fetomaternal ECG recordings were made between days 234 and 279 of pregnancy. Six recordings were conducted prepartum. Recordings were repeated at approximately 7-day intervals until 1 or 2 days before calving and one day after delivery. On each day, recordings were made for 7-10min, starting at 8 a.m. to 2 p.m. Neonatal recordings were made after birth and five times weekly. In cows as well as in fetuses and neonates, HRV was analyzed for a 5-min interval in the ECG recording. To remove trend components, the data were detrended, and artifact correction was performed using established procedures (Malik et al. 1996).

The electrocardiographic examinations were repeated at predefined times. Fetomaternal ECG was performed at six time points between day 35 and one or two days prepartum, resulting in 132 exams. Neonatal ECG was studied at six time points, from birth to 35 days of age; there were 108 recordings in total.

Maternal, fetal and neonatal ECG. Fetomaternal and neonatal ECG measurements were made with the Televet 100 recording system (version 4.2.3; Kruuse, Marslev, Denmark). After the cow entered the chute, 2 square inches of hair were shaved at the sites where the pads were to be attached with self-adhesive pads.

For all measurements, the green electrode was attached to the neck on the left side of the cow, 20cm cranial from the point of the shoulder. The yellow electrode was placed in the flank region, above the line from the hip joint to the shoulder, and the black electrode was placed on the left croup. On the right side of the animal, the red electrode was attached to the abdomen at the height of the knee, along the line from the stifle joint to the point of the elbow. The electrodes were connected to the Televet 100 recording device. The Televet 100 sent the data via Bluetooth to a computer.

The Televet 100 uses a filter that can extract and amplify the fetal ECG signal from the abdominal ECG signal of the mother. The maternal signal represents a modified apex-base recording, and the fetal signal depends on fetal position in the uterus, so it is not a standardized recording.

For the neonates, the Televet 100 was fixed onto an elastic band around the thorax, and the electrodes were attached to the coat with self-adhesive pads. The red and black electrodes were placed on the right side of the lower part of the thorax, and the green and yellow electrodes were placed on the left side in the precordial region.

Heart rate variability analysis. Kubios HRV software (Biomedical Signal Analysis Group, Department of Applied Physics, University of Kuopio, Finland) was used for HRV analysis. In agreement with generally accepted recommendations for HRV analysis in animals (Tarvainen & Niskanen 2008) a 5-min time interval of each recording was used to determine the HRV parameters. To remove trend components, the data were detrended, and an artifact correction was made. In the time domain, HR, beat-to-beat interval (RR), standard deviation of beat-to-beat interval (SDNN), and square root of the mean squared differences of successive RR intervals (RMSSD) were assessed. SDNN was used to reflect all cyclic components of the variability in recorded series of RR intervals. RMSSD was used as an estimate of high-frequency variations in short-term RR recordings. In the frequency domain (by fast Fourier transform analysis), the normalized power of the LF nu and the high-frequency band (HF nu) was calculated, as was the LF/HF quotient. LF Norm was used to examine sympathetic modulations, while HF Norm was used to evaluate parasympathetic modulations. LF/HF was used to assess sympathovagal balance. The divergent respiratory frequencies in calves and cows were taken into consideration by setting the limits

of the HF band to 0.3 Hz (lower limit) and 0.8 Hz (upper limit) for calves, 0.25 and 0.58 Hz for cows (Von Borell et al. 2007).

Statistical analysis. Kolmogorov-Smirnov tests were used to assess normality of distribution for each variable. Normally distributed data were evaluated with 1-way ANOVA for repeated measures to analyze significant changes, and a post hoc Bonferroni test was performed for each HRV index at all time points. In addition, a paired t-test was used for the comparison of sampling stages with the first time point. The data that did not pass the Kolmogorov-Smirnov test were analyzed with nonparametric tests (Friedman analysis). The results are presented as the mean $\pm$ SEM. A p value <0.05 was considered significant. The SPSS statistical package (SPSS 21 Inc., Chicago/IL, USA) was used for data analyses.

RESULTS

Table 1 shows the mean, standard error of the mean ($\pm$ SEM) and range of the fetal and neonatal HR and the time-domain HRV indexes RR interval, SDNN, RMSSD at six time points before calving and six time points after delivery.

The HRV time-domain indexes SDNN and RMSSD and the frequency-domain variables LF nu and HF nu were significantly different from the fetal to the neonatal stage. FHR and RR intervals differed significantly from the neonatal stage at day one before calving (161 ± 7.1 beats/min 381 ± 19.2 ms) and the day after calving (130 ± 4.8 beats/min; 471 ± 15.7 ms) (Table 1). The change from the last day of gestation to the first day after calving was statistically significant ($p<0.05$).

There was a significant change in RMSSD from the fetal to the neonatal stage, i.e., from the last day (day 1) before calving (26.9 ± 4.7 ms) to one day after delivery (29.2 ± 7.3 ms; $p<0.05$) (Fig.1). It was also observed a significant increase in the value of SDNN, from the day one before calving (23.3 ± 1.8 ms) to the last day of the study (47.1 ± 9.8 ms) (Table 1).

In the last month of gestation (days 244-279), the maternal mean SDNN reached a statistically significant change days 28 and 1. It was also observed a significantly decreased in maternal SDNN from 38.08 ± 2.6 ms (day 14 before calving) to 23.7 ± 2.5 ms (day 1 after calving), whereas the variable RMSSD decreased without a significant difference (Fig.1).

The maternal RR interval decreased from gestational day 244, with values of 849 ± 105 ms corresponding to an HR of 71 ± 9 bpm, to the lowest RR intervals, observed 1 day before calving, with values of 781 ± 149 ms, corresponding to an HR of 78 ± 12 bpm. Within 24 hours after delivery, the heart rate increased to 80 ± 10 bpm. No significant changes in maternal RR interval or HR were found.

Table 2 contains the fetal and neonatal values for the frequency-domain components LF nu, HF nu and the ratio of LF/HF. There was a significant change in LF before (41.1 ± 9.6 nu) and after (87.0 ± 2.5 nu) calving and HF before (79.2 ± 4.6 nu) and after (30.9 ± 5.9 nu) delivery ($p<0.05$). In the LF/HF ratio, there was no significant change seen in the transition from one stage to another.

Table 3 describes the results of recordings conducted at six time points during the 35 days before calving and one time point in the first 24 hours after calving in Holstein cows. The heart rate variability (HRV) indexes reported are in the time (HR, RR, SDNN and RMSSD) and frequency domains (LF, HF and LF/HF).

In the maternal frequency-domain indexes, LF and HF presented no statistically significant changes during the entire

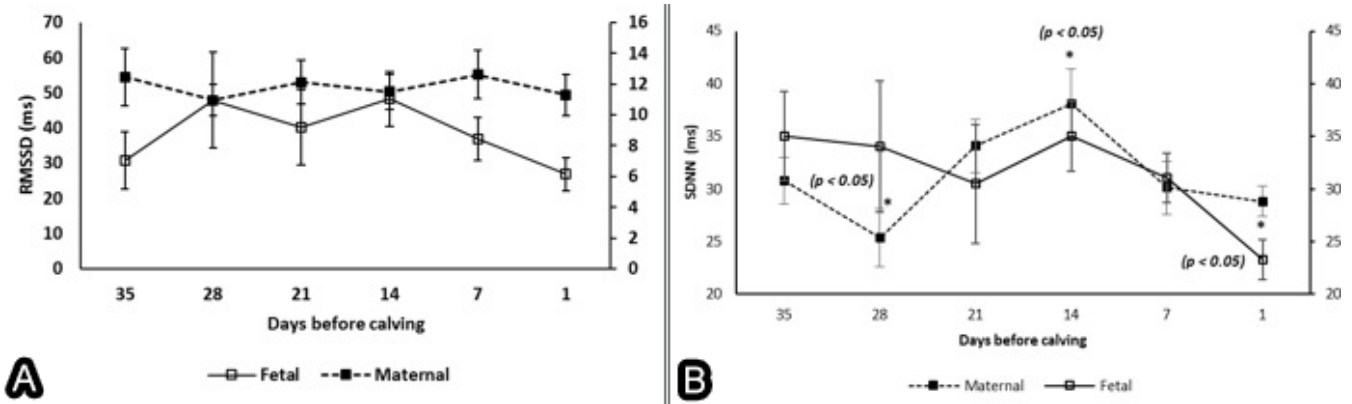


Fig.1. Maternal (■) and fetal (□) heart rate variability in the time domain: (A) root mean square of successive beat-to-beat differences (RMSSD) and (B) standard deviation of the beat-to-beat interval (SDNN) at six time points from day 35 to 1 day before delivery (n=16 to 17 per time point).

Table 1. Mean±SEM and range of HRV indexes in the time domain during the perinatal period (n=16 to 18 per time point)

	Day	RR Interval (ms)		Heart Rate (bpm)		RMSSD (ms)		SDNN (ms)	
		Mean±SEM	Range	Mean±SEM	Range	Mean±SEM	Range	Mean±SEM	Range
Records before calving	-35	413±25.8 ^{abc}	139	148±8.8 ^{bcd}	49	30.8±8.1 ^{ab}	44	35.1±4.2 ^{ab}	23
	-28	417±21.8 ^{abc}	160	147±8.1 ^{bcd}	60	47.9±13.4 ^b	106	34.0±6.2 ^{ab}	52
	-21	407±21.6 ^{abc}	137	150±8.6 ^{bcd}	53	40.2±10.7 ^{ab}	62	30.5±5.6 ^{ab}	36
	-14	435±17.3 ^{abcd}	135	139±5.8 ^{abcd}	47	48.2±7.8 ^b	67	35.0±3.3 ^{ab}	31
	-7	394±7.6 ^{ab}	77	153±2.9 ^{cd}	30	37.0±6.1 ^{ab}	55	31.0±2.3 ^{ab}	24
	-1	381±19.2 ^a	264	161±7.1 ^d	98	26.9±4.7 ^{ab}	56	23.3±1.8 ^a	22
Records after calving	1	471±15.7 ^{bc}	260	130±4.8 ^{ab}	74	18.4±6.4 ^a	96	33.7±4.5 ^{ab}	79
	7	441±14.9 ^{cd}	256	139±5.0 ^{abcd}	88	18.1±6.1 ^a	85	32.7±4.6 ^{ab}	66
	14	460±21.0 ^{abcd}	357	135±6.0 ^{abc}	109	21.4±4.5 ^a	67	33.5±4.2 ^{ab}	51
	21	461±23.5 ^{bcd}	279	136±6.5 ^{abc}	76	16.6±5.5 ^a	88	37.9±7.5 ^{ab}	98
	28	460±17.4 ^{bcd}	221	134±5.4 ^{abc}	72	23.8±7.0 ^a	85	35.4±6.1 ^{ab}	84
	35	502±18.8 ^d	246	123±5.2 ^a	65	28.4±9.8 ^{ab}	128	47.1±9.8 ^b	125

^{a,b,c,d} Within each column, different superscripts denote significant differences ($p < 0.05$); SEM = standard error of the mean, RR = beat-to-beat interval, RMSSD = root mean square of successive beat-to-beat differences, SDNN = standard deviation of beat-to-beat interval, ms = milliseconds, bpm = beats per minute. Bonferroni test.

Table 2. Mean±SEM and range of heart rate variability (HRV) indexes in the frequency domain during the perinatal period (n=16 to 18 per time point)

	Days	LF (nu)		HF (nu)		LF/HF	
		Mean±SEM	Range	Mean±SEM	Range	Mean±SEM	Range
Records before calving	35	65±16.4 ^{abcde}	50	42.6±24.2 ^{abc}	75	3.5±1.1	7.5
	28	74±10.5 ^{bcde}	83	51.4±12.2 ^{abc}	71	2.8±0.8	6.2
	21	54.3±5.8 ^{abc}	34	69.6±9.5 ^{bc}	61	0.8±0.2	1.4
	14	44.6±6 ^{ab}	31	75.4±5.7 ^{bc}	33	0.6±0.1	1
	7	56.8±10.8 ^{abcd}	65	67.6±10.6 ^{abc}	63	2.1±0.9	9.7
	1	41.1±9.6 ^a	64	79.2±4.6 ^c	34	0.9±0.1	2
Records after calving	1	87.0±2.5 ^e	34	30.9±5.9 ^a	77	6.0±1.6	25
	7	83.3±3.8 ^{de}	53	29.4±6.0 ^a	82	6.1±1.6	27.5
	14	81.7±3.7 ^{bcd}	44	35.6±5.2 ^{ab}	69	4.1±1.2	20.8
	21	89.9±2.7 ^e	44	28.1±4.7 ^a	57	6.0±1.4	20.1
	28	82.1±4.4 ^{cde}	70	36.9±6.5 ^{ab}	90	5.3±1.5	17.7
	35	80.5±4.4 ^{cde}	46	37.5±6.7 ^{ab}	85	3.7±1.2	17.5

^{a,b,c,d} Within each line, different superscripts denote significant differences ($p < 0.05$); SEM = standard error of the mean, LF = low frequency, HF = high frequency, LF/HF = ratio of low frequency to high frequency, nu = normalized units. Bonferroni test.

recording period before and after calving. Meanwhile, the LF/HF ratio had a statistically significant change ($p<0.05$) on day 14 (32.6 ± 28.6) before calving and 24 hours after delivery (10 ± 14.3) (Table 3).

DISCUSSION

At day 35 before calving, the mean FHR was 148 ± 8.8 bpm, and it exhibited a statistically significant increase to 161 ± 7.1 bpm by day 1 after delivery. During fetal life, there is a common increase in HR associated with the functional maturation of the cardiac muscle (Territo & Altimiras 1998). After calving, neonatal HR decreased significantly to 130 ± 4.8 bpm. After birth, HR tends to decrease with age due to structural and hemodynamic changes (Patteson 1996).

In the present study, the maternal HR increased from 71 ± 8.9 bpm to 80 ± 3 bpm and then decreased after delivery. During pregnancy, blood volume, heart rate and systolic volumen increase or increase the chronotropic overload to maintain homeostasis (Tejera et al. 2012). Thus, to supply a blood demand for the system, the autonomic nervous system will raise the heart rate (HR). This ability to adapt to different levels of stimuli is able to guarantee the safety of the autonomic nervous system (Kamath et al. 2013). The changes in maternal HR and RR interval during the days before delivery were not statistically significant, indicating that these parameters do not allow prediction of the delivery time.

In the fetal stage, recordings of RMSSD registered a statistically significant decrease from day 14 before calving, and SDNN decreased from day 35. Reductions in the values of the HRV variables SDNN and RMSSD reflect a shift toward greater sympathetic dominance (Aurich et al. 1993). Changes in fetal HRV observed during the last month of gestation indicate maturation of the ANS and its regulatory influence on HR.

After delivery, the neonates presented a declining trend in RMSSD and an increasing trend in SDNN, both statistically significant. These changes during the peripartum period

suggest that calving is stressful for the fetus. Based on the amounts of catecholamines and endorphins released in calves, birth has been characterized as the most stressful event in their lifetime (Mohr et al. 2002).

Elevated HR values were observed in the first weeks of life of the neonate, suggesting increased sympathoadrenal activity in this period, presumably caused by increasing demands on the cardiovascular system with adaptation to extrauterine life (Nagel et al. 2012). Thus, sympathetic innervation could have rapidly increased the heart rate of these animals through the release of adrenal hormones, such as cortisol, through sympathetic stimulation of adrenal cortex cells (UlrichLai & Herman 2009). The neonate has low blood pressure, blood volume and peripheral vascular resistance, so that, in order to maintain an adequate perfusion, it maintains a higher frequency and cardiac output, as well as central venous pressure compared to adults (Magrini 1978, Adelman & Wright 1985).

In the fetal-stage recordings, an increase of LF normalized units and a decreased in HF normalized units until birth were registered, indicating sympathetic predominance during the active state in fetuses near full term. On the day before delivery, LF normalized units rose while HF normalized units declined, indicating modulation of heart rate and ventilation by the vagal nerve (Malik et al. 1996). In the neonatal stage, the low frequency in normalized units (LF nu) and high frequency in normalized units (HF nu) showed a stable behavior.

Weeks before calving, the RR interval dropped, corresponding to an increase in HR, in cows. The cardiovascular system of cows in advanced pregnancy thus adapts to the increasing demands of gestation with a rise in HR. Physiological HR in healthy, late-pregnant cows may therefore be slightly higher. The HRV time-domain index SDNN and frequency-domain index LF/HF ratio recorded a statistically significant decrease between day 1 before and day 1 after calving. The decline in the sympathovagal balance (LF/HF ratio) implies a dominance of cardiac sympathetic tone, which was also

Table 3. Mean $\pm$ SEM heart rate variability (HRV) indexes in the time (HR, RR, SDNN and RMSSD) and frequency domains (LF, HF and LF/HF) during the last month of gestation and the first day after calving in Holstein cows (n=18 to 22 per time point)

Days		Before calving						After calving
		35	28	21	14	7	1	1
LH/HF	Mean $\pm$ SEM	13.8 $\pm$ 6.4 ^{ab}	19.7 $\pm$ 5 ^{ab}	21.7 $\pm$ 4.8 ^{ab}	32.6 $\pm$ 4.5 ^b	17.2 $\pm$ 4.2 ^{ab}	15.6 $\pm$ 4.1 ^{ab}	10.04 $\pm$ 4.3 ^a
	Range	28.8	44.8	60.5	106.5	62.4	67.6	61.4
RR	Mean $\pm$ SEM	849 $\pm$ 37	776 $\pm$ 25	783 $\pm$ 29	802 $\pm$ 26	790 $\pm$ 18	781 $\pm$ 33	749 $\pm$ 21
	Range	347	312	324	409	259	519	321
SDNN	Mean $\pm$ SEM	30.7 $\pm$ 3.7 ^{ab}	25.3 $\pm$ 2.9 ^a	34 $\pm$ 2.8 ^{ab}	38 $\pm$ 2.6 ^b	30.7 $\pm$ 3.7 ^{ab}	28.8 $\pm$ 2.7 ^{ab}	23.7 $\pm$ 2.5 ^a
	Range	26.9	16.9	31.8	42.4	40.2	50.4	27.1
HR	Mean $\pm$ SEM	71 $\pm$ 3	78 $\pm$ 3	77 $\pm$ 3	76 $\pm$ 2	76 $\pm$ 2	80 $\pm$ 3	79 $\pm$ 3
	Range	30	32	31	38	25	45	36
RMSSD	Mean $\pm$ SEM	12.4 $\pm$ 1.7	10.9 $\pm$ 1	12.1 $\pm$ 1.4	11.4 $\pm$ 1.1	11.7 $\pm$ 1.3	10.2 $\pm$ 1.6	13.9 $\pm$ 1
	Range	13.1	11.3	17.1	17.2	23.3	24	28.7
LF	Mean $\pm$ SEM	92.1 $\pm$ 2.7	91.3 $\pm$ 2.4	91.6 $\pm$ 2.2	91.5 $\pm$ 2.5	87.9 $\pm$ 3.7	85.2 $\pm$ 2.7	79.9 $\pm$ 3.5
	Range	23.5	31.7	30.4	38.5	55.2	39.7	41.5
HF	Mean $\pm$ SEM	9.3 $\pm$ 2.9	8.3 $\pm$ 2.3	8 $\pm$ 2.1	8.2 $\pm$ 2.5	14.6 $\pm$ 3.7	16.9 $\pm$ 4.5	19.8 $\pm$ 3.5
	Range	21.9	30.7	29.4	38.5	54.6	89.1	41.5

^{a, b} Within each line, different superscripts denote significant differences ($p<0.05$); SEM = standard error of the mean, RR = beat-to-beat interval, RMSSD = root mean square of successive beat-to-beat differences, SDNN = standard deviation of beat-to-beat interval, ms = milliseconds, BPM = beats per minute, LH = low frequency, HF = high frequency, LF/HF = ratio of low frequency to high frequency, nu = normalized units. Bonferroni test.

observed in lactating dairy cows by Kovács et al. 2014b. Changes in HRV with a declining trend in the last day might be associated with stressful situations (Schmidt et al. 2010). This study demonstrates that cows give birth under reduced parasympathetic tone.

CONCLUSIONS

Frequency heart rate (FHR) monitoring not only makes it possible to assess fetal health and viability but also can suggest information about the stage of development of the ANS. Reductions in the values of SDNN and RMSSD can reflect a shift toward greater sympathetic dominance.

After calving, the increase in HF and decrease in LF variables can indicate activation of the vagal nerve followed by heart and respiratory modulation.

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

REFERENCES

- Abbond S. & Sadeh D. 1990. Power spectrum analysis of fetal heart rate variability using the abdominal maternal electrocardiogram. *J. Biomed. Eng.* 12(2):161-164. <[http://dx.doi.org/10.1016/0141-5425\(90\)90138-D](http://dx.doi.org/10.1016/0141-5425(90)90138-D)> <PMid:2319767>
- Adelman R.D. & Wright J. 1985. Systolic blood pressure and heart rate in the growing beagle puppy. *Dev. Pharmacol. Ther.* 8(6):396-401. <<http://dx.doi.org/10.1159/000457064>> <PMid:4075938>
- Akselrod S., Gordon D., Ubel F.A., Shannon D.C., Berger A.C. & Cohen R.J. 1981. Power spectrum analysis of heart rate fluctuation: a quantitative probe of beat-to-beat cardiovascular control. *Science* 213(4504):220-222. <<http://dx.doi.org/10.1126/science.6166045>> <PMid:6166045>
- Aurich J.E., Dobrinski I., Petersen A., Grunert E., Rausch W.D. & Chan W.W. 1993. Influence of labor and neonatal hypoxia on sympathoadrenal activation and methionine enkephalin release in calves. *Am. J. Vet. Res.* 54(8):1333-1338. <PMid:8214906>
- Camm A.J., Malik M. & Bigger J.T. 1996. Heart rate variability: standards of measurement, physiological interpretation and clinical use. Task force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology. *Circulation* 93(5):1043-1065. <<http://dx.doi.org/10.1161/01.CIR.93.5.1043>> <PMid:8598068>
- Guyton A.C. & Hall J.E. 2000. The heart, p.95-142. In: Guyton A.C. & Hall J.E. (Eds), *Textbook of Medical Physiology*. 10th ed. W.B. Saunders, Philadelphia.
- Kamath M., Watanabe M. & Upton A. 2013. Heart Rate Variability Signal Analysis: clinical applications. CRC Press, New York. p.532.
- Kleiger R.E., Stein P.K., Bosner M.S. & Rottman J.N. 1995. Time domain measurements of heart rate variability, p.33-45. In: Malik M. & Camm A.J. (Eds), *Heart Rate Variability*. Futura Publishing Company Inc, Armonk, New York.
- Kovács L., Krisztina N., Szelényi Z., Szenci O. & Janos T. 2012. Heart rate variability as a measure of stress in cattle: biological background, methods of measurement and results, a review. *Magyar Allatorvosok Lapja* 134:515-523.
- Kovács L., Jurkovich V., Bakony M., Szenci O., Poti P. & Tozser J. 2014a. Welfare implication of measuring heart rate and heart rate variability in dairy cattle: literature review and conclusions for future research. *Animal* 8(2):316-330. <<http://dx.doi.org/10.1017/S1751731113002140>> <PMid:24308850>
- Kovács L., Tozser J., Szenci O., Poti P., Kezer F.L., Ruff F., Gabriel-Tozser G., Hoffmann D., Bakony M. & Jurkovich V. 2014b. Cardiac responses to palpation per rectum in lactating and nonlactating dairy cows. *J. Dairy Sci.* 97(11):6955-6963. <<http://dx.doi.org/10.3168/jds.2014-8327>> <PMid:25200771>
- Levy M.N. & Martin P.J. 1978. Neural control of the heart, p.337-354. In: Berne R.M. (Ed), *Handbook of Physiology*. American Physiological Society, Rockville.
- Magrini F. 1978. Haemodynamic determinants of the arterial blood pressure rise during growth in conscious puppies. *Cardiovasc. Res.* 12(7):422-428. <<http://dx.doi.org/10.1093/cvr/12.7.422>> <PMid:719655>
- Malik M., Camm A.J., Bigger J.T., Breithar D.T., Cerutti S., Cohen R.J., Coumel P., Fallen E.L., Kennedy H.L., Kleiger R.E., Lombardi F., Malliani A., Moss A.J., Rottman J.N., Schmidt G., Schwartz P.J. & Singer D.H. 1996. Heart rate variability: standards of measurement, physiological interpretation, and clinical use. *Eur. Heart J.* 17(3):354-381. <<http://dx.doi.org/10.1093/oxfordjournals.eurheartj.a014868>> <PMid:8737210>
- Moberg G.P. 2000. Biological response to stress: implications for animal welfare, p.1-21. In: Moberg G.P., Mench J.A. (Eds), *The Biology of Animal Stress*. CABI Publishing Wallingford, United Kingdom. <<http://dx.doi.org/10.1079/9780851993591.0001>>
- Mohr E., Langbein J. & Nurnberg G. 2002. Heart rate variability: a noninvasive approach to measure stress in calves and cows. *Physiol. Behav.* 75(1/2):251-259. <[http://dx.doi.org/10.1016/S0031-9384\(01\)00651-5](http://dx.doi.org/10.1016/S0031-9384(01)00651-5)> <PMid:11890975>
- Nagel C., Erber R., Bergmaier C., Wulf M., Aurich J., Möstl E. & Aurich C. 2012. Cortisol and progesterone release, heart rate and heart rate variability in the pregnant and postpartum mare, fetus and newborn foal. *Theriogenology* 78(4):759-767. <<http://dx.doi.org/10.1016/j.theriogenology.2012.03.023>> <PMid:22626780>
- Patteson M. 1996. *Equine Cardiology*. Oxford & Northampton, Blackwell Science, England. p.254.
- Reef V.B., Vaala W.E., Worth L.T., Sertich P.L. & Spencer P.A. 1996. Ultrasonographic assessment of fetal well-being during late gestation: development of an equine biophysical profile. *Equine Vet. J.* 28(3):200-208. <<http://dx.doi.org/10.1111/j.2042-3306.1996.tb03773.x>> <PMid:28976710>
- Schmidt A., Mostl E., Wehnert C., Aurich J., Müller J. & Aurich C. 2010. Cortisol release and heart rate variability in horses during road transport. *Horm. Behav.* 57(2):209-215. <<http://dx.doi.org/10.1016/j.yhbeh.2009.11.003>> <PMid:19944105>
- Schneider U., Frank B., Fiedler A., Kaehler C., Hoyer D., Liehr M., Haueisen J. & Schleussner E. 2008. Human fetal heart rate variability-characteristics of autonomic regulation in the third trimester of gestation. *J. Perinatal Med.* 36(5):433-441. <<http://dx.doi.org/10.1515/JPM.2008.059>> <PMid:18605969>
- Tarvainen M.P. & Niskanen J.P. 2008. Kubios HRV Version 2.0. User's Guide. Department of Physics, University of Kuopio, Kuopio, Finland.
- Tejera E., Areias J.M., Rodrigues A., Nieto-Villar J.M. & Rebelo I. 2012. Blood pressure and heart rate variability complexity in normal pregnancy. Influence of age, familiar history and parity. *Hypertension Pregnancy* 31:91-106. <<http://dx.doi.org/10.3109/10641955.2010.544801>> <PMid:21599453>
- Territo P.R. & Altimiras J. 1998. The ontogeny of cardio-respiratory function under chronically altered gas compositions in *Xenopus laevis*. *Respiratory Physiol.* 111(3):311-323. <[http://dx.doi.org/10.1016/S0034-5687\(97\)00117-5](http://dx.doi.org/10.1016/S0034-5687(97)00117-5)> <PMid:9628236>
- Ulrich-Lai Y.M. & Herman J. 2009. Neural regulation of endocrine and autonomic stress response. *Nat. Rev. Neurol.* 10:307-409.
- Van Leeuwen P., Lange S., Geue D. & Gronemeyer D. 2007. Heart rate variability in the fetus: a comparison of measures. *Biomed. Tech., Berlin*, 52(1):61-65. <<http://dx.doi.org/10.1515/BMT.2007.012>> <PMid:17313336>
- Von Borell E., Langbein J., Despres G., Hansen S., Lettieri C., Marchant-Forde J., Marchant-Forde R., Minero M., Mohr E., Prunier A., Valance D. & Veissier I. 2007. Heart rate variability as a measure of autonomic regulation of cardiac activity for assessing stress and welfare in farm animals, a review. *Physiol. Behav.* 92(3):293-316. <<http://dx.doi.org/10.1016/j.physbeh.2007.01.007>> <PMid:17320122>

Isolation, cultivation and immunofluorescence characterization of lamellar keratinocytes from equine hoof by using explants¹

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ABSTRACT- Pfeifer J.P.H., Santos V.H., Rosa G., Souza J.B., Watanabe M.J., Fonseca-Alves C.E., Deffune E. & Alves A.L.G. 2019. **Isolation, cultivation and immunofluorescence characterization of lamellar keratinocytes from equine hoof by using explants.** *Pesquisa Veterinária Brasileira* 39(4):292-298. Departamento de Cirurgia e Anestesiologia Veterinária, Faculdade de Medicina Veterinária e Zootecnia, Universidade Estadual Paulista "Julio de Mesquita Filho", Distrito de Rubião Junior s/n, Botucatu, SP 18618-681, São Paulo, Brazil. E-mail: ana.liz@unesp.br

The importance of the hoof to the horse health is clear, and the current knowledge regarding the cellular aspects of hoof keratinocytes is poor. Studies on equine keratinocyte culture are scarce. Developing keratinocyte cultures *in vitro* is a condition for studies on molecular biology, cell growth and differentiation. Some methods have already been established, such as those for skin keratinocyte culture. However, few methodologies are found for lamellar keratinocytes. The objective of this study was to standardize the equine hoof keratinocyte isolation and cultivation, and then characterize the cell immunophenotype. For this, the primary culture method used was through explants obtained from three regions of the equine hoof (medial dorsal, dorsal, and lateral dorsal). After the cell isolation and cultivation, the cell culture and its explants were stained with anti-pan cytokeratin (pan-CK) (AE1/AE3), vimentin (V9), p63 (4A4), and Ki-67 (MIB-1) antibodies. Cells were grown to third passage, were positive for pan-CK, p63 and Ki-67, and few cells had vimentin positive expression. As for the explants, the epidermal laminae were not stained for vimentin or Ki-67. However, some cells presented positive pan-CK and p63 expression. This study demonstrated the viability of lamellar explants of equine hooves as a form of isolating keratinocytes in primary cultures, as well as characterized the proliferation ability of such keratinocytes in monolayers.

INDEX TERMS: Isolation, cultivation, immunofluorescence, lamellar keratinocytes, equine hoof, cell migration, culture technology, skin, tissue engineering.

RESUMO.- [Isolamento, cultivo e caracterização por imunofluorescência de queratinócitos lamelares de casco equino utilizando explantes.] É notória a importância do casco na saúde dos equinos, mas o conhecimento em nível celular é pouco entendido. Estudos envolvendo o cultivo

de queratinócitos equinos são escassos. Sabe-se que o desenvolvimento de cultivos de queratinócitos *in vitro* é uma condição para estudos sobre a biologia molecular, crescimento e diferenciação celular. Alguns métodos já estão estabelecidos, como para cultivo de queratinócitos de pele, mas poucas metodologias são encontradas para queratinócitos lamelares. O objetivo desse estudo foi padronizar o cultivo de queratinócitos provenientes de casco equino visando futuramente associar ao estudo da medicina regenerativa para assim estabelecer um modelo experimental *in vitro* e indicar o uso criterioso de terapias regenerativas para a laminite equina. Desta forma, o cultivo em monocamada e a caracterização de queratinócitos lamelares foram realizados. Para isso, o método de cultura primária utilizado foi através de explantes obtidos de três regiões do casco (dorso-medial,

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dorsal e dorso-lateral). As células foram caracterizadas para os marcadores anti pan-cytokeratin (AE1/AE3), vimentin (V9), p63 (4A4) e Ki-67 (MIB-1) nos cultivos e nos explantes. As células foram cultivadas até terceira passagem, tendo marcação positiva para pan-CK, p63 e Ki-67 e fraca marcação para vimentina. Já as lâminas epidermais não tiveram marcação de vimentin e Ki-67, porém marcaram acentuadamente para pan-CK e p63. Este estudo demonstrou a exequibilidade do uso de explantes lamelares do casco de equinos, como forma de isolamento de queratinócitos em cultivos primários, bem como caracterizou a habilidade de proliferação desses queratinócitos em monocamada.

TERMOS DE INDEXAÇÃO: Isolamento, cultivo, imunofluorescência, queratinócitos lamelares, casco equino migração celular, tecnologia de cultivo, engenharia tecidual.

INTRODUCTION

Several studies have sought to elucidate and characterize healthy tissues of epithelial origin, such as the skin, the eyes, and the hoof, so that they can identify the mechanisms of disease development in these tissues (Parsa et al. 1999, Carter et al. 2010, 2011, Shimabukuro et al. 2014, Linardi et al. 2015). The difficulty in healing or repairing keratinized tissues in horses has led to *in vitro* investigations mainly on skin and hoof injuries, particularly laminitis. Such studies focus both on the behavior and on the expression of hoof cell protein patterns (Ekfalck et al. 1990, Wunn et al. 1999, Dahm et al. 2002, Visser & Pollitt 2010, Pawlak et al. 2014, Sharma et al. 2016).

The cultivation of equine keratinocytes has been researched for decades, and the studies are divided either into the distinct objectives, such as how to obtain the keratinocytes (methods of tissue collection) or by the diverse ways of isolation, culture media and expression of cytokeratins (Ekfalck et al. 1990, Wunn et al. 1999, Dahm et al. 2002, Visser & Pollitt 2010, Sharma et al. 2016). An *in vitro* analysis of the keratinocytes culture of the equine hoof performed by Dahm et al. (2002) demonstrated that these cells have cuboid/oval morphological characteristics.

For the characterization of the keratinocyte culture, antibodies are used to recognize the cytokeratins of these cells of epithelial origin, as specific cytokeratins according to the cellular source. For lamellar cells, the anti-cytokeratin type I and II (*Pan-Cytokeratin*) antibodies serve as markers of lamellar origin (Visser & Pollitt 2010). To carry out the negative control of these cultures, many researchers use anti-vimentin markers, which is a protein expressed in cells of mesenchymal origin, that is, cells of epithelial origin do not express it (Wunn et al. 1999, Visser & Pollitt 2010, Sharma et al. 2016).

The expression of Ki-67 together in p63 positive cells is similar to the expression pattern observed in skin and stratified epidermal cultures, where the expression of such proteins is present in cells that are proliferating or have proliferation capacity (Yang et al. 1998, Senoo et al. 2007).

The equine hoof is formed by epidermis in different stages of keratinization subdivided in stratum externum (or hoof wall), stratum medium, and stratum internum. It is in the latter that the primary epidermal laminae (PEL) and secondary epidermal laminae (SEL) are located, where the

keratinocytes are attached to the basement membrane (Pollitt 1995). In cases of endotoxemia, the target regions frequently affected are the dermo-epidermal interfaces of the secondary laminae, consequently causing the onset of laminitis in horses (French & Pollitt 2004, Belknap et al. 2007, Pollitt 2007).

Despite the great importance of the hoof in equine health, knowledge regarding the cells is still little studied (Carter et al. 2011), and the development of *in vitro* studies for keratinocyte cultures is essential for understanding the cell growth, differentiation, and cellular and molecular biology (Dahm et al. 2002).

The objective of this study was to standardize the equine hoof keratinocyte culture aiming to associate it with the study of regenerative medicine in the future in order to establish an experimental model *in vitro* and to indicate the wise use of regenerative therapies for equine laminitis.

MATERIALS AND METHODS

This was a pilot study performed according to the Brazilian Council for Animal Care and approved by the 'Ethics Committee in the Use of Animals' of the Faculdade de Medicina Veterinária e Zootecnia, Universidade Estadual Paulista - Unesp (Protocol number 151/2016).

Laminar tissue collection. Three samples of laminar tissue were collected from a five-year-old Mangalarga stallion that had died with no history or evidence of hoof or regional disease or alteration. The samples were collected one-hour *post mortem*.

The limb was sawn into the metacarpal middle third region and washed with soap and water to remove dirt. After cleaning, the hooves were subjected to antisepsis, and sterile surgical fields were positioned in the limb portion to the coronary band. The regions collected from each hoof were in the proximal-middle third in the dorsal (D), lateral dorsal (LD), and medial dorsal (MD) faces, all of them approximately two centimeters distal from the coronary band.

The fragments were collected according to Croser & Pollitt (2006) and Visser & Pollitt (2011) with modifications. Briefly, the dorsal, medial and lateral sample collection sites were 2cm distal to the hairline of the coronet, and the sites were 4cm equidistant. Tissue samples were obtained using a 1.5cm diameter hole saw (Starret, BRA) and a drill (Bosch, BRA), both sterile. The perforation was irrigated with sterilizing solution in order to avoid heating the sample until reaching approximately 2cm deep, according to the perception of the saw touching the dermis. Subsequently, still with the hole saw, the fragments were luxated from the dermis with proximal to distal movements, then removed and packed into conical tubes containing phosphate-buffered saline - PBS (Gibco, USA) for washing. After being washed in PBS three times, the fragments were packed in a carrier containing antibiotic-antimycotic (amphotericin B, penicillin and streptomycin) zwitterionic buffer HEPES (Gibco, USA) and then sent to the laboratory.

Keratinocyte isolation. The keratinocyte isolation using explants was based on the methodologies of Ekfalck et al. (1990) and Sharma et al. (2016). Concisely, the fragments were washed three times using iodopovidine with decreasing 4%, 2%, and 1% dilution, and then washed with 30 mL PBS. Afterwards, the fragments were packed into falcon-type tubes with HEPES (Gibco, USA) + 0,6% antibiotic-antimycotic (Gibco, USA) + amikacin (Teuto, BRA), and kept overnight (18h) at 4°C. After this period, the epidermal laminae were separated from the hoof matrix using a scalpel blade nº 24. Such epidermal laminae were cut, weighed, and distributed in duplicate with equal weights in Petri dishes.

Keratinocyte culture. A flask containing 500mL calcium free DMEM (Gibco, USA) culture medium was added supplements with 5% fetal bovine serum (FBS) (Gibco, USA), 10ng/mL epidermal growth factor (EGF) (Gibco, USA), 30µg/mL bovine pituitary extract (BPE) (Gibco, USA), 0.4µg/mL hydrocortisone (Blau Pharmaceuticals, BRA), 5µg/mL insulin (Sigma-Aldrich, USA), 0.6mM calcium (HalixIstar, BRA), 1% antibiotic-antimycotic (Gibco, USA), and 2.2µL amikacin (Teuto, BRA). The primary cultures were carried out in Petri dishes for 10 days in the presence of the 10 explants per dish, incubated in a humidified atmosphere of 5% carbon dioxide at 37°C. Every 48-72 hours, the culture medium was replenished, and the cell growth was monitored by using an inverted phase contrast microscope.

The explants were removed on day 10 and the primary cell culture was cultured until approximately 80% of confluence, when a selective trypsinization was performed with cold Trypsin-EDTA, according to Kisselbach et al. (2009). This procedure allowed the fibroblast detachment and then we trypsinized the remaining attached cells for 10 minutes using 0.25% Trypsin-EDTA at 37°C (Gibco, USA). The cells were then centrifuged at 251 x *g* for 10 minutes and washed with the media HEPES (Gibco, USA) and FBS (Gibco, USA).

During the subculture, the cells were grown in six well plates (Sarstedt, GER) and incubated in a controlled environment incubator (5% CO₂ at 37°C), replenishing the culture medium and monitoring cell growth in an inverted phase contrast microscope (Carl Zeiss Company, GER) every 48-72 hours. At the end of the third passage, the cultures were characterized by immunofluorescence.

Cell culture immunofluorescence. The immunofluorescence was used to characterize the markers following the recommendation of Guo & Jahoda (2009). In short, a cell-culture glass coverslip was inserted into each of the six wells of a plate, and culture medium was added. Each culture was carefully transferred in quadruplicate into each well of the plate, being dripped on top of the respective coverslips, and incubated at 37°C and 5% CO₂. After the cell adhesion to the coverslips, cell fixation was performed by adding 1 mL methanol (CH₃OH) to each well, and the samples were incubated at 4°C for 30 minutes. Thereafter, cell permeabilization was performed using 0.2% Tween 20 (DAKO, DK) for 10 min.

The non-specific proteins were blocked using a commercial protein block solution (Spring Bioscience, USA), and were incubated at 27°C for 20 minutes. Next, the primary antibodies were incubated overnight at 4°C. The primary antibodies used were pan-CK AE1/AE3 (sc81714, Santa Cruz Biotechnology, USA) and Vimentin (V9 - sc6260, Santa Cruz Biotechnology, USA), both at 1:300 dilution, in addition to p63 (4A4 - sc8431, Santa Cruz Biotechnology, USA) and Ki-67 (Mib-1-clone - M7240, DAKO, DK), primary antibodies, both at 1:100 dilution. The secondary antibody Alexa Fluor 532 (A11002, Life Technology, CAN) was used at 5µg/mL concentration and incubated in the dark for 30 minutes at room temperature. After that, diamidino-2-phenylindole, DAPI (Sigma-Aldrich, USA), was added at 1:1000 dilution to each coverslip. The samples were analyzed by confocal TCS SP5 laser scanning microscope (Leica Microsystems, GER). After the immunofluorescence labeling analysis, the positive cells were counted in each sample in a 15 mm-diameter area (coverslip area), with the mean of the count being defined as a percentage of the positive cells (positive cells/total cells).

Lamellar explant immunofluorescence. Lamellar explant fragments were fixed in 10% buffered formalin solution and embedded in paraffin. The tissue sections with 4µm were prepared in a manual microtome (Leica Microsystems, GER) and were placed on immunohistochemistry slides (Starfrost, Knittel, Germany). The antigen retrieval was performed using a citrate buffer solution

in a pressure cooker (DakoCytomation, Carpinteria, CA, USA). Consequently, the endogenous peroxidase blocking was performed with 8% peroxidase solution diluted in methanol for 10 minutes. The non-specific proteins were blocked using a commercial protein block solution (Spring Bioscience, USA) and then incubated at 27°C for 20 minutes. Afterwards, the primary antibodies were incubated overnight at 4°C. The antibodies anti-cytokeratin, anti-vimentin, anti-p63 (4A4, Santa Cruz Biotechnology, USA), and anti-Ki-67 (Mib-1-clone, DAKO, DK) were diluted at 1:300, 1:300, 1:50, and 1:50, respectively (Robertson et al. 2008). The secondary antibody Alexa Fluor 532 was used at 5µg/mL concentration and incubated in the dark for 30 minutes at room temperature. The samples were analyzed by confocal TCS SP5 laser scanning microscope (Leica Microsystems, GER).

RESULTS

Keratinocyte culture

After the explants were plated for three days, cells with ovoid/cuboidal morphology and variable size (20µm length and 15µm width, approximately) appeared under the explants adhered to the plate (Fig.1A). Within this time, some explants had some adherence to the plate, which later demonstrated low cellularity. In some of these explants, the surrounding cells were observed to come out from under the explants more slowly than others in the first days of culture. Seven days after plating, cells in proliferation with ovoid/cuboidal morphology and cells adhered were observed. At day 10, the explants were removed from the culture plates, which were maintained until reaching 80% confluence to make the passage.

Cells with fibroblastic characteristics were not observed in the cultures, neither when in subculture. A grouping of cells with stratified formation (Fig.1B) was observed, probably by an adaptation to the *in vitro* environment, since they are cells originating from the keratinized stratified epithelium. There was no difference in cell migration and proliferation among the collected regions (D, LD and MD).

Monolayer culture immunofluorescence

The pan-CK antibody had strong staining in the subculture cells of all three cultures (3/3). This positivity confirms that the cultured cells had epithelial origin (Fig.2A). The anti-vimentin antibody was positive in few cells and most of the cell population had no vimentin staining (Fig.2C). The basal cell marker p63 was positive in more than 90% of cells (Fig.2E). It was possible to identify the proliferation potential of cell cultures by Ki-67 (Fig.2G). It was justified by the explant immunofluorescence, as the cells coming out from under the explants and the axial cells were absent and negative for Ki-67, which maybe demonstrated that the cells might have migrated from the explant.

Explants immunofluorescence

The explant immunofluorescence analysis after the removal of the primary culture had a similar immunohistochemical pattern to the monolayer cultures. It was possible to identify positive pan-CK expression (Fig.3A) and negative vimentin expression in epithelial cells. Stromal cells of secondary dermal laminae were positive for vimentin (Fig.3B).

Interestingly, the p63 immunostaining of the explants revealed positive cells adjacent to the basement membrane of the SEL (Fig.3C). Thus, p63 positive cells were limited to

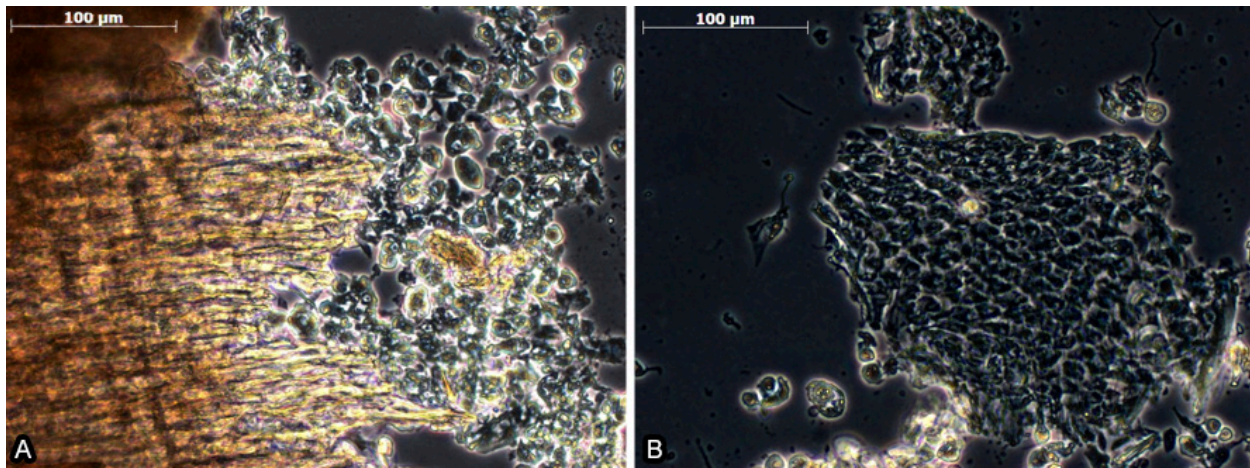


Fig.1. Inverted microscopy with phase contrast of primary cultures in monolayer. (A) Cells migrating to the culture plate and appearing beneath the explants. (B) Cell groupings in stratified epithelial formation typical of keratinocytes due to their epidermal origin. Ovoid cells are also observed. Obj.20x.

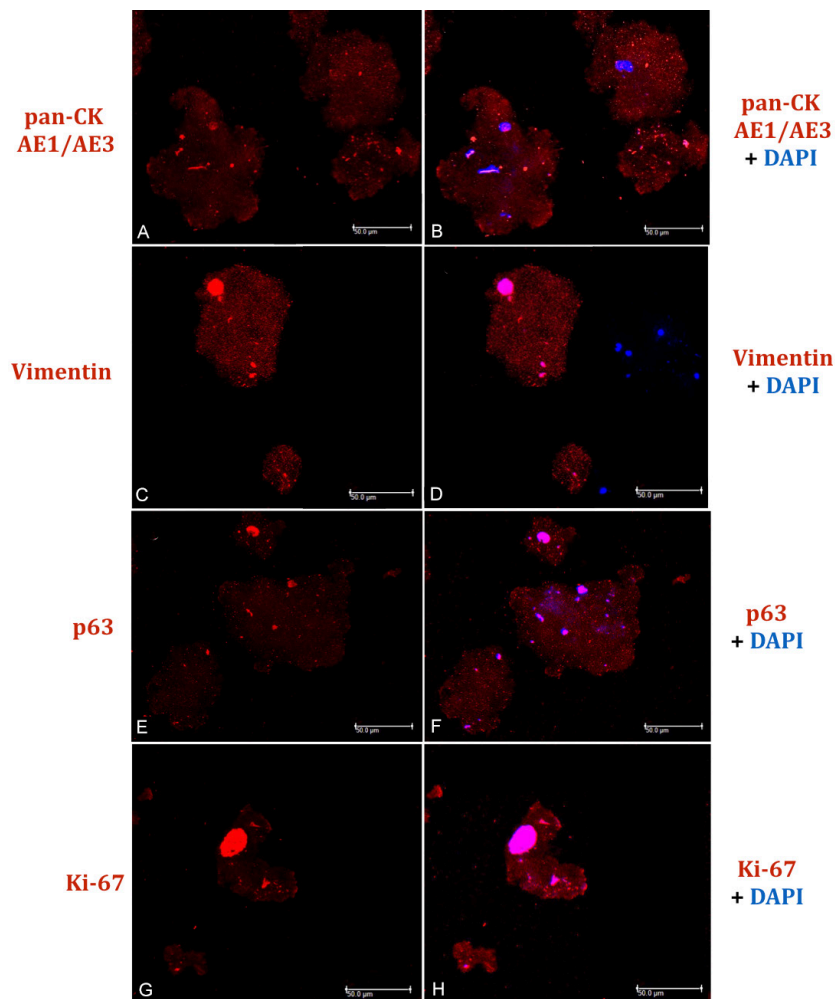


Fig.2. Keratinocyte culture immunofluorescence in monolayer. Red fluorophore labeling due to secondary antibody (Alexa Fluor 532) and blue due to nucleus labeling by DAPI. (A) Positivity for pan-CK due to cytoplasmic labeling in red. (B) Merged labeling image, in blue DAPI and red pan-CK. (C) Cytoplasmic labeling in red positive for vimentin. (D) Merged labeling image, in pink Alexa Fluor 532 + DAPI labeling cell nucleus, and negative labeling for vimentin in blue dots was also observed. (E) Positivity for p63. (F) Merged image, in pink Alexa Fluor 532 + DAPI labeling cell nucleus. (G) Ki-67 positivity evident by the red color in nucleus. (H) Merged image, the pink nucleus due to the sum of the secondary red for the Ki-67 nuclear antibody and the blue of DAPI, which marks the cellular nucleus. Some punctual markings can be observed in all images, yet with no relevance. Laser confocal microscopy, obj.40x.

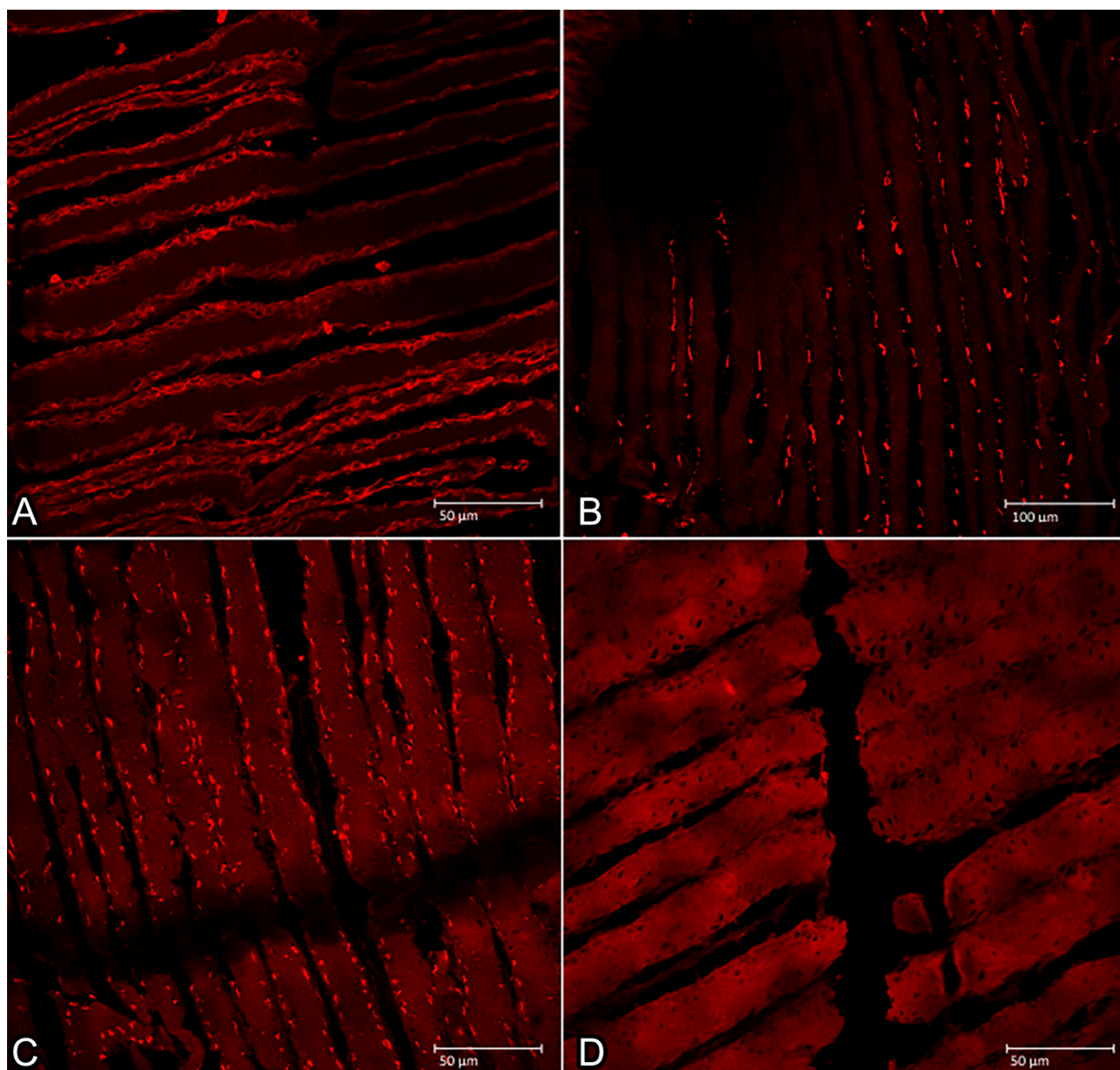


Fig.3. Immunofluorescence of the explants (epidermal laminae) after removal of the primary culture. (A) Cytoplasmic deep red pan-CK labeling in cells of the basal layer on the secondary laminae. Laser confocal microscopy, obj.40x. (B) Vimentin labeling was limited to the secondary dermal laminae region. Laser confocal microscopy, obj.20x. (C) Nuclear labeling of p63 in the cells present in the basal layer of the secondary laminae. Laser confocal microscopy, obj.40x. (D) Negative Ki-67 labeling where the cell nuclei appear dark. Laser confocal microscopy, obj.40x.

the germ cell layer, adjacent to the basal membrane of the SEL. The positive cells remained in these explants and did not migrate to the culture plate. Only the axial keratinocytes in the SEL of these explants might have migrated.

Ki-67 positive cells were identified in all analyzed explants (3/3) (Fig.3D). The positive cells were restricted to the basal membrane of the SEL.

DISCUSSION

From the standardization of equine hoof epidermal cell culture, an important step in the development of new therapies for equine laminitis can be taken by creating an experimental model *in*

vitro, thereby limiting the use and induction of such disease in animals for its study. In ours, the culture and characterization of lamellar keratinocytes of a healthy horse using explants as primary culture was successful. This method was based on previous research (Ekfalck et al. 1990, Sharma et al. 2016). The concentrations of the culture medium and supplements used were crucial to perform the lamellar keratinocyte culture in monolayer, especially the calcium concentration, which can influence the cellular proliferation and differentiation (Hennings et al. 1980). According to Dahm et al. (2002) and Visser & Pollitt (2010), the ideal culture medium for keratinocytes of lamellar origin is using DMEM medium with low calcium concentration (0.6mM).

Using 5% FBS since the primary culture was important, and it corroborates with our results, since the FBS supplementation around 2% can lead to cellular stress. Thus, 5% concentration was shown to be ideal for the keratinocytes culture by using explants (Sharma et al. 2016). Fibroblasts commonly appear in primary cultures and are separated from the plate along the trypsinizations or when specific enzymes are used to separate only this type of cell (Visser & Pollitt 2010). The absence of fibroblast cells was confirmed by the positive result in pan-CK and p63 expression assays, since fibroblasts do not express pan-CK or p63 (Lupatov et al. 2015). The absence of fibroblasts in the cultures may have occurred due to the caution in separating the epidermal laminae from the dermal tissue of the samples collected. Visser & Pollitt (2010) reported that such care is essential, so that there is no contamination by fibroblasts in the keratinocyte cultures. We did not perform flow cytometry to verify the cell immunophenotype due to the low number of cells isolated from each culture. Instead, we performed immunofluorescence to confirm the origin of each cell type that grew in culture conditions, according to Guo & Jahoda (2009). Even though the referred study was not performed with equine epithelial tissue, we found similar results, which prove that most of our cells had keratinocyte characteristics.

There was no variation on the cellular migration rate among the three different sites of sampling. This could be explained by the fact that all the sites were situated mainly in the dorsal aspect of the hoof wall. The nomenclature "LD" and "MD" was given comparing where they are to the central site of sampling, located in the central axis of the limb. Moreover, all the samples were obtained taking the same distance of the coronary region.

The keratinocyte culture characterization demonstrated the cell phenotype, indicating the origin and differentiation stage of these cells. The epithelial origin of cultured cells was confirmed by pan-cytokeratin expression, which recognized acidic and basic cytokeratins, types I and II, respectively. Cytokeratins are intermediate filaments of epithelial cells (Wilkinson et al. 1989) used to recognize cytokeratins from the hoof wall (Wattle 1998, Visser & Pollitt 2010). The cell morphology associated with the expression of pan-CK suggested these cells were keratinocytes. We also performed a fibroblast selective detachment using cold Trypsin-EDTA.

Vimentin is known to target cells or tissues of mesenchymal origin, like fibroblasts present in the dermis. Equine skin keratinocytes do not express vimentin (Dahm et al. 2002, Sharma et al. 2016). However, cells in culture condition can be submitted to different transcriptional modification, and vimentin expression by epithelial cells in culture conditions is widely reported (Coggi et al. 1989, Richard et al. 1990, Mahabal et al. 2016). Other studies evaluating lamellar keratinocytes have demonstrated that they can express vimentin when in culture (Wunn et al. 1999, Visser & Pollitt 2010), and such positive expression for vimentin may be an adaptation to the *in vitro* environment (Coggi et al. 1989, Richard et al. 1990). The positive result for vimentin in few cells can justify the adaptive condition of cells to the *in vitro* environment. Another result confirming the theory found in the literature was that the explants did not express vimentin, which is restricted to the secondary dermal laminae region.

The protein p63 in association with the other markers give evidence that the cells are keratinocytes. The p63 is a protein encoded by the *TP63* gene and represents a nuclear specific

marker for keratinocyte proliferation and differentiation, showing a decrease in its expression, mainly in prolonged cultures (Truong & Khavari 2007). The cytokeratin+/vimentin-/p63+ phenotype indicates the epidermal origin of the cells in our experiment, as this phenotype can be found in keratinocytes.

The evaluation of proliferation potential and maturation stage in which the keratinocytes are found, both *in vitro* and *in vivo*, is important so as to know how much the culture is still able to proliferate. The expression of p63 and Ki-67 was found in the cultured cells maintained until the third passage. It is also known that p63 is an epidermal stem cell (ESC) regulatory molecule, an intranuclear protein that is an expression marker related with the potential of cell proliferation (Parsa et al. 1999, Carter et al. 2011). Linardi et al. (2015) showed that in order to characterize and differentiate the presence of epidermal stem cells from the skin, coronary, and lamellar regions of equine hooves, there must be a combination of molecule p63 expression together with p63 phosphorylated molecule (pp63), which is a marker of the transition from ESC to transit-amplifying cell. On the other hand, Ki-67 is a protein present in the nucleolus of keratinocytes that are active in the cell cycle (Scholzen & Gerdes 2000), so its presence is a prerequisite for cultures performed for an extended period (Dahm et al. 2002).

The expression of Ki-67 together with p63 positive cells is similar to the pattern of expression observed in the skin and stratified epidermal cultures. The expression of p63 is present only in proliferating cells or those that have this ability (Yang et al. 1998, Senoo et al. 2007), which suggests what has been described by Blanpain & Fuchs (2009). They reported that the p63 positive cells in the coronary of the hoof, the periople region, continuously provide epithelium to generate hoof wall in rapid growth, while p63 positive cells in the lamellar function have action in the homeostasis maintenance and tissue repair, like their function in the skin.

However, Ki-67 labeling was not observed in the epidermal slides used as explants, only a poor labeling of p63, possibly due to the stage and stress to which those cells that did not migrate to the culture plate were subjected, as the labeling of these two molecules denote keratinocyte proliferative potential.

CONCLUSIONS

This study demonstrated the viability of lamellar explants of equine hooves as a form of keratinocyte isolation in primary culture, as well as characterized the proliferation ability of these keratinocytes in monolayers.

The results point to the need to implement the protocol used with techniques and methodologies that promote greater migration of explant cells to the culture environment. This implementation will be a tool as an experimental model for the evaluation of therapeutic studies that can elucidate cell behavior and evaluate the potential use of regenerative medicine as a treatment for equine laminitis.

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REFERENCES

- Belknap J.K., Giguère S., Pettigrew A., Cochran A.M., Van Eps A.W. & Pollitt C.C. 2007. Lamellar pro-inflammatory cytokine expression patterns in laminitis at the developmental stage and at the onset of lameness: innate vs. adaptive immune response. *Equine Vet. J.* 39(1):42-47. <PMid:17228594>
- Blanpain C. & Fuchs E. 2009. Epidermal homeostasis: a balancing act of stem cells in the skin. *Nat. Rev. Mol. Cell Biol.* 10(3):207-217. <http://dx.doi.org/10.1038/nrm2636> <PMid:19209183>
- Carter R.A., Shekk V., de Laat M.A., Pollitt C.C. & Galantino-Homer H.L. 2010. Novel keratins identified by quantitative proteomic analysis as the major cytoskeletal proteins of equine (*Equus caballus*) hoof lamellar tissue. *J. Anim. Sci.* 88(12):3843-3855. <http://dx.doi.org/10.2527/jas.2010-2964> <PMid:20622188>
- Carter R.A., Engiles J.B., Megee S.O., Senoo M. & Galantino-Homer H.L. 2011. Decreased expression of p63, a regulator of epidermal stem cells, in the chronic laminitic equine hoof. *Equine Vet. J.* 43(5):543-551. <http://dx.doi.org/10.1111/j.2042-3306.2010.00325.x> <PMid:21496086>
- Coggi G., Dell'Orto P., Braidotti P., Coggi A. & Viale G. 1989. Coexpression of intermediate filaments in normal and neoplasia human tissues: a reappraisal. *Ultrastruct. Pathol.* 13(5/6):501-514. <http://dx.doi.org/10.3109/01913128909074533> <PMid:2678645>
- Croser E. & Pollitt C.C. 2006. Acute laminitis: descriptive evaluation of serial hoof biopsies. 52nd Annual Convention of the American Association of Equine Practitioners, San Antonio, Texas, p.542-546. (Abstract)
- Dahm A.M., de Bruin A., Linat A., von Tscharn C., Wyder M. & Suter M.M. 2002. Cultivation and characterization of primary and subcultured equine keratinocytes. *Equine Vet. J.* 34(2):114-120. <http://dx.doi.org/10.2746/042516402776767187> <PMid:11902754>
- Eckfalk A., Rodriguez-Martinez H. & Obel N. 1990. Cultivation of tissue from the matrix of stratum medium of the equine and bovine hoof walls. *Am. J. Vet. Res.* 51(11):1852-1856. <PMid:2240811>
- French K.R. & Pollitt C.C. 2004. Equine laminitis: loss of hemidesmosomes in hoof secondary epidermal lamellae correlates to dose in an oligofructose induction model: an ultrastructural study. *Equine Vet. J.* 36(3):230-235. <http://dx.doi.org/10.2746/0425164044877125> <PMid:15147130>
- Guo A. & Jahoda C.A. 2009. An improved method of human keratinocyte culture from skin explants: cell expansion is linked to markers of activated progenitor cells. *Exp. Dermatol.* 18(8):720-726.
- Hennings H., Michael D., Cheng C., Steinert P., Holbrook K. & Yuspa S.H. 1980. Calcium regulation of growth and differentiation of mouse epidermal cells in culture. *Cell* 19(1):245-254. <http://dx.doi.org/10.1016/0092-8674(80)90406-7> <PMid:6153576>
- Kisselbach L., Merges M., Bossie A. & Boyd A. 2009. CD90 Expression on human primary cells and elimination of contaminating fibroblasts from cell cultures. *Cytotechnology* 59(1):31-44. <http://dx.doi.org/10.1007/s10616-009-9190-3> <PMid:19296231>
- Linardi R.L., Megee S.O., Mainardi S.R., Senoo M. & Galantino-Homer H.L. 2015. Expression and localization of epithelial stem cell and differentiation markers in equine skin, eye and hoof. *Vet. Dermatol.* 26(4):213-247. <http://dx.doi.org/10.1111/vde.12214> <PMid:25963063>
- Lupatov A.Y., Vdovin A.S., Vakhrushev I.V., Poltavtseva R.A. & Yarygin K.N. 2015. Comparative analysis of the expression of surface markers on fibroblasts and fibroblast-like cells isolated from different human tissues. *Bull. Exp. Biol. Med.* 158(4):537-543. <http://dx.doi.org/10.1007/s10517-015-2803-2> <PMid:25708341>
- Mahabal S., Konala V.B., Mamidi M.K., Kanafi M.M., Mishra S., Shankar K., Pal R. & Bhonde R. 2016. Sequential cultivation of human epidermal keratinocytes and dermal mesenchymal like stromal cells in vitro. *Cytotechnology* 68(4):1009-1018. <http://dx.doi.org/10.1007/s10616-015-9857-x> <PMid:25698160>
- Parsa R., Yang A., Mckee F. & Green H. 1999. Association of p63 with proliferative potential in normal and neoplastic human keratinocytes. *J. Invest. Dermatol.* 113(6):1099-1105. <http://dx.doi.org/10.1046/j.1523-1747.1999.00780.x> <PMid:10594758>
- Pawlak E.A., Geor R.J., Watts M.R., Black S.J., Johnson P.J. & Belknap J.K. 2014. Regulation of hypoxia-inducible factor-1alpha and related genes in equine digital lamellae and in cultured keratinocytes. *Equine Vet. J.* 46(2):203-209. <http://dx.doi.org/10.1111/evj.12092> <PMid:23663159>
- Pollitt C.C. 1995. *Color Atlas of The Horses's Foot*. Mosby-Wolfe, London, p.15-39.
- Pollitt C.C. 2007. Laminitis Pathophysiology, p.313-319 In: Floyd A.E. & Mansmann R.E. (Eds), *Equine Podiatry*. Saunders Elsevier, Saint Louis. <http://dx.doi.org/10.1016/B978-072160383-4.50019-0>
- Richard M.H., Viac J., Reano A., Gaucherand M. & Thivolet J. 1990. Vimentin expression in normal human keratinocytes grown in serum-free defined MCDB 153 medium. *Arch. Dermatol. Res.* 282(8):512-515. <http://dx.doi.org/10.1007/BF00371945> <PMid:2082832>
- Robertson D., Savage K., Reis-Filho J.S. & Isacke C.M. 2008. Multiple immunofluorescence labelling of formalin-fixed paraffin-embedded (FFPE) tissue. *BMC Cell Biol.* 9(1):13. <http://dx.doi.org/10.1186/1471-2121-9-13> <PMid:18366689>
- Scholz T. & Gerdes J. 2000. The Ki-67 protein: from the known and the unknown. *J. Cell Physiol.* 182(3):311-322. <http://dx.doi.org/10.1002/(SICI)1097-4652(200003)182:3<311::AID-JCP1>3.0.CO;2-9> <PMid:10653597>
- Senoo M., Pinto F., Crum C.P. & Mckee F. 2007. p63 is essential for the proliferative potential of stem cells in stratified epithelia. *Cell* 129(3):523-536. <http://dx.doi.org/10.1016/j.cell.2007.02.045> <PMid:17482546>
- Sharma R., Barakzai S.Z., Taylor S.E. & Donadeu F.X. 2016. Epidermal-like architecture obtained from equine keratinocytes in three-dimensional cultures. *J. Tissue Eng. Regen. Med.* 10(8):627-636. <http://dx.doi.org/10.1002/term.1788> <PMid:23897780>
- Shimabukuro J., Yamaoka A., Murata K., Kotani E., Hirano T., Nakajima Y., Matsumoto G. & Mori H. 2014. 3D co-cultures of keratinocytes and melanocytes and cytoprotective effects on keratinocytes against reactive oxygen species by insect virus-derived protein microcrystals. *Mater. Sci. Eng. C, Mater. Biol. Appl.* 42:64-69. <http://dx.doi.org/10.1016/j.msec.2014.05.013> <PMid:25063093>
- Truong A.B. & Khavari P.A. 2007. Control of keratinocyte proliferation and differentiation by p63. *Cell Cycle, Georgetown* 6(3):295-299. <http://dx.doi.org/10.4161/cc.6.3.3753> <PMid:17264679>
- Visser M.B. & Pollitt C.C. 2010. Characterization of extracellular matrix macromolecules in primary cultures of equine keratinocytes. *BMC Vet. Res.* 6(1):16. <http://dx.doi.org/10.1186/1746-6148-6-16> <PMid:20230631>
- Visser M.B. & Pollitt C.C. 2011. The time line of lamellar basement membrane changes during equine laminitis development. *Equine Vet. J.* 43(4):471-477. <http://dx.doi.org/10.1111/j.2042-3306.2010.00292.x> <PMid:21496071>
- Wattle O. 1998. Cytokeratins of the equine hoof wall, chestnut and skin: bio- and immunohisto-chemistry. *Equine Vet. J. Suppl.* 26(26):66-80. <PMid:9932096>
- Wilkinson J.E., Lee C.S., Lillie J.H., Suter M.M. & Lewis R.M. 1989. Ultrastructure of cultured canine oral keratinocytes. *Am. J. Vet. Res.* 50(7):1161-1165. <PMid:2476052>
- Wunn D., Wardrop K.J., Meyers K., Kramer J. & Ragle C. 1999. Culture and characterization of equine terminal arch endothelial cells and hoof keratinocytes. *Am. J. Vet. Res.* 60(1):128-132. <PMid:9918161>
- Yang A., Kaghad M., Wang Y., Gillett E., Fleming M.D., Dötsch V., Andrews N.C., Caput O. & Mckee F. 1998. p63, a p53 homolog at 3q27-29, encodes multiple products with transactivating, death-inducing, and dominant-negative activities. *Mol. Cell* 2(3):305-316. <http://dx.doi.org/10.1016/S1097-2765(00)80275-0> <PMid:9774969>

GUIDE FOR AUTHORS

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

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a) The **TITLE** should be concise and indicate the content of the article; details of scientific identification should be put into MATERIALS AND METHODS.

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S.L. Barros or Barros C.S.L., and Franklin Riet-Correa Amaral writes Franklin Riet-Correa or Riet-Correa F. **The papers should not have more than 8 (eight) authors.** Corresponding author should be one who guarantees the contact with the Editorial Board of PVB. Asterisks for call to the footnotes should be elevated once more, in order to appear larger.

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g) The **INTRODUCTION** should be short, with citation of the specific literature without assuming main importance, followed by the objective of the research.

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f) Use preferably scale bars for micrographs. For optical micrographs indicate at the legend finally the staining method and the objective used, for example: HE, obj.40x.

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Exemples for References:

➤ Articles published in scientific journals:

Ubiali D.G., Cruz R.A., De Paula D.A., Silva M.C., Mendonça F.S., Dutra V., Nakazato L., Colodel E.M. & Pescador C.A. 2013. Pathology of nasal infection caused by *Conidiobolus lamprauges* and *Pythium insidiosum* in sheep. J. Comp. Pathol. 149(2/3):137-145.

Hooiveld M., Smit L.A., Wouters I.M., Van Dijk C.E., Spreeuwenberg P., Heederik D.J. & Yzermans C.J. 2016. Doctor-diagnosed health problems in a region with a high density of concentrated animal feeding operations: a cross-sectional study. Environ. Health 17:15-24.

(Note: The first letters of the words in the title of papers published in journals are small. It is preferable to indicate the number of the respective issue.)

➤ Books:

Marsh P. & Martin M. 1992. Oral Microbiology. 3rd ed. Chapman and Hall, London, p.167-196.

Tokarnia C.H., Brito M.F., Barbosa J.D., Peixoto P.V. & Döbereiner J. 2012. Plantas Tóxicas do Brasil para Animais de Produção. 2ª ed. Helianthus, Rio de Janeiro, p.305-348.

(Note: The first letter in the words of the title of books should be capital.)

➤ Chapters of books:

Uzal F.A., Plattner B.L. & Hostetter J.M. 2016. Alimentary system, p.1-257. In: Maxie M.G. (Ed.), Jubb, Kennedy and Palmer's Pathology of Domestic Animals. Vol.2. 6th ed. Elsevier, St Louis, Missouri.

Barros C.S.L. 2007. Doenças víricas: leucose bovina, p.159-169. In: Riet-Correa F., Schild A.L., Lemos R.A.A. & Borges J.R.J. (Eds), Doenças de Ruminantes e Equídeos. Vol.1. 3ª ed. Pallotti, Santa Maria, RS.



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

➤ Dissertations and Theses:

Rech R.R. 2007. Alterações no encéfalo de bovinos submetidos à vigilância das encefalopatias espongiformes transmissíveis. Tese de Doutorado, Universidade Federal de Santa Maria, Santa Maria. 228p.

(Note: Use articles which originated from dissertations or theses instead of these).

➤ Abstracts published in Events:

Massa A.T., Potter K.A. & Bradway D. 2016. Epizootic bovine abortion outbreak in Eastern Nevada cattle. Annual Meeting American College of Veterinary Pathologist (ACVP), New Orleans, Louisiana. (Abstract D-50)

Mendonça F.S., Almeida V.M., Albuquerque R.F., Chaves H.A.S., Silva Filho G.B., Braga T.C., Lemos B.O. & Riet Correa F. 2016. Paralisia laríngea associada à deficiência de cobre em caprinos no semiárido de Pernambuco (IX Endivet, Salvador, BA). Pesq. Vet. Bras. 36(Supl.2):50-51. (Resumo)

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

(Note: Consult entire papers instead of only Abstracts)

LIVESTOCK DISEASES

- Phytotoxins eliminated by milk: a review** [Fitotoxinas eliminadas através do leite: uma revisão]. Lopes J.R.G., Riet-Correa F. & Medeiros R.M.T. 231-237
- Experimental reproduction of cyanogenic poisoning by star grass (*Cynodon nlemfuensis* Vanderyst var. *nlemfuensis* cv. "Florico") in cattle** [Reprodução experimental de intoxicação cianogênica por grama estrela (*Cynodon nlemfuensis* Vanderyst var. *nlemfuensis* cv. "Florico") em bovinos]. Molossi F.A., Ogliari D., Melchiorretto E., Hugen G.F.G.P., Quevedo L.S., Vettori J.M. & Gava A. 238-243
- Neurological diseases in cattle caused by plants and mycotoxins in Santa Catarina state, Brazil** [Enfermidades neurológicas em bovinos causadas por plantas e micotoxinas no estado de Santa Catarina]. Morais R.M., Wicpolt N.S., Molossi F.A., Ogliari D., Mori A.M., Surkamp V. & Gava A. 244-250
- High frequency of cutaneous squamous cell carcinoma in Friesian Milchschaaf sheep in Uruguay** [Alta frequência de carcinoma cutâneo de células escamosas em ovinos da raça Frisona Milchschaaf no Uruguai]. Costa R.A., Schild C., Caffarena D., Giannitti F., Banchemo G., DelPino L. & Riet-Correa F. 251-254

SMALL ANIMAL DISEASES

- Diagnosis of canine leptospirosis: evaluation of two PCR assays in comparison with the microagglutination test** [Diagnóstico de leptospirose canina: avaliação de dois ensaios de PCR em comparação com o teste de microaglutinação]. Martin P.L., Stanchi N.O., Brihuega B.F., Bonzo E., Galli L. & Arauz M.S. 255-262
- A new experimental model of intrinsic denervation in ileum from wistar rats through intramural microinjections of benzalkonium chloride** [Um novo modelo experimental de desnervação intrínseca em íleo de ratos wistar através de micro injeções intramurais de cloreto de benzalcônio]. Ribeiro J.O., Ferreira M.G.P.A., Marques I.C.S., Alvarenga C., Nardi A.B., Garcia S.B., Montanhim G.L. & Moraes P.C. 263-270

WILDLIFE MEDICINE

- Comparison between total intravenous anesthesia with propofol and intermittent bolus of tiletamine-zolazepam in capuchin monkey (*Sapajus apella*)** [Comparação entre anestesia total intravenosa com propofol e *bolus* intermitente de tiletamina-zolazepam em macaco-prego (*Sapajus apella*)]. Galante R., Carvalho E.R., Muniz J.A.C., Castro P.H.G., Gris V.N., Amora Júnior D.S. & Vilani R.G.D'O.C. 271-277
- Morphometry of the mandibular foramen applied to local anesthesia in hoary fox (*Lycalopex vetulus*)** [Morfometria do forame mandibular aplicada à anestesia local em raposa-do-campo (*Lycalopex vetulus*)]. Magalhães H.I.R., Romão F.B., Paula Y.H., Luz M.M., Barcelos J.B., Silva Z., Carvalho-Barros R.A. & Ribeiro L.A. 278-285

ANIMAL MORPHOPHYSIOLOGY

- Maternal, fetal and neonatal heart rate and heart rate variability in Holstein cattle** [Frequência cardíaca e variabilidade da frequência cardíaca materna, fetal e neonatal em bovinos da raça Holstein]. Quevedo D.A.C., Lourenço M.L.G., Bolaños C.D., Alfonso A., Ulian C.M.V. & Chiacchio S.B. 286-291
- Isolation, cultivation and immunofluorescence characterization of lamellar keratinocytes from equine hoof by using explants** [Isolamento, cultivo e caracterização por imunofluorescência de queratinócitos lamelares de casco equino utilizando explantes]. Pfeifer J.P.H., Santos V.H., Rosa G., Souza J.B., Watanabe M.J., Fonseca-Alves C.E., Deffune E. & Alves A.L.G. 292-298