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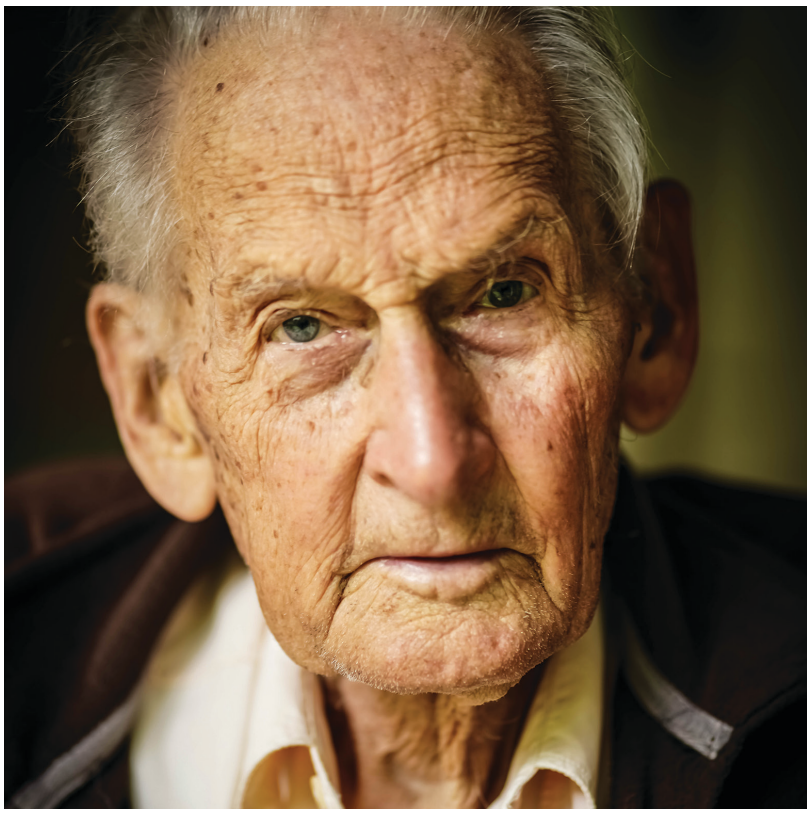

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Cover illustration: Dr. Jürgen Döbereiner, CBPA president (1978-2018), Pesq. Vet. Bras. Editor General (1981-2018).

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Topic of General Interest

Jürgen Döbereiner: a life dedicated to science[1]

Jürgen Döbereiner: uma vida dedicada à ciência[1]

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ABSTRACT.- Dutra I.S., Colling A., Driemeier D., Brito M.F., Ubiali D.G., Schild A.L., Riet-Correa F. & Barros C.S.L. 2019. **Jürgen Döbereiner: a life dedicated to science.** *Pesquisa Veterinária Brasileira 39(1):1-11*. Setor de Anatomia Patológica, Departamento de Epidemiologia e Saúde Pública, Instituto de Veterinária, Universidade Federal Rural do Rio de Janeiro, Seropédica, RJ 23890-000, Brazil. E-mail: iveraldo.dutra@unesp.br

Dr. Jürgen Döbereiner was born in Germany, on the 1st of November 1923, and lived in Brazil for 68 years during which time he developed a range of scientific projects in veterinary pathology and related disciplines. His main interests were the identification of new poisonous plants and mineral deficiencies and the causes of "cara inchada" ("swollen face" a periodontal disease) and botulism in livestock. This research has resulted in the improved health and saving of hundreds of thousands of animals, mainly cattle, annually, and is consequently of enormous economic value to the country. This contribution remains largely under appreciated. He was also involved in organizing diagnostic methods for identifying infectious diseases such as African swine fever and glanders in horses. One of his other major achievements has been the foundation and editing of specialized scientific journals for the documentation of veterinary science research results. At the beginning of his career in the 1950s, he and colleagues from the Institute for Animal Biology (IBA) were struggling to find a national scientific journal where research results from veterinary medicine could be published with practical application to the Brazilian reality. In consequence, the team founded "Arquivos do Instituto de Biologia Animal" and published three volumes (1959-1961). He then founded and edited "Pesquisa Agropecuária Brasileira" (The Brazilian Journal of Agricultural Research") that included a veterinary section. A series of veterinary volumes were published (1966-1976). Finally, in 1978 he helped create the Brazilian College of Veterinary Pathology (CBPA) that published "Pesquisa Veterinária Brasileira" (The Brazilian Journal of Veterinary Research) from 1981. The main goal was to communicate the most relevant disease problems of Brazilian livestock, in particular pathology and related subjects such as epidemiology, clinical study series and laboratory diagnosis to field veterinarians and academics. Dr. Jürgen Döbereiner was president of CBPA (1978-2018) and chief editor of "Pesquisa Veterinária Brasileira" (1981-2018). He passed away on the 16th of October, 2018, at the age of 94 at his home in Seropédica/RJ, Brazil.

INDEX TERMS: Science, livestock diseases, diagnosis, veterinary pathology, poisonous plants, mineral deficiencies, periodontal disease, botulism.

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RESUMO.- [Jürgen Döbereiner: uma vida dedicada à ciência]. Dr. Jürgen Döbereiner nasceu na Alemanha em 1 de novembro de 1923, durante 68 anos viveu no Brasil e desenvolveu trabalhos científicos no campo da patologia veterinária *latu sensu*. Sua contribuição científica de destaque foi em temas como plantas tóxicas de interesse pecuário, deficiências minerais em animais de produção, cara inchada (doença periodontal) dos ruminantes, botulismo e diagnóstico de doenças infecciosas. Estas pesquisas resultaram na melhoria da saúde e de centenas de milhares de animais, principalmente bovinos e, consequentemente, foram de enorme valor econômico para o país. Esta contribuição ainda permanece em grande parte subestimada. De grande destaque para a ciência brasileira foi ainda a sua atuação profissional na documentação científica de resultados de pesquisa. No início de sua carreira na década de 1950, Dr. Döbereiner e outros pesquisadores do Instituto de Biologia Animal (IBA) detectaram a necessidade de um periódico científico nacional para publicar resultados de pesquisas com aplicação pratica à realidade brasileira. Dessa iniciativa surgiram os Arquivos do Instituto de Biologia Animal, que publicou três fascículos (1959-1961), em seguida o Dr. Jürgen Döbereiner participou na fundação da revista Pesquisa Agropecuária Brasileira que publicou a Série Veterinária (1966-1976) e finalmente em 1978, houve a fundação do Colégio Brasileiro de Patologia Animal (CBPA) que publica desde 1981 a revista Pesquisa Veterinária Brasileira. Este periódico científico foi criado para apresentar à comunidade, principalmente veterinários de campo e professores, os principais problemas de saúde em animais de produção no Brasil, ou seja, patologia em seu sentido amplo, envolvendo as áreas de epidemiologia, clínica e diagnóstico laboratorial. Dr. Jürgen Döbereiner, que foi presidente do CBPA (1978-2018) e Editor-Chefe da revista Pesquisa Veterinária Brasileira (1981-2018), faleceu em casa, em 16 de outubro de 2018, aos 94 anos, no município de Seropédica/RJ.

TERMOS DE INDEXAÇÃO: Ciência, pecuária, doenças de rebanhos, diagnóstico, patologia veterinária, plantas tóxicas, deficiências minerais, doença periodontal, botulismo.

Fig.1. Jürgen Döbereiner (1923-2018), German-born, Brazilian citizen graduated as Veterinarian from "Universidade Federal Rural de Rio de Janeiro" (1954); scientific researcher and founder of the journal "Pesquisa Veterinária Brasileira". Photo Jeann Leal, October 2018.

Fig.1. Jürgen Döbereiner (1923-2018), alemão naturalizado brasileiro, graduou-se em Medicina Veterinária pela atual Universidade Federal Rural do Rio de Janeiro (1954), dedicou-se à pesquisa científica durante sua vida e fundou a revista Pesquisa Veterinária Brasileira. Foto de Jeann Leal, outubro de 2018.

Jürgen Döbereiner (Fig.1) was born in Königsberg, former capital of East Prussia, Germany, 1st November 1923 (today Kaliningrad, Russia). From 1935 he went to secondary school in Berlin and graduated from high school in 1942. Being an excellent skier he served in the Austrian mountain artillery from 1942 to 1945 and was promoted to lieutenant during World War II.

After the war he studied veterinary medicine at the University of Munich, from 1947 to 1950. In March 1950 as a young student Jürgen married Johanna Kubelka, who just had obtained her degree in Agricultural Science. Johanna's family belonged to the Sudeten German speaking population of Czechoslovakia, who suffered from being displaced after the Second World War. In the same year Jürgen accepted an invitation from his father in law, Dr. Paul Kubelka (who had

Jürgen Döbereiner (Fig.1) nasceu em 1 de novembro de 1923, na cidade de Königsberg, na Prússia Oriental, Alemanha (hoje com nome de Kaliningrad, sob domínio russo). De 1935 a 1942 frequentou em Berlim escolas secundárias, finalizando-as com a madureza. Na Segunda Guerra Mundial, serviu na artilharia das montanhas austríacas, de 1942 a 1945, por ser um bom esquiador e após ter sido promovido a tenente.

Após a guerra estudou medicina veterinária de 1947 a 1950, na Universidade de München, Alemanha. Em março de 1950, ainda como estudante casou-se com a recém-formada Engenheira Agrônoma Johanna, cuja família pertenceu à população de língua alemã na Checoslováquia, que foi sumariamente expulsa após a Segunda Guerra Mundial. No mesmo ano aceitou o convite do sogro, Dr. Paul Kubelka (que tinha emigrado ao Brasil em 1948) de vir para o Rio

emigrated to Brazil in 1948) to come to Rio de Janeiro. After transferring from the University of Munich to the Veterinary School of the Rural University of Brazil at Km 47 of the old Rio-Sao Paulo road in 1951, Jürgen met Carlos Hubinger Tokarnia and they began their close, productive and long lasting professional collaboration. Jürgen graduated from the National Veterinary School (today Universidade Federal Rural de Rio de Janeiro, UFRRJ) in 1954. Jürgen was registered as number 52 in the Regional Council of Veterinary Medicine of the state of Rio de Janeiro (CRMV/RJ).

From 1955 to 1976, he worked as a researcher at the Ministry of Agriculture in the "Seção de Anatomia Patológica" of the "Institute of Animal Biology" (IBA) at "Km 47" in Seropédica/RJ. From 1976 to 2010, he worked for the recently founded Embrapa (Brazilian Agency for Agriculture Research), which absorbed the IBA. Recently the collection of the former "Seção de Anatomia Patológica" was incorporated into the "**Setor de Anatomia Patológica** (SAP)" of the "Instituto de Veterinária" of UFRRJ.

During the late 1950s, Dr. Jürgen Döbereiner and his colleagues Carlos Tokarnia, Camilo Canella and Jerome Langenegger became research pioneers in Brazilian veterinary medicine. Tokarnia et al. (1959) contributed significantly to the de-mystification of a disease called "mal dos chifres", a purulent sinusitis idiosyncratically inflicted on bovines when cutting horns too deep. During field visits, clinical examinations and post mortems were performed on bovines from 17 farms in the states of Bahia, Pernambuco, Paraíba, Rio Grande do Norte, Ceará, Piauí, and Maranhão (Fig.2). They concluded that the popular term "mal dos chifres" did not describe a specific disease or a group of diseases but rather clinical signs and lesions of diverse causes. At the same time Döbereiner & Tokarnia (1959a) described an outbreak of malignant catarrhal fever (=coryza gangrenosa) in cattle in the state of Rio Grande do Norte and an outbreak of glanders in equines in the state of Rio de Janeiro (Langenegger et al. 1960).

de Janeiro. Assim, Jürgen transferiu-se da Universidade de München para a Universidade Rural do Brasil, no Km 47 da antiga Estrada Rio-São Paulo, em 1951. Neste mesmo ano ainda como estudante de medicina veterinária, Jürgen Döbereiner conheceu Carlos Hubinger Tokarnia, o que resultou em estreita colaboração profissional. Döbereiner formou-se em 1954 pela Escola Nacional de Veterinária (hoje, Universidade Federal Rural do Rio de Janeiro, UFRRJ). Jürgen foi registrado como número 52 no Conselho Regional de Medicina Veterinária do estado do Rio de Janeiro (CRMV/RJ).

De 1955 a 1976, trabalhou como pesquisador do Ministério da Agricultura na "Seção de Anatomia Patológica" do "Instituto de Biologia Animal" (IBA) no "Km 47", em Seropédica/RJ. De 1976 a 2010, trabalhou para a recém-fundada Embrapa, que absorveu o IBA. Recentemente a coleção e acervo da antiga "Seção de Anatomia Patológica" foi incorporada ao "**Setor de Anatomia Patológica** (SAP)" do Instituto de Veterinária da UFRRJ.

Já na segunda metade da década de 1950, Dr. Jürgen Döbereiner e seus colegas de pesquisa, Carlos Tokarnia, Camilo Canella e Jerome Langenegger realizaram trabalhos de pesquisa pioneiros. Tokarnia et al. (1959) participaram da elucidação de uma crença popular, principalmente na região Nordeste do Brasil, de uma condição conhecida como "mal dos chifres", que na realidade era uma condição causada pelas pessoas que perfuravam o chifre de bovinos e cursava com sinusite purulenta. Nesse trabalho, realizaram o exame clínico e necropsias de bovinos em 17 fazendas nos estados da Bahia, Pernambuco, Paraíba, Rio Grande do Norte, Ceará, Piauí e Maranhão (Fig.2). Concluíram que o "mal dos chifres" era um termo popular que não designava uma doença ou certo grupo de doenças, mas sim era usado para explicar sinais clínicos e lesões de diversas origens. Na mesma viagem tiveram a oportunidade de abordar e descrever um surto de febre catarral maligna (=coriza gangrenosa) em bovinos no estado do Rio Grande do Norte (Döbereiner & Tokarnia 1959a). Em seguida relataram um surto de mormo em equinos no estado do Rio de Janeiro (Langenegger et al. 1960).

Fig.2. Dr. Jürgen Döbereiner and Carlos Tokarnia travelled over 9,000 km through the Northeast region of Brazil during the 1950s to investigate the condition popularly known as "mal dos chifres".

Fig.2. Dr. Jürgen Döbereiner e Carlos Tokarnia viajaram mais de 9.000 km na região Nordeste do Brasil, durante a década de 1950, para investigar a condição popularmente conhecida como "mal dos chifres".

Fig.3. Jürgen Döbereiner travelling in Fazenda Recreio, Marília/SP, to investigate about infectious bovine periodontitis. October 1987.

Fig.3. Jürgen Döbereiner em uma viagem na Fazenda Recreio, Marília/SP, para investigação da periodontite infecciosa dos bovinos. Outubro de 1987.

From 1959 to 1961 he was invited to be the chief editor of **Arquivos do Instituto de Biologia Animal** by the director of IBA.

From 1961 to 1963 Jürgen obtained his Master of Science at the University of Wisconsin, Madison, USA supported by a grant from the Rockefeller foundation. His dissertation was on the poisoning of bovines with "samambaia" braken fern plant, *Pteridium aquilinum* (=*P. arachnoideum*), which causes lesions in the bladder (Döbereiner et al. 1966). Further manuscripts were published later that described neoplastic lesions in the bladder and squamous cell carcinoma in the superior digestive tract of bovines consuming samambaia (Döbereiner et al. 1967, Tokarnia et al. 1969).

He founded the journal **Pesquisa Agropecuária Brasileira** and was chief editor 1966-1976 on the invitation of the Director General of the National Department of Research and Experimental Agriculture (DNPEA). Today this journal is published by Embrapa.

After returning from the USA he was offered a position at UFRRJ but preferred the better opportunity to work at IBA, the former Brazilian reference laboratory for animal health.

During the late 1960s until the mid 1990s he undertook 52 scientific expeditions (Fig.3) to 12 Brazilian states, in particular to the Midwest, North and Southeast. Jürgen documented all his trips in his travel diary (Döbereiner 2005). He was always accompanied by his necropsy box (Fig.4) and meticulously took notes of any relevant findings related to animal health problems, post mortems, farmers and observations from veterinarians and other professionals (Fig.5). Jürgen and collaborators worked on *Solanum malacoxylon* (=*S. glaucophyllum*), a plant that was associated with a disease called "Espichamento" after being consumed by bovines, in particular in the Brazilian Pantanal (Döbereiner et al. 1971, Tokarnia & Döbereiner 1974). As an example that illustrates Jürgen's early endeavour to foster international collaboration and scientific networking, he extended his research on the pathological aspects and pathogenesis of poisoning with *S. glaucophyllum* at the *Royal Veterinary College*, London, UK, 1970-1971 supported by a Queen's scholarship (Sansom et al. 1971, Döbereiner & Dämmrich 1974, Döbereiner et al. 1975a, 1977, Dämmrich et al. 1975, Done et al. 1976a, 1976b).

In recognition of his research in Brazil he received the title *Doctor Honoris Causa* from the Justus-Liebig-Universitaet Giessen, Germany as part of their 200 years anniversary of the Faculty of Veterinary Medicine in 1977.

He lectured courses for post-graduates at Master and PhD level at UFRRJ and University of Sao Paulo (USP) for many years.

In 1978 Tokarnia, Jürgen and others collaborated on the diagnosis of African Swine Fever (ASF) during an outbreak in Paracambi/RJ, close to IBA, which resulted in a swift containment and eradication of the epidemic in Brazil. Later, it was shown that a series of wrong interpretations of serological test results, suggested ASF was present in several Brazilian States (Tokarnia et al. 2004, Viana 2008).

From 1969 his research focused on the etiology, pathogenesis and epidemiology of a disease generally known as "**swollen face of bovines**" (Döbereiner et al. 1974). Supported by grants from the National Research Council (CNPq) and German Academic Exchange Service (DAAD) he undertook

De 1959 a 1961, foi Editor-Chefe dos **Arquivos do Instituto de Biologia Animal** a convite do diretor do IBA.

De 1961 a 1963, cursou o mestrado na Universidade de Wisconsin em Madison, como bolsista da Fundação Rockefeller, e obteve o título de *Master of Science*, com a defesa de dissertação relacionada às lesões em bexigas de bovinos intoxicados pela planta "samambaia", *Pteridium aquilinum* (=*P. arachnoideum*) (Döbereiner et al. 1966). Posteriormente foram publicados outros trabalhos sobre essa planta e associaram-se lesões neoplásicas de bexiga e carcinomas de células escamosas no trato digestório superior de bovinos com o consumo de samambaia (Döbereiner et al. 1967, Tokarnia et al. 1969).

Foi fundador da revista **Pesquisa Agropecuária Brasileira** e Editor-Chefe, de 1966 a 1976, a convite do diretor-geral do Departamento Nacional de Pesquisa e Experimentação Agropecuária (DNPEA). Esta revista hoje está sendo publicada pela Embrapa.

Após o regresso dos Estados Unidos, fez concurso na Universidade Federal Rural do Rio de Janeiro, mas não tomou posse por considerar as condições de pesquisa melhores no IBA, o antigo laboratório de referência em sanidade animal do Brasil.

A partir do final da década de 1960 e até meados da década de 1990, realizou 52 expedições científicas (Fig.3) a diferentes municípios de doze estados brasileiros, com destaque aos localizados nas regiões Centro-Oeste, Norte e Sudeste, todas registradas nos seus Diários de Viagem (Döbereiner 2005). Sempre acompanhado da sua caixa de necropsia (Fig.4), tinha o cuidado de anotar todas as informações relacionadas aos problemas de saúde animal, objeto das suas linhas de pesquisa, das necropsias realizadas e dos contatos com proprietários rurais, médicos veterinários e outros profissionais a quem recorria (Fig.5).

Em 1971, o grupo de pesquisas do qual o Dr. Döbereiner participava associou o consumo de *Solanum malacoxylon* (=*S. glaucophyllum*) com a doença popularmente conhecida como "espichamento" e que na ocasião afetava bovinos, sobretudo no Pantanal do Brasil (Döbereiner et al. 1971, Tokarnia & Döbereiner 1974). Em 1970/71, realizou pelo *Queen's Scholarship* estudos no *Royal Veterinary College* em Londres sobre a intoxicação por *S. malacoxylon*. Em estreita colaboração internacional, o que sempre caracterizou a sua atuação como pesquisador, envolveu-se com os aspectos patológicos da intoxicação e a patogênese desta doença (Sansom et al. 1971, Döbereiner & Dämmrich 1974, Döbereiner et al. 1975a, 1977, Dämmrich et al. 1975, Done et al. 1976a, 1976b).

De 1976 a 2010, atuou como pesquisador da recém-criada Embrapa.

Em 1977, foi contemplado com o título de *Doctor Honoris Causa* pela Justus-Liebig-Universitaet Giessen, Alemanha, por ocasião da comemoração de 200 anos da Faculdade de Medicina Veterinária em face dos trabalhos de pesquisa realizados no Brasil.

Lecionou nos cursos de pós-graduação de Mestrado e Doutorado da UFRRJ e Universidade de São Paulo (USP).

Em 1978 Jürgen, Tokarnia e outros pesquisadores realizaram o diagnóstico de um surto de peste suína africana que ocorreu no município de Paracambi/RJ, próximo ao IBA, o que resultou na rápida extinção deste foco no Brasil. Após a realização deste diagnóstico, houve uma série de interpretações equivocadas de exames sorológicos que consideraram

studies to explain this unusual periodontitis in collaboration with the University of Giessen and Berlin from 1984 to 1989. In particular the collaboration with Prof. Dr. Hans Blobel, Director of the Institute for Bacteriology and Immunology of the Justus-Liebig-Universität Giessen, resulted in the discovery of bacterial involvement in the etiology and pathogenesis of the disease. Some epidemiological aspects of the disease, such as the improvement of calves after being moved to healthy, balanced soils, were observed (Döbereiner et al. 1975b, 1976). Studies on bacterial involvement showed the relevance of specific genera such as *Treponema, Porphyromonas* and *Prevotella* (=*Bacteroides melaninogenicus*) (Blobel et al. 1984, 1987, Botteon et al. 1993, Dutra et al. 2000, Döbereiner et al. 2004, Borsanelli et al. 2015a, 2015b). Subsequently a series of experiments provided evidence that "swollen face" was a multifactorial, bacterial disease rather than a specific mineral deficiency (Döbereiner et al. 1990, Moraes et al. 1999). An important diet-related factor for "swollen face" to occur is a disturbed balance of bacteria in the soil following forest clearing, which facilitates the production of *in situ* antibiotics. These have a direct or indirect impact on the buccal microbiotic flora (Döbereiner et al. 1987, 2000, Dutra et al. 1993a, diagnóstico de peste suína africana em diversas regiões do Brasil (Tokarnia et al. 2004, Viana 2008).

Desde 1969 dedicou-se à documentação da etiologia, patogênese e epidemiologia da doença popularmente conhecida como "**cara inchada dos bovinos**" (Döbereiner et al. 1974). Sob financiamento do CNPq e do Serviço de Intercâmbio Acadêmico Alemão (DAAD) realizou em 1984 e 1989 estudos sobre esta periodontite dos ruminantes nas Universidades de Giessen e Berlin. Um destaque especial nesses estudos foi a sua colaboração com o Prof. Dr. Hans Blobel, Diretor do Instituto de Bacteriologia e Imunologia da Universidade Justus-Liebig, de Giessen, Alemanha, que resultou nos estudos inéditos sobre a participação de bactérias na etiopatogenia da cara inchada. Além dos avanços no estudo da doença, esta colaboração possibilitou a formação de um considerável e efetivo quadro de pesquisadores brasileiros e alemães (Dutra et al. 1993a, Kopp et al. 1996, Schmitt et al. 1996, Döbereiner & Dämmrich 1997, Grassmann et al. 1997). Alguns aspectos epidemiológicos desta doença foram elucidados como a recuperação de bezerros transferidos para pasto de área considerada indene (Döbereiner et al. 1975b, 1976). Os estudos bacteriológicos resultaram na identificação de uma ampla

Fig.4. Dr. Jürgen sitting on a necropsy box after finishing a post mortem and taking specimens for diagnostic examination and research of bovine diseases. September 1975.

Fig.4. Dr. Jürgen sentado sobre a caixa de necropsia após ter realizado a necropsia e a coleta de material de pesquisa para diagnóstico de doença em bovinos. Setembro de 1975.

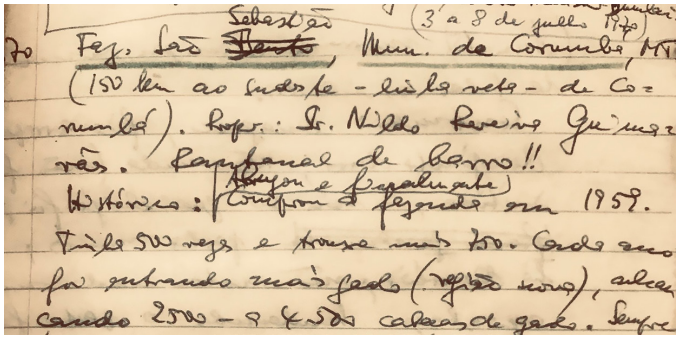

Fig.5. Notes from Jürgen's research diary, Corumbá/MS, 1970.

Fig.5. Anotações de Jürgen em seu diário de pesquisa, Corumbá/MS, 1970.

Fig.6. Jürgen Döbereiner telling stories about his research at his resort "Porangaba", Itaguaí/RJ. Beside him a specimen of *Palicourea marcgravii*, an important poisonous plant for Brazilian livestock. April 2018.

Fig.6. Jürgen Döbereiner em seu sítio Porangaba, em Itaguaí/RJ contando suas histórias de pesquisa ao lado de um exemplar de *Palicourea marcgravii*, importante planta tóxica para a pecuária brasileira. Abril de 2018.

Kopp et al. 1996, Schmitt et al. 1996, Döbereiner & Dämmrich 1997, Grassmann et al. 1997). Results further contributed to the prophylaxis (Döbereiner et al. 1990) and control (Rosa et al. 1985, Tims et al. 1992) of the disease. In addition to the scientific advances, collaboration facilitated the development of a new generation of scientists from Brazil and Germany (Dutra et al. 1993a, Kopp et al. 1996, Schmitt et al. 1996, Grassmann et al. 1997).

Döbereiner was also co-author of two books titled: **Plantas Tóxicas da Amazônia a Bovinos e Outros Herbívoros** (Tokarnia et al. 1979, 2007) and **Plantas Tóxicas do Brasil para Animais de Produção** (Tokarnia et al. 2000a, 2012) and co-author of a book entitled: "**Deficiências Minerais em Animais de Produção**" (Tokarnia et al. 2010). The diagnosis and systematic study of poisoning plants for ruminants, carried with Tokarnia, showed the importance of plant poisoning in Brazil and allowed the scientific advance on measures of control and prophylaxis. Using data from diagnostic laboratories from different regions in Brazil, Pessoa et al. (2013) calculated the loss of livestock due to the ingestion of poisonous plants and estimated the following annual losses: 820,761 to 1,755,763 cattle, 399,800 to 445,309 sheep, 52,675 to 63,292 goats and 38,559 horses. In April 2008, Dr. Jürgen Döbereiner talked about poisonous plants when he was being filmed (Fig.6). The picture shows him on his smallholding "Porangaba", near Itaguaí/RJ, next to *Palicourea marcgravii*, a poisonous plant causing sudden death in cattle, known as "cafezinho", or "erva-de-rato". This specimen had originally been planted and raised by Prof. Carlos Tokarnia in his house and both had used it to teach their students about the morphology and characteristic scent of *P. marcgravii* during practical sessions on poisonous plants. This is the most important poisonous plant in the country and is thought to be responsible for something like 90% of sudden death in the Brazilian herd. It has a wide geographic distribution but is not found in the South or in the Pantanal. It is found in the state of Rio de Janeiro in the region above of Serra das Araras (altitude above 550 meters). It is not found at lower altitudes, for example, in the Baixada Fluminense (Tokarnia et al. 2012).

Thiloa glaucocarpa (=*Combretum glaucocarpum*), also known as "sipaúba" or "vaqueta", causes outbreak of poisoning shortly after the beginning of the rainy season in the Northern Caatinga. Bovines develop clinical signs after 5-8 days and get sick 10-25 days after the first rainfall. *T. glaucocarpa* poisoning happen during or soon after this initial fast growth. After that period there are other plants available for consumption. (Tokarnia et al. 1981, 1994, 2002, 2012). This research on *Palicourea marcgravii* (Döbereiner & Tokarnia 1959b) and *T. glaucocarpa* (Tokarnia et al. 1981, 1994) illustrate the team's pioneering research to discover the many poisonous plants affecting livestock.

After the diagnosis of **botulism** in the "agreste" of Piauí (Tokarnia et al. 1970), Dr. Döbereiner and colleagues undertook a variety of epidemiological investigations into **epizootic botulism** in diverse regions of Brazil (Langenegger & Döbereiner 1988, Döbereiner et al. 1992, Dutra et al. 2001, 2005). The team described the connection between outbreaks of botulism and osteophagy, the eating of the bones of dead cattle in regions with very low soil phosphate contents. The role of water and food as vectors provided crucial information to bridge the gap

Fig.7. Jürgen Döbereiner with Daniel Guimarães Ubiali, October 10, 2018. While they were discussing the January 2019 edition for the "Pesquisa Veterinária Brasileira" journal, there was a fall in electric energy and they kept on working by the light of a candle! Photo Jeann Leal, October 2018.

Fig.7. Jürgen Döbereiner e Daniel Guimarães Ubiali no dia 10 de outubro de 2018. Ao programar juntos o fascículo de janeiro de 2019 da revista Pesquisa Veterinária Brasileira, acabou a energia e trabalharam à luz de velas! Foto de Jeann Leal, outubro de 2018.

microbiota de patógenos potenciais associada à doença. Dentre os micro-organismos destacam-se os gêneros *Treponema*, *Porphyromonas* e *Prevotella* (=*Bacteroides melaninogenicus*) (Blobel et al. 1984, 1987, Botteon et al. 1993, Dutra et al. 2000, Döbereiner et al. 2004, Borsanelli et al. 2015a, 2015b). Por meio de uma série de estudos, estabeleceram que a "cara inchada" tratava-se de uma doença multifatorial bacteriana e não relacionada com deficiência mineral específica (Döbereiner et al. 1990, Moraes et al. 1999). Um importante fator ambiental que estaria relacionado com a ocorrência da cara inchada seria decorrente do desequilíbrio na microbiota do solo e que envolveria a formação *in situ* de antibióticos, atuando assim direta ou indiretamente na disbiose da microbiota bucal por meio da dieta (Döbereiner et al. 1987, 2000, Dutra et al. 1993a, Kopp et al. 1996, Schmitt et al. 1996, Grassmann et al. 1997). Nesta linha de estudos os trabalhos resultaram ainda no desenvolvimento de medidas profiláticas (Döbereiner et al. 1990) e de controle (Rosa et al. 1985, Tims et al. 1992) da cara inchada dos bovinos.

Döbereiner foi ainda coautor das duas edições dos livros **Plantas Tóxicas da Amazônia a Bovinos e outros Herbívoros** (Tokarnia et al. 1979, 2007) e **Plantas Tóxicas do Brasil para Animais de Produção** (Tokarnia et al. 2000a, 2012), e também do livro **Deficiências Minerais em Animais de Produção** (Tokarnia et al. 2010). O estudo sistemático sobre o diagnóstico e os quadros clínico patológicos de intoxicação por plantas em ruminantes, realizado com o Professor Tokarnia, mostrou a importância da intoxicação por plantas no Brasil e permitiu o avanço científico sobre medidas de controle e profilaxia. Pessoa et al. (2013) calcularam através de dados dos laboratórios de diagnóstico de diferentes regiões do país a quantidade de mortes de animais de produção atribuídas ao consumo de plantas tóxicas. As perdas anuais por mortes de animais foram estimadas em 820.761 a 1.755.763 bovinos,

Fig.8. Jürgen Döbereiner at his residence, Seropédica/RJ, Km 47. Photo Jeann Leal, October 2018.
Fig.8. Jürgen Döbereiner em sua residência, em Seropédica/RJ, Km 47. Foto de Jeann Leal, outubro de 2018.

between research and extension and has turned Brazil into the largest global market for anti-botulism vaccines.

According to Dr. Döbereiner the historic experiences with botulism as well as other public health issues revealed the need to develop an integrated animal health system for the country. This should be based on combining practical information from field veterinarians with on and off farm laboratory diagnostics integrated by modern information technology. Diverse discussions about the etiology of the "mysterious disease" or "fallen cow disease", although legitimate delayed the extension of proper diagnosis, control and prevention which has led to the unnecessary loss of millions of bovines during many decades (Tokarnia et al. 2010).

In the 1990s, when Dr. Döbereiner was looking for a diagnostic test for botulism with good sensitivity and specificity, he was involved in an international collaboration with Embrapa, the Department of Preventive Veterinary Medicine of the Faculty of Agriculture and Veterinary Science (Unesp/Jaboticabal) and Dr. Hans-Erich Weiss, a veterinarian from a diagnostic institute in Heidelberg, Germany. They adapted the Complement Fixation Test for the diagnosis of botulism. The scientific impact of their achievements led to a breakthrough for consolidating modern diagnosis and the resulting control of the disease in Brazil

399.800 a 445.309 ovinos, 52.675 a 63.292 caprinos e 38.559 equinos.

Em abril de 2018, Dr. Jürgen Döbereiner contava histórias de pesquisa sobre plantas tóxicas enquanto estava sendo filmado (Fig.6). O exemplar de *Palicourea marcgravii*, conhecida como "cafezinho" ou "erva-de-rato", está plantada no sítio "Porangaba", em Itaguaí/RJ; este exemplar estava na casa do professor Carlos Tokarnia. Em aulas práticas sobre plantas tóxicas, os pesquisadores mostravam aos estudantes a morfologia e o aroma característico de *P. Marcgravii*. Esta planta tóxica tem larga distribuição geográfica no Brasil, com exceção da região Sul e do Pantanal. No estado do Rio de Janeiro *P. marcgravii* está presente na região acima da Serra das Araras (altitude 550 metros ou mais) e está ausente na Baixada Fluminense (Tokarnia et al. 2012).

O pioneirismo no campo das plantas tóxicas de interesse pecuário pode ser exemplificado entre outros, pelo estudo de *Palicourea marcgravii* (Döbereiner & Tokarnia 1959b) e pelo estudo de *Thiloa glaucocarpa* (=*Combretum glaucocarpum*) (Tokarnia et al. 1981, 1994). Esta planta, conhecida como "sipaúba" ou "vaqueta", causa intoxicação sob a forma de surtos no começo da estação chuvosa em regiões de Caatinga. Os bovinos adoecem entre o 10º e 25º dias, após a primeira chuva, a evolução clínica é de 5-8 dias. *T. glaucocarpa* tem crescimento rápido no início da brotação e após esse período o crescimento é mais lento. A intoxicação ocorre na fase inicial da brotação, após esse período há disponibilidade de outras plantas para o consumo dos bovinos (Tokarnia et al. 1981, 1994, 2002, 2012).

Após o diagnóstico do **botulismo** no agreste do Piauí (Tokarnia et al. 1970), Dr. Döbereiner e colaboradores realizaram diversos estudos epidemiológicos sobre o **botulismo epizoótico** em diversas regiões brasileiras (Langenegger & Döbereiner 1988, Döbereiner et al. 1992, Dutra et al. 2001, 2005). Assim, descreveram surtos da doença associados à osteofagia, à veiculação hídrica e aos alimentos; sempre conjugando as atividades de pesquisa às de extensão e que resultaram hoje em dia no maior mercado mundial de vacinas anti-botulínicas. De importante contexto histórico o botulismo, assim como diversos outros problemas sanitários, revelava a necessidade na visão do Dr. Döbereiner de um sistema de saúde animal em que a base seria o diagnóstico realizado nas propriedades rurais por médicos veterinários e um sistema de informação. As diversas discussões sobre a etiologia da "doença misteriosa", ou "doença da vaca caída", embora legítimas, atrasaram a divulgação das medidas de diagnóstico, controle e prevenção dessa enfermidade, que no seu curso natural causou a perda de milhões de bovinos nas décadas de 1980-1990.

Na década de 1990, diante da necessidade de se buscar um teste diagnóstico com boa sensibilidade e especificidade novamente o Dr. Döbereiner procurou a colaboração internacional. Nesse contexto, com o auxílio da Embrapa e do Departamento de Medicina Veterinária Preventiva da Faculdade de Ciências Agrárias e Veterinárias da Unesp/Jaboticabal estabeleceu uma parceria com o Dr. Hans-Erich Weiss, veterinário de um instituto de diagnóstico de Heidelberg, Alemanha, que havia adaptado a técnica de fixação de complemento para o diagnóstico do botulismo. Os trabalhos científicos resultantes dessas ações e da equipe foram de grande valor para consolidar uma nova etapa no diagnóstico e controle da doença no país

(Dutra et al. 1993b, Menegucci et al. 1998, Silva et al. 1998, Souza et al. 2006, Curci et al. 2007).

Research into mineral deficiencies had a breakthrough with the investigation of an outbreak of enzootic ataxia of ovines in Piauí state (Döbereiner et al. 1966). Evaluation of a series of results demonstrated the importance of field veterinarians with experience in the diagnosis of animal disease based on clinical and pathological findings and confirmatory testing by analysis of minerals in animal tissues (Tokarnia et al. 1988, 1999, Pilati et al. 1996, Moraes et al. 1999). The authors further provided evidence that increased levels of minerals in the diet, mainly phosphorus as well as mineral deficiencies of specific elements such as phosphorus, sodium, copper and cobalt, are the main reasons for economic losses (Tokarnia et al. 2000b, 2012, Malafaia et al. 2014).

From undergraduate level and throughout many years of his research career, Dr. Jürgen Döbereiner was supported by grants from **CNPq**. He published 174 papers in national and international science journals and supervised many postgraduates.

From 2000 to 2004 he was president of the **Brazilian Association of Scientific Editors** (ABEC) during two consecutive periods (2000-2001 and 2002-2003) and collaborated tirelessly for the growth of the organization.

Dr. Jürgen together with other colleagues (Carlos Hubinger Tokarnia, Severo Sales de Barros, Jerome Langenegger, Hugo Edson Barbosa de Rezende, Rubens Pinto Melo and Laerte Grisi) founded the **Brazilian College of Animal Pathology** (CBPA) with the goals of promoting the importance of Veterinary Medicine and having a national scientific journal of veterinary medicine. Dr. Jürgen was president of CBPA from 1978 to 2018.

In 1981, CBPA published the first volume of the journal **Pesquisa Veterinária Brasileira**. The main objective of this scientific journal was to communicate to the public, in particular to field veterinarians and academics the main health problems of livestock in Brazil, in particular pathology and related subjects such as epidemiology, clinical findings and laboratory diagnosis. The journal was edited monthly from 2007 and included additional topics such as *Small Animals, Wildlife Medicine* and *Animal Morphophysiology.* From the first edition **Pesq. Vet. Bras.** was indexed by the prestigious international database, Thomson Reuters, and recently as Qualis A2 by CAPES. Another goal of Dr. Jürgen was to extend the indexation of Pesq. Vet. Bras. to the global PubMed database. CBPA has signed the terms for cooperation with UFFRJ and the University of Brasilia (UnB) to improve publication and visibility of the journal. From 2019 onwards, all manuscripts will be published in English with a summary in Portuguese. A new service is now available that allows the publishing of high quality images similar to other international scientific journals in the field of veterinary pathology. Jürgen worried about the future of the journal and engaged in many philosophic discussions to encourage young junior academics to carry his ideas and journal forward (Fig.7).

Jürgen lived on the University Campus, Km 47 (Fig.8), also known as "Ecology" and lived in the same house from 1952-2018. Together with Johanna (his wife) and their three children Maria Luisa, Christian and Lorenz the family enjoyed the proximity between their home and IBA.

It is worthwhile mentioning that Dr. Döbereiner enjoyed to relax and recover during weekends at his smallholding "**Sítio Porangaba**", located in the maritime mountains of the

(Dutra et al. 1993b, Menegucci et al. 1998, Silva et al. 1998, Souza et al. 2006, Curci et al. 2007).

O início dos estudos com deficiências minerais ocorreu com a descrição de um surto de ataxia enzoótica em ovinos no Piauí (Döbereiner et al. 1966) e a continuação de uma série de avaliações detectaram que o diagnóstico clínico patológico de deficiências minerais deve ser realizado por veterinário com experiência em diagnóstico de doenças e a confirmação deve ser realizada sobretudo com dosagens de minerais em tecidos do animal (Tokarnia et al. 1988, 1999, Pilati et al. 1996, Moraes et al. 1999). Verificaram também que as perdas econômicas na pecuária ocorrem tanto em função de dietas com excesso de elementos (principalmente fósforo) e quanto pela falta de determinado elemento na dieta (deficiência mineral, principalmente sódio, fósforo, cobre e cobalto) (Tokarnia et al. 2000b, 2012, Malafaia et al. 2014).

Desde estudante e nos muitos anos de pesquisa Dr. Jürgen Döbereiner, foi bolsista do **CNPq**, publicou 174 artigos em periódicos científicos nacionais e internacionais e orientou várias teses de pós-graduação.

Jürgen foi presidente da **Associação Brasileira de Editores Científicos** (ABEC) por dois mandatos consecutivos (2000-2001 e 2002-2003) e colaborou incansavelmente para o crescimento da ABEC.

Em 1978, Dr. Jürgen e outros colegas (Carlos Hubinger Tokarnia, Severo Sales de Barros, Professor Langenegger, Professor Hugo Edson Barbosa de Rezende, Rubens Pinto Melo e Professor Laerte Grisi) fundaram o **Colégio Brasileiro de Patologia Animal** (CBPA), com o objetivo da edição de um periódico científico de medicina veterinária de âmbito nacional. Dr. Jürgen foi presidente do CBPA de 1978 a 2018.

Em 1981, o CBPA publicou o primeiro fascículo da revista **Pesquisa Veterinária Brasileira**. Este periódico científico foi criado para apresentar à comunidade, principalmente veterinários de campo e professores, os principais problemas de saúde em Animais de Produção/*Livestock Diseases* no Brasil, ou seja, patologia em seu sentido amplo, envolvendo as áreas de epidemiologia, clínica e diagnóstico laboratorial. A partir de 2007, a revista passou a ter periodicidade mensal e ampliou a sua abrangência ao publicar artigos das áreas de Pequenos Animais/*Small Animal Diseases*, Animais Selvagens/*Wildlife Medicine* e Morfofisiologia/*Animal Morphophysiology*. A **Pesq. Vet. Bras.** foi desde o primeiro fascículo, indexada pela prestigiada base de dados internacional Thomson Reuters e, recentemente, foi classificada pela CAPES como Qualis A2. A partir de 2019, todos os artigos serão publicados em inglês e com um resumo em português. Recentemente foi incorporado o serviço de edição de imagens como exemplo de importantes periódicos científicos internacionais da área de Patologia Veterinária. Um dos objetivos de Dr. Jürgen era indexar a Pesq. Vet. Bras. na prestigiada base de dados PubMed. O CBPA firmou termos de cooperação técnica com a Universidade Federal Rural do Rio de Janeiro e com a Universidade de Brasília para aperfeiçoar a publicação e conquistar maior visibilidade para a Pesq. Vet. Bras. Jürgen se preocupava com a segurança da continuidade da publicação da revista, por isso, dedicou-se a ensinar várias filosofias sobre a publicação científica aos jovens professores com quem mantinha amizade (Fig.7).

Jürgen sempre residiu de 1952 a 2018 no bairro residencial da Universidade Rural, Km 47 (Fig.8), também conhecido como Ecologia; a proximidade de sua residência com o IBA permitiu uma qualidade de vida excelente a Jürgen, Johanna

Mata Atlântica, near Itaguaí/RJ. He was a founding member and president of the **Association of Environmental Heritage** (APN) and his Sítio became one of the first **Environmental Reserves for Environmental Heritage** (RPPN) in the state of Rio de Janeiro. These examples demonstrate his sensibility and early awareness for the need to conserve and promote biodiversity.

Together with journalist Kristina Michahelles, Jürgen recently concluded the publication of a biography of his wife titled: "Hanne, Johanna Döbereiner: a life dedicated to science" (Michahelles 2018).

In 2010 after 55 years of service, Dr. Jürgen retired at the age of 86. But he continued as unpaid general editor of the Pesq. Vet. Bras. from 1981 until the last day of his life.

In memory of his philosophy he left a document entitled: "**Carta de Porangaba**", where together with his collaborators he had developed a modern approach to assist and improve the pubic animal health service in Brazil, in particular the field of veterinary science through an **Integrated Animal Health System** (SISA).

Jürgen loved to talk about the adventures of his multiple field trips and there is plenty of cinematographic material available, which will be edited to produce a documentary titled: **Stories on Animal Health Research in Brazil**.

Dr. Jürgen Döbereiner passed away suddenly and peacefully on the 16th of October 2018 at the age of 94 in his house.

Acknowledgments.- We thank Prof. Jeann Leal de Araújo for the outstanding quality and artistic images.

(sua esposa) e seus três filhos Maria Luisa, Cristian e Lorenz. Convém mencionar que Dr. Döbereiner estava concentrando suas energias nos finais de semana no seu "**Sítio Porangaba**" (Fig.4), umas das primeiras **Reservas Particulares de Patrimônio Natural** (RPPN) do Estado do Rio de Janeiro, situado na **Mata Atlântica** da Serra do Mar, em Itaguaí/RJ. Ele foi um dos fundadores e o presidente da **Associação do Patrimônio Natural** (APN) e demonstrava exemplo em contribuir na conservação da biodiversidade.

Recentemente Dr. Jürgen Döbereiner desenvolveu a publicação do livro "Hanne, Johanna Döbereiner: uma vida dedicada à ciência" sobre a vida de sua esposa, que foi escrito pela jornalista Kristina Michahelles (2018).

Após 55 anos de serviço, em 2010 Dr. Jürgen se aposentou com 86 anos de idade, mas continuou atuando, desde 1981 como editor geral da revista Pesq. Vet. Bras. até o último dia de sua vida. Ele deixou um documento intitulado "**Carta de Porangaba**", em homenagem ao seu sítio, onde desenvolveu junto com colaboradores um projeto para o aperfeiçoamento dos serviços veterinários no Brasil por meio de um **Sistema Integrado de Saúde Animal** (SISA).

Jürgen gostava de contar sobre suas aventuras durante viagens a campo, portanto realizaram-se filmagens de grande parte dessas histórias, as quais serão editadas para a preparação de um documentário intitulado por Jürgen como **Histórias de Pesquisa em Saúde Animal no Brasil**.

Dr. Jürgen Döbereiner com 94 anos, faleceu em casa no dia 16 de outubro de 2018.

Agradecimentos.- Agradecemos o Prof. Jeann Leal de Araújo pela excelente qualidade artística das imagens.

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Original Article
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Current trends in bovine abortion in Argentina[1]

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ABSTRACT.- Morrell E.L., Campero C.M., Cantón G.J., Odeón A.C., Moore D.P., Odriozola E., Paolicchi F. & Fiorentino M.A. 2019. **Current trends in bovine abortion in Argentina.** *Pesquisa Veterinária Brasileira 39(1)12-19.* Animal Health Group, Instituto Nacional de Tecnología Agropecuaria (INTA), Ruta 226 Km73, 5 (7620), Balcarce, Argentina. E-mail: morrell.eleonora@inta.gob.ar

Bovine abortion is an important cause of significant economic losses in beef and dairy herds. This syndrome is usually difficult to diagnose. The aim of this study was to characterize bovine abortion causes in Argentina by standard diagnosis procedures (histology, bacterial and viral isolation) and other diagnostic tests like direct fluorescent antibody test (DFAT), fetal serology, immunohistochemistry (IHC), and PCR, showing their specific advantages and limitations. Necropsies were performed in 150 aborted bovine fetuses submitted to the diagnostic laboratories of Instituto Nacional de Tecnología Agropecuaria (INTA) Balcarce, Argentina. Etiological diagnosis was confirmed in 78 fetuses (52% of the cases). Most causes of abortion were of infectious origin, being *Neospora caninum* (14.67%), *Campylobacter fetus* sp. (9.33%), *Leptospira* spp. (7.33%) and *Brucella abortus* (6.65%) the main microorganisms identified. Bovine viral diarrhea virus (BVDV) and bovine herpes virus (BHV) were diagnosed in 2 (1.33%) and 3 (2%) cases, respectively. This study showed a better characterization of bovine abortion compared with previous researches done on this topic.

INDEX TERMS: Current trends, bovine, abortion, fetuses, bovine, diagnosis, PCR, Argentina, surgery.

RESUMO.- [**Tendências atuais do aborto bovino na Argentina.**] O aborto bovino é uma causa importante de perdas econômicas significativas em rebanhos bovinos e leiteiros. Esta síndrome é geralmente difícil de diagnosticar. O objetivo deste estudo foi caracterizar o aborto bovino na Argentina por procedimentos diagnósticos de rotina (histologia, isolamento viral e bacteriana) e outros testes diagnósticos como ensaio directo de anticorpos fluorescentes (DFAT), sorologia fetal, imuno-histoquica (IHC), e PCR; mostrando suas vantagens e limitações específicas. As necropsias foram realizadas em 150 fetos bovinos abortados submetidos aos laboratórios de diagnóstico do Instituto Nacional de Tecnologia Agropecuária (INTA) de Balcarce, na Argentina. O diagnóstico etiológico foi confirmado em 78 fetos (52% dos casos). A maioria das causas de aborto foram de origem infecciosa, sendo *Neospora caninum* (14,67%), *Campylobacter fetus* sp. (9,33%), *Leptospira* spp. (7,33%) e *Brucella abortus* (6,65%) os principais microrganismos identificados. O vírus da diarréia viral bovina (BVDV) e o herpesvírus bovino (BHV) foram diagnosticados em 2 (1,33%) e 3 (2%) casos, respectivamente. Este estudo mostrou uma melhor caracterização do aborto bovino em comparação com pesquisas anteriores feita sobre este tema.

TERMOS DE INDEXAÇÃO: Tendências atuais, aborto, bovinos, fetos, diagnóstico, PCR, Argentina, cirurgia.

INTRODUCTION

Bovine abortion is one of the most important problems of livestock production and health, causing severe economic loss. The worldwide diagnosis of bovine abortion is difficult and costly (Anderson et al. 1990, Kirkbride 1990, Campero et al. 2003, Anderson 2007, Holler 2012). Several studies reported that a definitive etiologic diagnosis of bovine abortion was achieved in 23.3 to 45.5% of the cases (Campero et al. 2003, Anderson 2007, Campero et al. 2017).

Amongst the causes of bovine abortion, infectious causes are the most prevalent (Hubbert et al. 1973, Corbellini et al. 2006,

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Anderson 2007, Bon Durant 2007). Factors such as production type, climate, management practices and vaccination programs are able to determine differences in the frequency of various infectious abortigenic agents identified (Anderson 2007). Non-infectious bovine abortions had been described, but these losses are difficult to establish (Kirkbride 1990).

Traditional diagnostic tools include macroscopic examination, histology, and bacterial and viral isolation (Campero et al. 2003, Anderson 2007). Pathogen isolation is considered the "gold standard" test for detection of infectious agents; however these techniques are laborious, time-consuming and in some cases had low sensitivity (Smith et al. 1994, Seleem et al. 2010). The application of immunohistochemistry (IHC) and PCR had improved the identification of infectious agents in bovine fetuses (Richtzenhain et al. 2002, Van Maanen et al. 2004, Cortez et al. 2006, Silva et al. 2009, Tramuta et al. 2011). This study assesses the most important, bacterial, viral, fungal and protozoal causes of bovine abortion in Argentina, focusing on the methods used to reach an accurate diagnosis.

MATERIALS AND METHODS

Diagnostic criteria. The causes of bovine abortion were classified as determined (including infectious and non- infectious origin) and undetermined (with or without histological lesions), respectively.

Specimens, origin and macroscopic examination procedures. Samples were collected from 150 bovine aborted fetuses that had undergone spontaneous abortion from 2004 to 2006. Fetuses were submitted by veterinary practitioners or private veterinary diagnostic laboratories to the Specialized Veterinary Diagnostic Service at INTA Balcarce, Argentina. Only two fetuses were sent with their placenta. Full necropsies were performed in all fetuses, including the examination of both placentas. Herd origin and other epidemiological information were collected in most of the cases. The differentiation between abortion and perinatal mortality was evaluated by the hydrostatic pulmonary docimasy test (presence or absence of aerated lungs) and by the presence of hemorrhage and/or thrombosis surrounding the umbilical vessels. Fetal deaths due to dystocia were diagnosed based on the presence of head swelling, subcutaneous edema, and/or fractures of the ribs and limbs.

Fetal age was estimated based on the crown-rump length (Hubbert et al. 1973, Kirkbride 1986). The state of post-mortem decomposition was estimated using a subjective score from 1 (slight) to 3 (severe). A score of 2 indicated moderate decomposition.

Histology and special staining. Samples of cerebrum, lung, heart, liver, spleen, kidneys, adrenal glands, thymus, lymph-nodes, skeletal muscle, abomasum, small intestine and colon from all fetuses and samples from two placentas, were fixed in 10% neutral buffered formalin, embedded in paraffin, processed routinely and stained with hematoxylin and eosin (HE) (Campero et al. 2003). In addition, selected sections were stained with Gram, Grocott and Warthin-Starry (Campero et al. 2003).

Microbiological cultures. Samples of fetal lung and abomasal content were cultured for aerobic and microaerophilic bacteria (Campero et al. 2003). *Campylobacter fetus* was isolated by culture of abomasal content on blood agar and incubating the plates under microaerophilic conditions (Campero et al. 2003). Further additional phenotypic test were employed to identify the species and subspecies. The criteria to confirm opportunistic bacteria as a cause of abortion were: 1) isolation of the bacteria in pure culture from abomasal content and/or lung; 2) presence of pathological lesions consistent with bacterial infection in the fetus; 3) exclusion of other common abortigenic agents (Kirkbride 1993, Yaeger & Holler 2007).

For virus isolation, samples of fetal spleen were inoculated into Madin-Darb Bovine Kidney (MDBK) cells. After 4 blind passages, cultures were tested for BVDV and BHV antigens by IFAT procedure with two commercially available polyclonal antibodies, respectively (American Bio-Research, Sevierville/TN, USA).

The identification of *Tritrichomonas foetus* was performed inoculating fetal abomasal content into the liver broth medium. The samples were incubated at 37°C and examined under optic microscope at 20x during 7 days (Campero et al. 1986).

Fetal abomasal content and lung was cultured on Sabouraud's dextrose agar from fetuses that had macroscopic pathological findings compatible with mycotic infections.

Direct fluorescent antibody test (DFAT). Smears of liver, lung, kidney and aqueous humor were performed routinely for *Leptospira* spp. identification by DFAT. Fluorescein labeled rabbit polyclonal antibodies (Lepto Multivalent FA Conjugate, National Veterinary Services Laboratories, Ames/IA, USA) at a 1:20 dilution was used. Samples were examined under a fluorescence microscope at 40x. Smear from a culture of *L. interrogans* was prepared as a positive control.

Similarly, *C. fetus* DFAT was performed routinely from fetal abomasal content as previously described (Campero et al. 2003). A *C. fetus venerealis* conjugate (Conjugado-campy, Laboratorio Biologico de Tandil S.R.L, Tandil, Argentina) at a 1:20 dilution was employed. Samples were examined under a fluorescence microscope (Nikon Fluophot). Smears from cultures of *C. fetus venerealis* and *C. sputorum bubulus* were employed as positive and negative controls, respectively.

Fetal serology (FS). Fetal fluids from thoracic and abdominal cavities were tested for *Neospora caninum* antibodies as previously described (Dubey et al. 1988). Titers ≥1:25 were considered as evidence of *N. caninum* infection (Wouda et al. 1997).

The presence of antibodies against BVDV and BHV were performed from samples of thoracic and abdominal fetal fluids using the microtitre technique, as previously described (Odeón et al. 2001). Serial dilutions of the samples were incubated with a viral inoculum containing 100 TCID50/50µl for 1 hour at 37°C. A suspension of MDBK cells was added and incubation continued at 37°C. Antibody titer was determined as the highest dilution that showed complete inhibition of the cytopathic effect after 72 hours of incubation. Titers ≥1:8 were considered indicative of fetal infection (Odeón et al. 2001).

Immunohistochemistry (IHC). Selected samples of brain with microscopic lesions (focal or multifocal lymphohistiocytic encephalitis with gliosis and/or necrosis) compatible to *N. caninum* infection were processed. Polyclonal antibodies against this protozoon at a 1:300 dilution (kindly provided by Dr. M. Anderson, UCDavis, USA) were employed. An Avidin Biotin commercial kit (ABC Elite ABC Peroxidase Complex Vector PK6101, Vector, Burlingame, USA) was employed according to the instruction of the manufacturer. AEC substrate chromogen K3464 (Dako, Carpinteria, CA, USA), was used as previously described (Campero et al. 2003).

PCR. Fetal tissues were taken during the necropsy and stored at -80°C until processing. Selected tissues and PCR protocols (Table 1) were collected for detection of *N. caninum* (Baszler et al. 1999), *Leptospira* spp. (Mérien et al. 1992), *Brucella abortus* (Morrell 2010), *C. fetus venerealis* (Schulze et al. 2006), *T. fetus* (Bon Durant et al. 2003), BHV (Takiuchi et al. 2005) and BVDV (Ridpath et al. 1994). The PCR was applied in 38 fetuses that only had microscopic lesions suggestive of infectious etiology. In addition, the PCR was employed to confirm

the cause of abortion in fetuses positive to *Leptospira* spp. by DFAT (11 fetuses); and in fetuses with serology to *N. caninum* (11), BHV (14) and BVDV (15). DNA was extracted using a commercial kit (DNeasy, Blood and Tissue kit, QIAGEN, Frankfurt, Germany) and PCR conditions was applied accordingly (Table 1). PCR products were revealed by electrophoresis on a 2% agarose gel and stained with ethidium bromide. Positive (DNA from infectious agent) and negative (bi-distilled water) controls were included appropriately.

Statistics. A generalized linear model with Poisson distribution (Proc. Gen. Mod) (SAS 2002) was applied in order to establish association between etiologic diagnosis, herd origin and trimester of gestation. Chi^2 test (Proc Freq) (SAS 2002) was used to identify significant differences between herd type (beef versus dairy), determined and undetermined causes of abortion, and the presence or absence of lesions. Differences were considered significant for P <0.05.

RESULTS

The etiology of bovine abortion was identified in 78 of 150 (52%) fetuses. Infectious causes were determined in 72 of 150 bovine fetuses; being 32 of 72 identified by standard diagnosis procedures, and 40 of 72 by other diagnostic test (Table 2).

Non-infectious causes of bovine abortion were determined in four fetuses that had congenital malformations, and in two fetuses with iatrogenic origin suspicion (Table 2). Congenital malformations consisted of arthrogryposis (Fig.1), hydrocephalus, cheiloschisis and lumbar spina bifida. The first iatrogenic abortion was related with *Bacillus anthracis* (Stern strain) vaccination and subsequent abortion during late gestation. In this case, *B. anthracis* was isolated from abomasal content and fetal lung and microscopic lesions was observed in the fetus. The second iatrogenic abortion was attributed to generalized hypersensitivity reaction, after the administration of a parenteral multivitamin complex injected to the dam.

In a total of 72 abortions the cause was undetermined (Table 2). In all these cases, infectious agents were not detected but 38 of 72 fetuses had microscopic lesions suggestive of infectious etiology (Table 2).

Specimens, origin and macroscopic examination procedures. A total of 150 bovine fetuses from 77 herds from Buenos Aires province were analyzed; 104 (69.2%) were submitted from beef (Aberdeen Angus, Hereford and their crosses) and 35 (23.4%) from dairy herds (Holstein and Jersey). Information regarding type of herd was not received for 11 fetuses (7.4%). The number of fetuses submitted for diagnosis from beef herds was significantly higher (p<0.05) than from dairy herds. Seventy six (50.6%) and 58 fetuses (38.6%) were male and female (p<0.05), respectively, and in 16 (10.6%) cases the gender was not recorded. The gestational ages of 80 fetuses (53.3%) corresponded to the third trimester of the pregnancy. A total of 57 fetuses (38%) aborted in the second trimester of the pregnancy, and five fetuses (3.33%) in the first trimester. In eight fetuses (5.33%) the gestational age could not be recorded.

A high degree of autolysis (score 2 to 3) was observed in 61% of the fetuses, and the remaining fetuses had slight autolysis (score 1) (p<0.05).

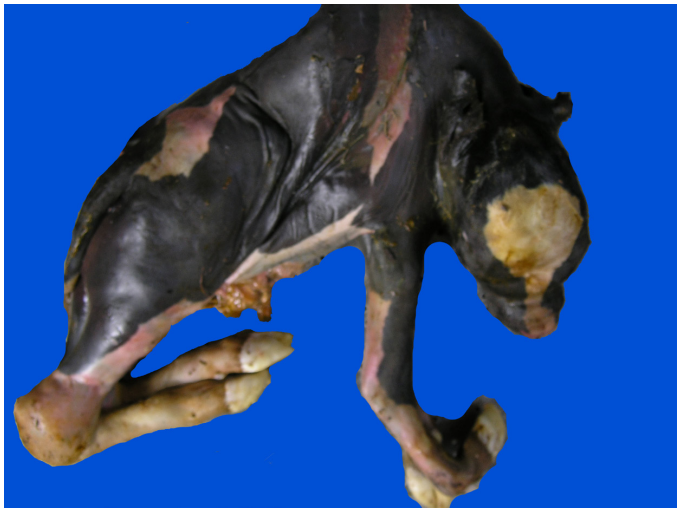

Fig.1. Aborted bovine fetus with arthrogryposis.

Table 1. PCR conditions of *Brucella abortus, Campylobacter fetus venerealis, Leptospira* spp., BVDV, BHV, *Neospora caninum* and *Tritrichomonas foetus* for diagnosis of bovine abortion

Infectious agent	Analyzed tissues	Primers	Annealing (°C)	Cycles	Product (bp)	References
B. abortus	Lung, AC[a]	tgccgatcacttaagggccttcat gacgaacggaatttttccaatcc	58	40	498	Morrell 2010
C. fetus venerealis	Lung, AC	ggtagccgcagctgctaagat tagctacaataacgacaact	55	35	142	Schulze et al. 2006
Leptospira spp.	Liver, lung, kidney, aqueous humor	ggcggcgcgtcttaaacatg ttagaacgaagttacccccctt	63	29	331	Mérien et al. 1992
BVDV	Spleen	ccatgtgccatgtacag catgcccatagtaggac	48	25	283	Ridpath et al. 1994
BHV	Spleen	aacatgcaagggccgacattgg gaccgtgccgtcgatgtacagc	55	30	552	Takiuchi et al. 2005
N. caninum	CNS[b]	cagtcaacctacgtcttct gggtgaaccgagggagttg	56	35	227	Baszler et al. 1999
T. foetus	Lung, AC	cgggtcttcctatatgagacagaacc cctgccgttggatcagtttcgttaa	67	40	347	Bon Durant et al. 2003

[a] AC = Abomasal content, [b] CNS = central nervous system.

Table 2. Diagnosis of bovine abortions in 150 fetuses

Diagnostic	SDP [a]	Other diagnostic test*					Percentage of total
		PCR[b]	FS[c]+PCR	DFAT[d]+PCR	IHC[e]	Total	
Determined causes						78	52.0
Infectious							
a) Bacterial						42	28.0
Ccampylobacter fetus sp.	14						9.33
Leptospira spp.				11			7.33
Brucella abortus	6	4					6.65
Streptococcus α hemolytic	3						2.0
Trueperella pyogenes	2						1.33
Aeromonas hydrophila	1						0.68
Listeria monocytogenes	1						0.68
b) Protozoal						24	16.0
Neospora caninum		5	6		11		14.67
Tritrichomonas foetus	2						1.33
c) Fungal						1	0.68
Aspergillus fumigatus	1						0.68
d) Viral						**5**	**3.33**
BVDV	2		0				1.33
BHV			3				2.0
Non infectious						6	4.0
Malformations	4						2.67
Iatrogenic	2						1.33
Undetermined causes						72	48.0
With histological lesion	38						**25.33**
Without histological lesion	34						22.67

* Include the histology in all cases; [a] SDP = standard diagnostic procedures (include histology, bacterial, and viral isolation), [b] PCR = polymerase chain reaction, [c] FS = fetal serology, [d] DFAT = direct fluorescence antigen test, [e] IHC = Immunohistochemistry.

Macroscopic lesions like jaundice caused by *Leptospira pomona*, fibrinous pericarditis and/or pleuritis caused by *B. abortus*, circumscribed plaques on the skin, and necrotic placentitis caused by *Aspergillus fumigatus* were observed in some fetuses.

Histology and special staining. The most common microscopic lesions associated with the infectious agent identified were: neutrophilic bronchopneumonia, lymphohistiocytic meningitis with *B. abortus* and *Campylobacter fetus venerealis,* lymphohistiocytic bronchopneumonia and occasional presence of giant cells with *Tritrichomonas foetus* (Fig.2), neutrophilic bronchopneumonia with *Trueperella pyogenes*, lymphohistiocytic folliculitis, lymphohistiocitic bronchopneumonia, necrotic placentitis with *A. fumigatus*, lymphohistiocytic interstitial nephritis, lymphohistiocytic meningitis, lymphohistiocytic periportal hepatitis with *L. interrogans*, neutrophilic multifocal hepatitis with *Listeria monocytogenes,* focal lymphohistiocytic encephalitis with necrosis and/or gliosis, lymphohistiocytic myocarditis and/or pericarditis, lymphohistiocitic interstitial pneumonia, and portal lymphohistiocitic hepatitis with foci of necrosis with *N. caninum*, lymphohistiocytic interstitial myocarditis, lymphohistiocytic vasculitis with BVDV and multifocal hepatic necrosis with BHV.

The presence of fungal-like structures (hiphas) from skin sections were identified by Grocott stain. *Leptospira*-like organisms were stained by Warthin-Starry from sections of kidneys and/or liver. Intra-alveolar cluster of *T. pyogenes* was stained by Gram.

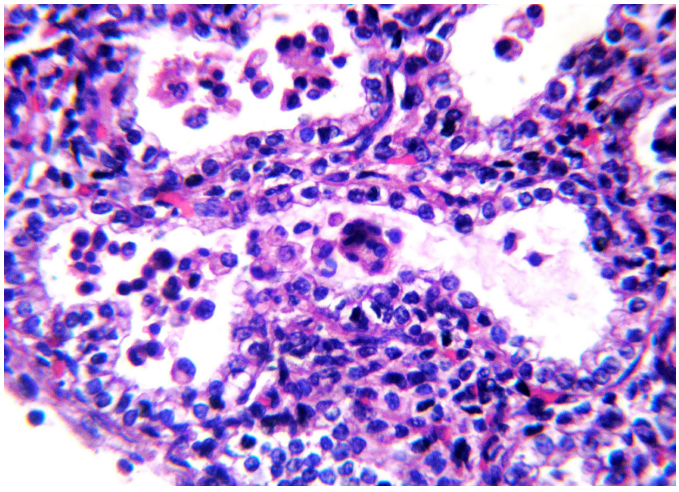

Fig.2. Lymphohistiocytic bronchopneumonia with occasional presence of giant cell from a bovine fetus positive to *Tritrichomonas foetus*. HE, obj.40x.

Microbiological cultures. A total of 129 samples of abomasal content or lung were available for bacteria, and *T. foetus* culture. Bacterial infectious agents were identified in 29 of 129 of those samples: *C. fetus venerealis* (14), *B. abortus* (6), *T. pyogenes* (2), *Aeromonas hydrophila* (1),

L. monocytogenes (1), *Streptococcus α hemolytic* (3) (Table 2). *T. foetus* was identified in two opportunities from abomasal contents. For BVDV and BHV isolation 129 spleens were available. BVDV was isolated in 2 of 129 samples, while BHV was not isolated (Table 2). *A. fumigatus* was isolated from abomasal content and lung in one fetus.

DFAT. Fluorescent *Leptospira*-like structures were observed in 11 of 150 (Table 2) smears of kidneys (3), lung (1), liver and kidney (3), aqueous humor (3) and aqueous humor and kidney (1). *Campylobacter*-like structures were observed by DFAT in 14 of 150 samples of abomasal content processed (data not show).

Fetal serology. Fetal fluids from 110 fetuses were examined. Antibodies against *N. caninum*, BHV and BVDV were detected in 11, 14 and 15 fetuses respectively.

IHC. Positive immunostaining to *N. caninum* structures (tachyzoites and tissue cysts) were observed in the 11 fetuses with microscopic lesions compatible with this parasite (Fig.3). Only two of those fetuses had antibodies to *N. caninum.*

PCR. This technique was positive to *N. caninum* and *B. abortus* in 5 of 38 CNS and 4 of 38 lungs (Fig.4), respectively. These 38 fetuses had microscopic lesions suggestive of infectious etiology (see discussion).

The inclusion of PCR confirmed the etiologic diagnosis in 11 of 11 fetuses positive to *Leptospira* spp. by DFAT. In addition, PCR confirmed the cause of abortion in 6 of 11 and 3 of 14 fetuses with serology to *N. caninum* and BHV, respectively; but PCR was negative in 15 fetuses that had antibodies to BVDV (Table 2). In each one of these cases, the fetuses had inflammatory lesions compatible to the agent involved.

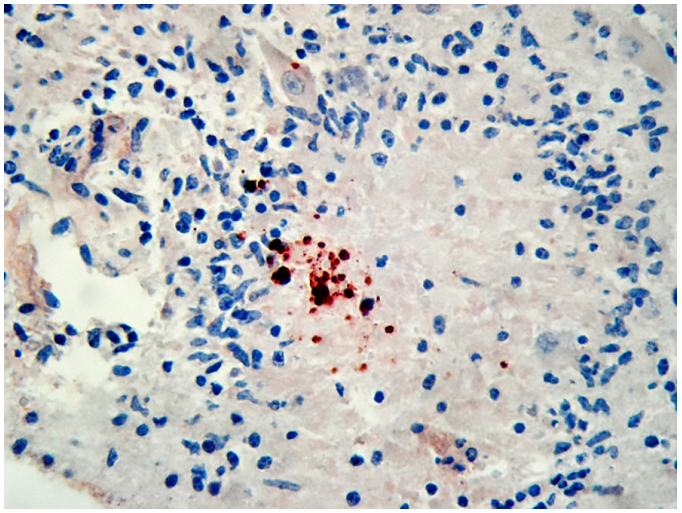

Fig.3. Positive immunostaining to *Neospora caninum* tachyzoites in central nervous system of bovine fetus. Mayer haematoxylin, obj.40x.

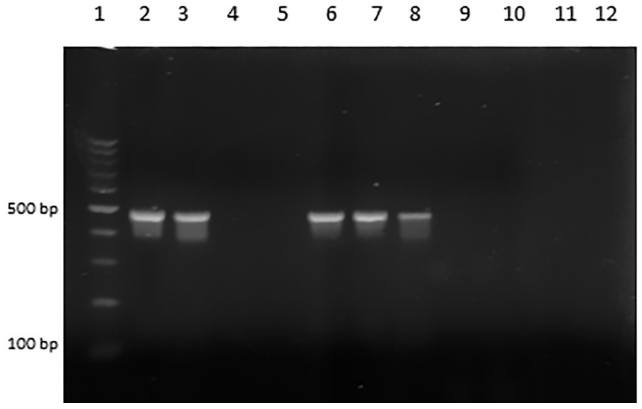

Fig.4. *Brucella abortus* PCR, products obtained using primers pairs IS 711/All Bru (498 pb). Vertical lanes = size marker, 100pb (1), positive control (2), *B. abortus* positives fetuses (3, 6, 7, 8), *B. abortus* negatives fetuses (4, 5), negative control (10), without samples (11, 12).

DISCUSSION

The etiology of bovine abortion is identified in less than half of the fetuses submitted (Campero et al. 2003, Campero 2006, Anderson 2007), being the degree of decomposition, fetal contamination, and absence of the placenta the main limiting factors for this low sensitivity (Kirkbride 1986, Holler 2012). In addition, laboratory procedures available, as well as sampling, are critical factors for the successful of the diagnosis (Thurmond et al. 1994, Holler 2012).

In the current work, the etiology of bovine abortion was identified in 52% of the fetuses submitted. This result was higher than previous works (Kirkbride, 1990, 1993, Campero et al. 2003) in which PCR was not included.

Most of the fetuses analyzed came from beef herds and were recovered during the last third of gestation. Estimation of fetal gestational age is a useful parameter when abortions are of infectious origin. Diseases like *Tritrichomonas foetus*, *Leptospira* spp. serovar Hardjo and BVDV can cause early fetal losses. Most of abortions due to *Neospora caninum* occur between five to seven months of gestation. *Campylobacter fetus* and HVB are frequently identified in fetuses of five to nine month of gestation, while *Brucella abortus,* and *L. pomona* cause abortions during the last third of gestation.

Coincidently to previous reports (Anderson et al. 1990, 1991, Kirkbride 1993, Campero et al. 2003, Campero 2006, Jamaludin et al. 1996, Morrell 2010) in this study, infectious etiology was the main cause of abortion.

Brucelosis is a zoonotic and chronic disease that causes important losses due to abortion, stillbirth and infertility in ruminants (Campero et al. 2017). Despite the inclusion of vaccination, serology test and slaughter policy, brucellosis is still present in Argentina. *B. abortus* was identified in six fetuses by culture isolation and in four fetuses by PCR. In the last case, the fetuses had been negative by culture, but the presence of microscopic lesions (neutrophilic bronchopneumonia and/or lymphohistiocytic meningitis or pericarditis) and PCR, confirmed the etiologic diagnosis.

Coincidently with Campero et al. (2003) *C. fetus* spp. was the second cause of abortion. Bovine campylobacteriosis is a venereal disease caused by *C. fetus venerealis,* while *C. fetus fetus,* an habitant of the intestine, cause sporadic abortions (Campero et al. 2017).Special culture and DFAT are routine diagnostic techniques for the disease, but don't allow to differentiate Campylobacter species (Schulze et al. 2006). The inclusion of PCR identified *C. fetus venerealis* species

as the cause of abortion in 14 fetuses with diagnosis of campylobacteriosis.

L. pomona is the most important serovar associated with abortion storms in cattle (Campero et al. 2017). Despite this, leptospirosis is probably under-diagnosed. The culture is not practical because is costly and time-consuming. Silver stained histologic sections and DFAT are frequently used, but their sensibilities are low and experienced personnel are required (Anderson 2007, Holler 2012, Borel et al. 2014). Nevertheless, 11 cases of leptospirosis were diagnosis by DFAT and subsequently confirmed by PCR. The high incidence of the disease (Table 2) was probably related to the favorable climatic conditions registered during the period of this study.

Oportunistic bacteria (*Escherichia coli, Pasteurella* spp., *Salmonella* spp., *Staphylococcus* spp., *Streptococcus* spp., *Listeria* spp., etc) and mycotic abortions cause sporadic bovine abortion at any stage of gestation, but more frequently during the last trimester (Campero et al. 2003, Anderson 2007, Borel et al. 2014). These microorganism are commonly found on mucosal surfaces of the host or its environments (poorly fermented silages, moldy stored or processed feedstuffs), but in some circumstances they can produce septicemia into the dam causing abortion. Opportunistic bacteria were isolated in pure culture from fetuses with consistent microscopic lesions due to *Trueperella pyogenes* and *Listeria monocytogenes* in the lung (neutrophilic bronchopneumonia) and liver (neutrophilic multifocal hepatitis), respectively. In addition, *T. pyogenes* was confirmed by the presence of intra-alveolar clusters of bacteria gram negative stained.

Aspergillus fumigatus, Candida spp., *Absidia* spp. and *Mucor* spp. are the main fungi implicated in sporadic bovine abortion. The former, was isolated in one fetus from abomasal content and lung. Circumscribed plaques on the skin, the presence of microscopic lesions (lymphohistiocitic bronchopneumonia, necrotic placentitis), and hiphas Grocott positive, confirmed the mycotic abortion in this study.

N. caninum was the most common infectious agent identified (Table 2). This result is not surprising, and consistent with previous reports of Argentina that shows the association between the high seroprevalence to *N. caninum* and the presence of abortions in beef and dairy cattle herds (Moore et al. 2002, Fernández et al. 2007, Morrell 2010). The IHC confirm the diagnosis of *N. caninum* as the cause of abortion when characteristic microscopic lesions and detectable antigens are both present in tissues, and no other infectious causes are identified (Anderson 2007, Borel et al. 2014). In this study, positive immunostaining to *N. caninum* tachyzoites was detected in the brain of 11 fetuses with focal lymphohistiocytic encephalitis with necrosis and/or gliosis. In addition, some fetuses had compatible inflammatory lesions in other organs. Only two of these 11 fetuses had antibodies to *N. caninum*; however, positive serology means the fetus mounted an immune response to the microorganism, but not proved that this microorganism caused the abortion (Anderson 2007, Campero et al. 2017). In addition, fetal serology depends on fetal age (fetal immunocompetence), duration of infection, and autolysis (Moore et al. 2003, Anderson 2007, Holler 2012).

PCR of *N. caninum* is more sensitive than histology and IHC (Baszler et al. 1999). However, PCR positive to *N. caninum* without fetal lesions is indicative of infection, but cannot explain the etiology of abortion. Eleven fetuses were *N. caninum* PCR positive, and six of them had positive serology. No multifocal necrosis and/or gliosis were present in the brain of any of these specimens, but moderate mononuclear cell infiltrates compatible to *N. caninum* infection were present in the meninges and/or myocardium, pericardium, lung and liver.

T. foetus usually produces early embryonic losses; however, occasional late term abortion can be produced by this parasite (Bon Durant 2007). Surprisingly, this agent was identified in one fetus of seven month from a beef herd. Campero et al. (2003) associate the poor diagnosis of trichomonosis from fetuses from beef herds of Argentina, to the difficulty of recover the specimens during the first trimester of gestation in those extensive production systems. The identification of trichomonads from abomasal fetal content by dark field microscopy and culture, and the lymphohistiocytic bronchopneumonia with occasional presence of multinucleated giant cells confirmed the diagnosis of abortion.

In Argentina BVDV and BHV are endemic diseases (Odeón et al. 2001). Virus isolation is the goal standard method and has the advantage that the isolate can be used for regional vaccine production; however, is a time consuming procedure and sometimes the sensitivity of viral isolation is reduced due to autolysis (Anderson 2007, Holler 2012). A proof of that was the absence of BHV isolation in all fetuses analyzed in this study. The PCR is a sensitive method for identifying BHV; mainly in the liver. (Takiuchi et al. 2005, Holler 2012). Three of 14 fetuses with serology to BHV were PCR positive from spleen. In addition, these fetuses had multifocal foci of hepatic necrosis compatible to herpesvirus.

Fetal infection by BVDV has variable outcomes depending on the moment of the infection, the biotype involved and others factors (Anderson 2007, Campero et al. 2017). Infertility, embryonic death, fetal resorption, mummification, malformations, abortion and birth of persistently infected lives calves had been associated to this virus. We isolated BVDV in two fetuses from beef and dairy herds, respectively. Both fetuses had characteristic microscopic lesions in the heart and other tissues, without vasculitis, but fetal lesions by this virus are quite variable (Anderson 2007).

None of 15 fetuses with serology to BVDV were PCR positive; being absent the microscopic lesions in those fetuses. This result indicates fetal viral infection, but excludes BVDV as the cause of abortion.

CONCLUSIONS

Bacterial and viral isolation are the "gold standard" test for the diagnosis of bovine abortion, however one of their main limitation is the low sensibility. Direct antigen and/or DNA detection are the currently preferred methods used for this purpose. Regardless of the techniques used, in all cases, the results must to be confirmed by the presence of compatible microscopic lesions. Failure of these conditions indicates fetal infection, but do not prove the cause of the abortion.

In this study, the fetuses diagnosed by PCR alone, or PCR combined to others test (fetal serology or DFAT), had histopathological lesions corresponding to the agent involved.

The criteria employed in the present study should be considered for future investigations of bovine abortion in order to avoid false-positive results.

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

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Original Article
Livestock Diseases

Cyanogenic poisoning by spontaneous ingestion of star grass (*Cynodon nlemfuensis* var. *nlemfuensis* cv. 'Florico') in cattle[1]

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ABSTRACT.- 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.** *Pesquisa Veterinária Brasileira 39(1):20-24*. 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

This study reports the epidemiological data and the clinical-pathological condition of five outbreaks of cyanogenic poisoning in cattle spontaneously ingesting star grass (*Cynodon nlemfuensis* Vanderyst var. *nlemfuensis* cv. 'Florico'). In all outbreaks, the areas where the plant was previously fertilized with high concentrations of nitrogen and the properties adopted the silvipastoral system. The first clinical signs appeared between 10 and 15 minutes after the first introduction of cattle and were characterized by muscular tremors, dyspnea, moderate tympanism, staggering gait, forced breathing with open mouth, sternal recumbency followed by death after 15 to 30 minutes and/or recovery in a few hours after the signs started. In total, 43 cows have become ill and 18 died. Two necropsies were performed and no significant changes were found except for the presence of the plant near the esophageal sphincter region. No histological lesions were seen through microscopy. Green leaves of the star grass were collected from all properties where the outbreaks occurred and the test of the picro-sodium paper was performed, revealing red-brick coloration in 20 minutes after maceration of the leaves.

INDEX TERMS: Cyanogenic poisoning, star grass, *Cynodon nlemfuensis*, hydrocyanic acid, star grass cv. Florico, cattle, toxic plants, toxicoses.

RESUMO.- [**Intoxicação cianogênica pela ingestão espontânea de grama estrela (*Cynodon nlemfuensis* var. *nlemfuensis* cv. 'Florico') em bovinos.**] Descrevem-se os dados epidemiológicos e quadro clínico-patológico de cinco surtos de intoxicação cianogênica em bovinos que ingeriram espontaneamente grama estrela (*Cynodon nlemfuensis* Vanderyst var. *nlemfuensis* cv. 'Florico'). Em todos os surtos, as áreas onde a planta se encontrava haviam sido previamente adubadas com altas concentrações de nitrogênio e as propriedades adotavam o sistema silvipastoril com *Eucaliptus* sp. Os primeiros sinais clínicos surgiram entre 10 e 15 minutos após a primeira introdução dos bovinos e caracterizou-se por tremores musculares, dispneia, timpanismo moderado, andar cambaleante, respiração forçada com a boca aberta, decúbito esternal seguido de morte após 15 a 30 minutos e/ou, recuperação em poucas horas após início dos sinais. No total, adoeceram 43 vacas e destas 18 morreram. Duas necropsias foram realizadas e não foram encontradas alterações significativas, exceto a presença da planta próxima a região do esfíncter esofágico. Através da microscopia não foram visualizadas lesões histológicas. Folhas verdes da grama estrela foram coletadas de todas as propriedades onde os surtos ocorreram e realizadas o teste do papel picro-sódico, o qual revelou coloração vermelho-tijolo em 20 minutos após maceração das folhas.

TERMOS DE INDEXAÇÃO: Intoxicação cianogênica, grama estrela, *Cynodon nlemfuensis*, bovinos, ácido cianídrico, grama estrela cv. Florico, plantas tóxicas, toxicoses.

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INTRODUCTION

Grasses of the genus *Cynodon*, belonging to the Poaceae family, came from Africa and were introduced in North America in the mid-eighteenth century (Harlan 1970). For more than 50 years, many breeding works have been carried out mainly at the universities of Georgia and Florida in the United States in order to better use the forage potential of this genus and to adapt it to the subtropical conditions of Southeastern United States. In Brazil, it is believed that this grass was introduced by private initiative to evaluate its behavior under Brazilian conditions; however, there are no records of how and where the genus *Cynodon* was introduced (Vilela & Alvim 1998). There are also many doubts about the recognition of the species used.

The genus *Cynodon* is divided into two groups, the Bermuda grass group (*Cynodon dactylon* (L) Pers.), in which several hybrids are available, such as Coastal, Alicia, Callie, Tifton 44, Tifton 68, Tifton 78, Tifton 85, Coastcroos and more recently the Florakirk. In the star grass group (*Cynodon nlemfuensis* Vanderyst, *Cynodon aethiopicus* Clayton and Harlan), the most used cultivars are McCaleb, Ona, Florico and Florona (Vilela & Alvim 1998). Florico (*Cynodon nlemfuensis* Vanderyst var. *nlemfuensis* cv. 'Florico') and Florona cultivars (*Cynodon nlemfuensis* Vanderyst var. *nlemfuensis* cv. 'Florona') are the most recent commercial star grass launches by the University of Florida in the USA (Mislevy et al. 1989a, 1989b). Florona cultivar was collected at the Experimental Station of the University of Florida in 1974 and submitted to evaluation trials from 1975 (Mislevy et al. 1989b). Florico cultivar, native to Kenya, was introduced in Puerto Rico in 1957 and later brought to Florida in 1972 (Mislevy et al. 1993a). Both cultivars were recorded in 1993 by Mislevy et al. (1993a, 1993b). Florico cultivar can be distinguished from other star grasses of the *nlemfuensis* variety by its hairiness and purplish-green color (Mislevy et al. 1989a). It presents high dry mass production and good response when it receives high levels of fertilization, but it has great potential for accumulation of cyanogenic glycosides, especially under high Nitrogen doses (N), especially during the early stages of plant development. In 16 years of testing in Ona, Florida, hydrocyanic acid (HCN) poisoning in cattle grazing in Florico grass (Mislevy et al. 1993a) was not observed. In Brazil, the presence of HCN in Florico grass was verified as a function of the age of grass cut, but the HCN dose did not exceed 109.01mg/kg (Castro 1998). In Santa Catarina, cattle breeding in a silvopastoral system recommended by Garcia et al. (2013) have been developed and star grass is one of the grasses used. The use of this system seems to favor the accumulation of hydrocyanic acid in the plant similar to what occurs with *Sorghum* spp. described by Vetter & Haraszti (1977).

In South Africa, other star grasses (*C. nlemfuensis* Vanderyst var. *robustus*, *C. aethiopicus* Clayton and Harlan) were also identified as cyanogenic (Kellerman et al. 1988). Most cyanogenic plants are harmless due to the low concentration of glycosides and low palatability. However, toxic grasses that are widely cultivated are highlighted due to their good acceptance by animals (Youssef & Maxie 2004).

In Brazil, several other cyanogenic plants of animal interest have been described, such as *Cnidoscolus phyllacanthus* (Oliveira et al. 2008), *Manihot glaziovii* (Tokarnia et al. 1994a) *M. glaziovii* Muell. Arg. (Amorim et al. 2005), *Manihot piauhyensis* (Canella et al. 1968), *Sorghum sudanense* (Juffo et al. 2012), *Sorghum halepense* (Nobrega Junior et al. 2006), *Passiflora foetida* (Carvalho et al. 2011), *Piptadenia macrocarpa* (Tokarnia et al. 1994b), *Piptadenia viridiflora* (Tokarnia et al. 1999), *Prunus sphaerocarpa* (Saad & Camargo 1967), *Prunus sellowii* (Gava et al. 1992) and Tifton 68 (Gava et al. 1997, Galindo et al. 2017).

The toxic principle of cyanogenic plants is the presence of cyanogenic glycosides, which through the action of intracellular plant enzymes and ruminal digestion are converted into hydrocyanic acid (HCN), one of the most known toxic compounds (Egekeze & Oehme 1980). HCN reversibly binds to the cytochrome c oxidase enzyme (CcOX) and disrupts oxygen transfer from red blood cells to somatic cells (Hibbs 1979, Way 1984, Leavesley et al. 2008). When ingested under the form of glycoside, the minimum lethal HCN dose for cattle and sheep is about 2mg/kg body weight (Radostits et al. 2002). Signs of histotoxic anoxia resulting from cyanogenic poisoning are observed within a few minutes after plant ingestion and death occurs within a few minutes to one hour after the onset of signs. Clinical signs are characterized by dyspnoea, anxiety, salivation, muscle tremors, incoordination, staggering gait, reddish mucous membranes, depression, tachycardia, seizure, decubitus and opisthotonus in the terminal phase (Kellerman et al. 1988, Radostits et al. 2002, Youssef & Maxie 2004). No significant macro and microscopic lesions are observed. The finding of leaves of the plant in the cardic region, not yet mixed with the ruminal content (Tokarnia et al. 2012), is fundamental for diagnosis.

The picrate test qualitatively evaluates the presence of HCN in plants, and is a fundamental tool for diagnosis. Slower reactions should not be disregarded, since there are cyanogenic glycosides of slower development. Treatment based on sodium nitrite and sodium thiosulphate is effective, but in most poisoning events, the timing for administration is short due to the rapid evolution of cases, but whenever possible, treatment should be performed as it is important in confirmation of diagnosis by cyanogenic plants (Tokarnia et al. 2012).

The aim of the present study was to evaluate the epidemiological, clinical and pathological aspects of five outbreaks of cyanogenic poisoning by spontaneous ingestion of star grass Florico cultivar in cattle.

MATERIALS AND METHODS

The collection of epidemiological data, the observation of clinical signs and the necropsy of two cows were carried out in visits to the five properties where the disease outbreak occurred. Viscera samples were collected and fixed in 10% formalin and processed for histological evaluation. Green star grass leaves were collected from all the properties where the outbreaks occurred and the picrate test described by Henrici (1926), quoted by Tokarnia et al. (2012) was performed. The test consisted of a strip of white paper immersed in solution composed of 5g sodium carbonate and 0.5g picric acid dissolved in 100ml distilled water. Leaves were macerated and placed in glass jars with lid, where the paper strip was fixed, which remained freely suspended above the plant material. Glass jars were held upright and the reaction was observed for 1 hour. The intensity of the reaction to the picrate test was classified taking into account the time of appearance of the red-brick color.

RESULTS

Five HCN poisoning outbreaks in cattle, four in Santa Catarina, in the municipalities of Água Doce (Properties 1 and 2), Santa Terezinha (Property 3) and Braço do Norte (Property 4) were diagnosed from 2015 to 2017. An outbreak occurred in Paraná, in the municipality of União da Vitória (Property 5). Epidemiological data from the five outbreaks are shown in Table 1. In four of the five properties, owners were informed that the cultivated grass was purchased from another property as being tifton specimens.

In all outbreaks, the areas where the plant was cultivated had been previously fertilized with high nitrogen concentrations and properties adopted the silvopastoral system with *Eucalyptus* sp. (Fig.1). The first clinical signs appeared between 10 and 15 minutes after the first introduction of the cattle in pasture exclusively constituted of star grass Florico cultivar and was characterized by muscle tremors, dyspnea, moderate tympanism, staggering gait, forced breathing with open mouth, sternal decubitus followed by death after 15 to 30 minutes and/or recovery within a few hours after the onset of signs. Two necropsies were performed and no significant changes were found except for the presence of the plant near the esophageal sphincter region. No histological lesions were observed in the various organs.

In all properties, star grass Florico cultivar samples collected to perform the picrate test showed a red-brick color change in 20 minutes after maceration of leaves.

Table 1. Data referring to outbreaks of spontaneous poisoning by Florico star grass

Outbreaks	County	Total cows in the picket/lot	Diseased	Deaths
1st	Água Doce/SC	11	11	3
2nd	União da Vitória/PR	10	10	4
3rd	Santa Terezinha /SC	16	16	8
4th	Braço do Norte/SC	30	3	2
5th	Água Doce/SC	27	3	1
Total		94	43	18

Fig.1. Star grass Florico cultivar, fresh and in silvopastoral system in the municipality of Santa Terezinha/SC.

DISCUSSION

The clinical-pathological condition observed in spontaneous poisoning by star grass Florico cultivar is in agreement with that described by other authors for cyanogenic plants (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 & Méndez 2007, Oliveira et al. 2008, Carvalho et al. 2011, Juffo et al. 2012, Galindo et al. 2017).

In the present study, the majority of cows with clinical signs recovered spontaneously within a few hours. These animals probably stopped star grass consumption spontaneously due to the appearance of mild clinical signs, consuming doses lower than those consumed by animals that became seriously ill and died, or may be related to the management system adopted in properties. According to Radostits et al. (2002), hungry animals and/or those not used to ingesting cyanogenic plants are more prone to poisoning. Studies indicate that animals in contact with cyanogenic plants can tolerate increasing HCN doses due to the increased production of the rhodanase enzyme, which is important in the organism detoxification. In the present study, all outbreaks were the first contact of cattle with the plant and morbidity ranged from 10% to 100% and mortality from 3.7% to 50%, evidencing the susceptibility of animals to poisoning.

The use of the silvopastoral system seems to show that shadow associated with excess nitrogen on star grass Florico cultivar favors the synthesis of hydrocyanic acid. Vetter & Haraszti (1977) observed that the production of HCN in *Sorghum* spp. decreases gradually during its vegetative development, as the photosynthesis intensity increases, that is, the less photosynthesis the more HCN. Mislevy et al. (1993a) reported that the hydrocyanic acid potential of Florico star grass is high under heavy N fertilization, especially during the early stages of plant development.

The response to the picrate test occurred within 20 minutes, which differs from that obtained from green *Prunnus sellowii* leaves, a cyanogenic plant found in the Southeastern and Southern regions of Brazil, which occurred within 3 to 5 minutes (Gava et al. 1992). This is probably due to the fibrous character of Florico leaves, which makes it difficult to macerate them and consequently delay the reaction between the enzyme and the glycoside.

The differential diagnosis of cyanogenic poisoning should be made mainly in relation to other diseases or poisonings with super acute clinical evolution and without macroscopic and microscopic alterations, especially nitrate/nitrite poisoning. This poisoning is commonly observed in the southern region of Brazil in cattle that graze on grasses, mainly oat and ryegrass, well fertilized and under favorable climatic conditions (dry seasons followed by rains). The grazing time on these grasses is also an important data for differential diagnosis. While for poisoning with plant containing hydrocyanic acid, the grazing time is 10 to 20 minutes, nitrate/nitrite poisoning requires grazing time at least greater than one hour. Both conditions show clinical signs of respiratory difficulty and staggering gait; however, in nitrate/nitrite poisoning, there is a marked tachypnea, not observed in the same proportion in HCN poisoning. In addition, in HCN poisoning, mucous membranes are bright red and in nitrate/nitrite poisoning

brown (Hibbs 1979). In these cases, it is interesting to apply the picrate test to identify HCN and/or the diphenylamine test for nitrate in pastures. Another difference is that pastures with high nitrate/nitrite content maintain their toxicity after desiccation (Jönck et al. 2013) and grasses with HCN, such as Tifton 68, lose their toxic action after phenation (Galindo et al. 2017).

Among the toxic plants that cause rapid death in cattle in the southern region of Brazil, *Amorimia exotropica* stands out in the coastal region of Santa Catarina and Rio Grande do Sul. However, it is possible to distinguish them among epidemiological aspects (Gava et al. 1998, Pavarini et al. 2011). Urea poisoning is also included in the differential diagnosis, which also produces rapid death. Epidemiological data on the accidental access of cattle to urea and the alkalinity of the ruminal content should also be evaluated.

CONCLUSION

When cultivated in the shade, star grass (*Cynodon nlemfuensis* Vanderyst var. *nlemfuensis* cv. 'Florico') can accumulate cyanide acid and cause cyanogenic poisoning in cattle.

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

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Original Article
Livestock Diseases

Serological response to *Neospora caninum* infection in goats and agreement between three diagnostic techniques to detect caprine neosporosis[1]

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ABSTRACT.- Kim P.C.P., Melo R.P.B., Almeida J.C., Silva J.G., Ribeiro-Andrade M., Porto W.J.N., Pinheiro Junior J.W. & Mota R.A. 2019. **Serological response to *Neospora caninum* infection in goats and agreement between three diagnostic techniques to detect caprine neosporosis.** *Pesquisa Veterinária Brasileira 39(1):25-31.* Laboratório de Doenças Infecto-Contagiosas dos Animais Domésticos, Departamento de Medicina Veterinária, Universidade Federal Rural de Pernambuco, Rua Dom Manoel de Medeiros s/n, Dois Irmãos, Recife, PE 52171-900, Brazil. E-mail: rinaldo.mota@hotmail.com

The present study aimed to measure the serological response of goats infected with *Neospora caninum* by assessing the diagnostic performance and agreement between three techniques (indirect immunofluorescent antibody test, IFAT; *Neospora* agglutitation test, NAT; enzyme-linked immunosorbent assay, ELISA). The panel of sera were comprised of 500 samples of goats, and 60 reference serum samples. These reference and field serum samples were tested by ELISA, NAT, and IFAT. In the field serum samples tested, the seroprevalences of anti-*N. caninum* antibodies were 3.2%, 4.6%, and 6.4% in the NAT, IFAT and ELISA, respectively. Using the IFAT as the gold standard, the NAT and the ELISA agreement was considered weak (k=0.28) and strong (k=0.75), respectively. When the IFAT performance was used for comparison purposes, the ELISA showed 91.3% sensitivity and 97.7%, specificity with a PPV of 65.2% and a NPV of 99.6%; The NAT presented sensitivity of 26.1% and specificity of 97.9% with a PPV of 37.5% and a NPV of 96.5%. Accordingly, the IFAT should remain the assay of choice for studies about *N. caninum* infection in goats in individual serum samples. A combination of serological assays with high sensitivity and specificity is recommended in serosurveys of caprine neosporosis.

INDEX TERMS: Serology, *Neospora caninum*, infection, goats, diagnostic techniques, caprine, neosporosis, serological screening tests, goats, parasitoses.

RESUMO.- [**Resposta sorológica à infecção por *Neospora caninum* em cabras e concordância entre três técnicas diagnósticas para detecção de neosporose caprina.**] Objetivou-se avaliar a resposta sorológica de caprinos infectados com *Neospora caninum* mediante o estudo da performance e concordância de três técnicas sorológicas (RIFI, NAT e ELISA). O painel de soros testes foi composto por 500 amostras de caprinos e ainda 60 soros classificados como de referência. Todos os soros de referência e de campo foram testados por ELISA, NAT e RIFI. Nos soros de campo, as soroprevalências de anticorpos anti-*N. caninum* foram de 3,2% no NAT, 4,6% na RIFI e 6,4% no ELISA. Utilizando a RIFI como técnica de referência, a concordância de NAT e ELISA foi considerada fraca (k=0,28) e substancial (k=0,75), respectivamente. Ainda utilizando a RIFI como comparação, foram obtidos valores de sensibilidade de 91,3% e 97,7% de especificidade no ELISA, e valores preditivos positivo de 65,2% e negativo de 99,6%; NAT apresentou resultados de sensibilidade de 26,1% e de especificidade de 97,9% com valores preditivos positivo de 37,5% e negativo de 96,5%. Com base nos resultados deste trabalho, sugerimos que a RIFI permaneça como técnica de escolha no estudo da neosporose caprina em amostras individuais, resguardando as recomendações e pontos de

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corte adotados neste estudo. Indicamos a associação de técnicas sorológicas de alta sensibilidade e especificidade.

TERMOS DE INDEXAÇÃO: Sorologia, infecção, *Neospora caninum*, caprinos, técnicas de diagnóstico, neosporose, testes de triagem sorológica, parasitoses.

INTRODUCTION

Neospora caninum is an obligate intracellular coccidian parasite that belongs to the phylum Apicomplexa and is considered an important cause of abortion in cattle around the globe (Dubey et al. 2007). This protozoal organism has a complex heteroxenous life cycle in which the domestic dog and other canids act as definitive hosts and a number of ungulates play a role as intermediate hosts in the transmission of the disease agent (Dubey & Schares 2011).

In non-pregnant animals, neosporosis is usually a latent asymptomatic infection (Buxton et al. 2002). Persistent infection throughout life is an important feature of bovine neosporosis. *N. caninum* can be transmitted vertically from the dam to the fetus in successive pregnancies resulting in repeated abortions and unthrifty, weak calves at birth. Congenitally infected animals transmit the protozoan parasite to their offspring in the next generation (Williams et al. 2000, Buxton et al. 2002). Over the years, neosporosis have been extensively studied in cows as the bovine is the most important farm animal species in economic terms (Reichel et al. 2013). Cases of neosporosis in small ruminants have also been reported worldwide, and studies on the transplacental transmission of *N. caninum* have been conducted by numerous researchers (Dubey & Schares 2011, Varaschin et al. 2012, Moreno et al. 2012, Nunes et al. 2017).

The occurrence of reproductive disorders in goats that are seropositive for *N. caninum* suggest that neosporosis is a significant cause of abortion and neonatal deaths in caprine herds (Moreno et al. 2012, Mesquita et al. 2018). *N. caninum* congenital transmission rates in goats may be as high as those reported to cattle. The prevalence of congenitally infected offspring is similarly high (Mesquita et al. 2013). Neosporosis should be included in the differential diagnosis of endemic or epidemic abortions along with other toxic and infectious causes of abortion that commonly affect farm animals (McAllister 2016). Diagnosis of abortion due to *N. caninum* infection in production animals is based on the clinical history and epidemiological data of the affected herd, serological screening of female animals, and a comprehensive diagnostic workup on aborted fetuses including necropsy, histopathology, and serology of fetal fluids (Ortega-Mora et al. 2006). The definitive diagnosis of abortion due to *N. caninum* can be tricky and relatively expensive. Asymptomatic *N. caninum* congenital infections are common. The presence of the pathogen DNA in tissues of aborted fetuses does not necessarily mean that this protozoan parasite was the cause of the abortion (Dubey & Schares 2011).

A number of serological assays which include commercially available kits have been used to detect specific anti-*N. caninum* antibodies in cattle. These ancillary tests are used primarily to distinguish between infected animals and non-infected ones. Each of these diagnostic tools has its particular features and pros and cons (Blumröder et al. 2004, Aguado-Martínez et al. 2008, Álvarez-García et al. 2013). The Indirect fluorescent antibody test (IFAT) is routinely used for the detection of specific IgM and IgG in serum samples. This assay was further optimized for the use in sera from small ruminants and other animal species (Buxton et al. 1998). Other techniques that have also been used in serological surveys of antibodies to *N. caninum* in large animals include the Enzyme-linked immunosorbent assays (ELISA) using recombinant antigens or intact tachyzoites, Immunoblotting (IB) (an immunoprotein technique), and the *Neospora* Agglutination Test (NAT). These assays should be carefully chosen according to the needs of the researcher and diagnostician (Ortega-Mora et al. 2006, Aguado-Martínez et al. 2008, Guido et al. 2016).

The evaluation of the degree of concordance (agreement) between tests to detect *N. caninum* infection and the comparative assessment of the performance of different assays used for the detection of *N. caninum* infections can be challenging, especially because a gold standard technique for the serological diagnosis of neosporosis is lacking (Ortega-Mora et al. 2007, Guido et al. 2016).

The aim of the present study was to evaluate the serological response of goats to *N. caninum* by assessing the performance and agreement between three different serological techniques used to detect specific IgG antibodies against *N. caninum* in serum samples of naturally and experimentally infected animals.

MATERIALS AND METHODS

Panel of sera and experimental design. The present survey consisted of a panel of serum samples for testing and a panel of reference (control) sera. Sample selection criteria used in this study were those available in the section about analytical and diagnostic performance characteristics of laboratory tests published in the OIE Guideline "Principles and Methods of Validation of Diagnostic Assays for Infectious Diseases" (OIE 2013). A total of 560 caprine serum samples were analyzed. Serum samples were divided into two categories: reference sera (positive controls and negative controls), and samples from naturally infected animals raised for subsistence in extensive goat farms (sera for testing) from semi-arid region of Pernambuco State, Brazil. A total of 500 field samples were collected from female goats, aged between one and three years, from different races. Four properties were chosen for convenience (ease of access). These collections were approved by the Committee on Ethics in the Use of Animals (CEUA) of the Federal University of Alagoas, under license number 78/2017. *Neospora caninum* serostatus of these caprine herds was unknown.

A total of 60 reference sera were divided as follows, 30 serum samples positive to *N. caninum*, and 30 serum samples negative to *N. caninum.* These sera were originally collected during a experimental *N. caninum* inoculation study previously published by Porto et al. (2016). Negative samples were collected from goats serologically negative to *N. caninum*, *Toxoplasma gondii*, Caprine Arthritis Encephalitis virus (CAEV), and *Coxiella burnetii*. These animals had no clinical history of reproductive problems, and did not seroconvert for neosporosis in three consecutive tests. Serum samples were tested by IFAT and ELISA at intervals of 30 days.

All data regarding the maintenance of *N. caninum* strains in the laboratory, selection of animals for this study, inoculation dose, sampling, and analysis of the immunological dynamics associated with *N. caninum* infection were previously published by Porto et al. (2016).

Serological tests (IFAT, ELISA, and NAT)

Antigen preparation and antigen production. The *N. caninum* Nc-Spain 7 isolate used in this study was maintained in a monolayer culture of Marc-145 cells under specific standardized laboratory conditions previously used in research conducted by Regidor-Cerrillo et al. (2008). Tachyzoites were stained with Tripan blue and resuspended in sterile PBS 1X (pH 7.4). The number of viable tachyzoites was determined with a Neubauer counting chamber. The protocol published by Álvarez-García et al. (2003) was followed for the preparation and production of the finished antigen that was used in the three serological techniques that were assessed in the present study (i.e., IFAT, ELISA, and NAT).

Indirect fluorescent antibody test (IFAT). In the present study, the IFAT was used as a reference technique (gold standard) to detect anti-*N. caninum* IgG antibodies in goat sera. The IFAT was performed according to the guidelines provided by Porto et al. (2016). The protocol established by Álvarez-García et al. (2003) was followed with some minor modifications. In summary, 10µL of the *N. caninum* tachyzoite suspension in formalin solution at a concentration of 10^7 tachyzoites/mL was inoculated into each slide well and then dried at room temperature. Slides were immersed for 10 min in acetone at -20°C in order to finish antigen fixation. A cut-off point of 1:50 was used with dilutions of caprine sera in 1X PBS (pH 7.2). Diluted sera were inoculated into each slide well, incubated at 37°C for 30 min, and washed twice in 1X PBS for 10 min. Anti-goat IgG solution conjugated with fluorescein isothiocyanate was added to a 1:400 dilution in 0.002% Evans Blue (Sigma-Aldrich Corp., St Louis/MO, USA) and then incubated at 37°C for 30 min, followed by 2 washes with 1X PBS and 1 final wash with distilled water. After drying, slides were coverslipped using glycerin solution and visualized under a fluorescence microscope. Positive controls and negative controls were included in all the slides examined. Samples were considered positive when total peripheral fluorescence was detected in more than 50% of the tachyzoites in different fields of each well. Samples in which tachyzoites did not fluorescence or that tachyzoites displayed irregular fluorescence were interpreted as negative.

In-house ELISA. Levels of anti-*N. caninum* specific IgG antibodies were measured by an in-house ELISA technique developed by González-Warleta et al. (2014) and modified for this study by the use of lyophilized antigen of *N. caninum* in a concentration of $5x10^7$ tachyzoites/mL. For such purpose, the antigen was used in a concentration of 10^5 tachyzoites per well diluted in a carbonate-bicarbonate buffer solution (0.1M, pH=9.6). A final volume of 100µL was inoculated into each well. Elisa microplates (Greiner Bio-One GmBH, Germany) were incubated overnight at 4°C. After the plates were sensitized, each well was blocked using 300µL of a bovine serum albumin solution (Sigma-Aldrich Corp., St Louis, MO, USA) diluted 3% in 1X phosphate buffer saline (pH=7.4) containing 0.05% Tween 20 (PBS-T). This step was followed by 2 hours incubation at room temperature. Plates were washed 3 times with PBS-T. The sera used were diluted 1:100 in the blocking solution, adding 100µL of this dilution into each corresponding plate well, and incubated for 1h at 37°C. The plates were washed 3 additional times with PBS-T; 100µL of G-Biotin Protein were added as the conjugate (Sigma-Aldrich Corp., St Louis, MO, USA), diluted in 1:10,000 PBS-T, and incubated for 1h at 37°C. The plates were then washed 3 times, and 100µL of ABTS Solution substrate (Roche, Indianapolis, USA) were inoculated into these plates. The reaction was stopped after 20 min at room temperature by adding 0.3M oxalic acid solution. ELISA plate reading was performed on a spectrophotometer (Multiskan RC, Thermo Labsystems) using 405nm wavelength (OD405). Optical density values were converted to percent relative index (IRPC) using the following formula: IRPC = (OD405 sample-OD405 negative control)/(OD405 positive control-OD405 negative control)×100. An IRPC value ≥10 meant a positive result. Duplicate serum sets consisting of positive and negative controls for *N. caninum* were used for the validation of the reactions.

***Neospora* agglutination test (NAT).** The NAT assay was used according to the guidelines provided by Romand et al. (1998) with some modifications. In the present study, an antigen from *N. caninum* isolate Nc-Spain 7 was used. The initial dilution of sera used was 1:25 and the final dilution was 1:50 (cut-off point); 96-well plates were used. These NAT plates had a U-shaped background. Results were interpreted as follows: samples were considered negative if a compact dot or button was formed at the bottom of the microplate well, filling more than 50% of this well. Samples were considered positive if an opaque mesh (web) was formed in at least 50% of the microplate well. Positive controls and negative controls were added to all microplates.

Data analysis. In order to compare the three serological assays performed in the present study, the IFAT was defined as the reference test (gold standard). The following parameters were calculated: Kappa coefficient (k), sensitivity (Se), specificity (Sp), positive predictive value (PPV), negative predictive value (NPV), and accuracy (ACU) (Cohen 1960, Gart & Buck 1966). The k-values were interpreted according to the criteria established by Landis & Koch (1977) as follows: <0, without agreement; 0.00-0.19, poor agreement; 0.20-0.39, weak agreement; 0.40-0.59, moderate agreement; 0.60-0.79, substantial agreement; 0.80-1.00, almost perfect agreement.

RESULTS

Analysis of reference sera

The tests of all reference sera (30 positive samples and 30 negative samples) showed 100% Se, Sp, PPV, NPV, ACU, and k values equal to 1 in the ELISA and NAT, when compared to the IFAT (reference technique/gold standard) (Table 1).

Antibody search (survey) in field serum samples (seroprevalence)

Seroprevalences of anti-IgG *Neospora caninum* antibodies in the three different serological tests assessed in this study were: 3.2% (1.98-5.13) in the NAT, 4.6% (3.05-6.84) in the IFAT, and 6.4% (4.57-8.90) in the ELISA. The highest prevalence was estimated in the ELISA with a cut-off point at 1:100 whereas the lowest prevalence was estimated in the NAT with a cut-off point at 1:50.

Results of agreement (concordance) between serological tests

The results of the agreement between the three serological tests assessed in the present study and the values of Se, Sp, PPV, NVP, and ACU associated with the detection of anti-*N. caninum* IgG antibodies for the 500 serum samples tested are provided in Table 2. The agreement between the NAT and the ELISA when compared to the IFAT was considered weak and substantial, respectively, with kappa (k) coefficients of 0.28 for the NAT and 0.75 for the ELISA. When the ELISA technique was compared with the IFAT, the values of 91.3% of sensitivity and 97.7% of specificity were found, with a positive predictive value of 65.2% and a negative predictive value of 99.6%. The NAT, when compared to the IFAT, showed a sensitivity of 26.1% and a specificity of 97.9% with a positive predictive value of 37.5% and a negative predictive value of 96.5%.

Table 1. Comparative concordance between three serodiagnostic assays using reference samples of goat sera tested for anti-*Neospora caninum* antibody detection

Reference sample panel	Total (n)	Serodiagnostic tests				Sensitivity (CI 95%)	Specificity (CI 95%)	Positive predictive value (CI 95%)	Negative predictive value (CI 95%)	ACU (CI 95%)	*k* (CI 95%)
Primo-infeccion model (experimental inoculation)		IFAT[R]	Pos.	Neg.	Total						
Positive goats	30	ELISA	Pos.	30	0	30	100% (100-100)	100% (100-100)	100% (100-100)	100% (100-100)	100% (100-100)	1.0 (P) (1,000-1,000)
Negative goats	30		Neg.	0	30	30						
			Total	30	30	60						
			IFAT[R]	Pos.	Neg.	Total						
		NAT	Pos.	30	0	30	100% (100-100)	100% (100-100)	100% (100-100)	100% (100-100)	100% (100-100)	1.0 (P) (1,000-1,000)
			Neg.	0	30	30						
TOTAL	60		Total	30	30	60						

IFAT = Indirect immunofluorescence antibody test, NAT = *Neospora* agglutination test, ELISA = enzyme-linked immunosorbent assay, Pos. = positive, Neg. = negative, ACU = accuracy; [R] reference test, k = Kappa coefficient: no agreement (<0.0), p = poor (0.0-0,19), W = weak (0.20-0.39), M = moderate (0.40-0.59), S = substantial (0.60–0.79), P = almost perfect (0.80-1.00) (Landis & Koch 1977).

Table 2. Comparative concordance between three serodiagnostic assays demonstrated by frequency of anti-*Neospora caninum* IgG antibodies in samples sera of 500 goats

Serodiagnostic assays					Sensitivity (CI 95%)	Specificity (CI 95%)	Positive predictive value (CI 95%)	Negative predictive value (CI 95%)	ACU (CI 95%)	*k* (CI 95%)
	IFAT[R]	Pos.	Neg.	Total						
ELISA	Pos.	21	11	32	91.3% (79.8-102.8)	97.7% (96.3-99.0)	65.2% (49.2-82.1)	99.6% (99-100.2)	97.4% (96-98.8)	0.750 (S) (0.620-0.880)
	Neg.	2	466	468						
	Total	23	477	500						
	IFAT[R]	Pos.	Neg.	Total						
NAT	Pos.	6	10	16	26.1% (8.1-44)	97.9% (96.6-99.2)	37.5% (13.8-61.2)	96.5% (94.8-98.1)	94.6% (92.6-96.6)	0.281 (W) (0.088-0.473)
	Neg.	17	467	484						
	Total	23	477	500						
	ELISA[R]	Pos.	Neg.	Total						
IFAT	Pos.	21	2	23	65.6% (49.2-82.1)	99.6% (99-100.2)	91.3% (79.8-102.8)	97.7% (96.3-99)	97.4% (96-98.8)	0.750 (S) (0.620-0.880)
	Neg.	11	466	477						
	Total	32	468	500						
	ELISA[R]	Pos.	Neg.	Total						
NAT	Pos.	12	4	16	37.5% (20.7-54.3)	99.1% (98.3-100)	75% (53.8-96.2)	95.9% (94.1-97.6)	95.2% (93.3-97.1)	0.478 (M) (0.301-0.654)
	Neg.	20	464	484						
	Total	32	468	500						
	NAT[R]	Pos.	Neg.	Total						
IFAT	Pos.	6	17	23	37.5% (13.8-61.2)	96.5% (94.8-98.1)	26.1% (8.1-44)	97.9% (96.6-99.2)	94.6% (92.6-96.6)	0.281 (W) (0.088-0.473)
	Neg.	10	467	477						
	Total	16	484	500						
	NAT[R]	Pos.	Neg.	Total						
ELISA	Pos.	12	20	32	75% (53.8-96.2)	95.9% (94.1-97.6)	37.5% (20.7-54.3)	99.1% (98.3-100)	95.2% (93.3-97.1)	0.478 (M) (0.301-0.654)
	Neg.	4	464	468						
	Total	16	484	500						

IFAT = Indirect immunofluorescence antibody test, NAT = *Neospora* agglutination test, ELISA = enzyme-linked immunosorbent assay, Pos. = positive, Neg. = negative. ACU = accuracy; [R] reference test, k = Kappa coefficient: no agreement (<0.0), p = poor (0.0-0.19), W = weak (0.20-0.39), M = moderate (0.40-0.59), S = substantial (0.60–0.79), P = almost perfect (0.80-1.00) (Landis & Koch 1977).

DISCUSSION

Experimental studies have shown that goats are susceptible to *Neopsora caninum*. Abortion occurs after pregnant does are inoculated with this protozoan parasite (Lindsay et al. 1995, Porto et al. 2016). In some countries, reports of abortion and neonatal death in naturally infected goats due *N. caninum* have been publshed (Barr et al. 1992, Dubey et al. 1996, Corbellini et al. 2001, Eleni et al. 2004, Moreno et al. 2012, Nunes et al. 2017). Abortions occur most often in seropositive goats, and culling is widely practised in eradication programs (Altbuch et al. 2012). Serodiagnosis is the first step towards initiating any surveillance or control program for neosporosis in a goat herd (Guido et al. 2016).

Our findings corroborate those of previous studies about caprine neosporosis carried out by Brazilian researchers in which low seroprevalences of *N. caninum* infection in goats were found. In Northeastern Brazil, seroprevalences of caprine neosporosis vary between 2.9% (Arraes-Santos et al. 2016) and 3.3% (Faria et al. 2007) in the IFAT assay with a cut-off point of 1:50 and 15% in the IFAT assay with a cut-off point of 1:100 (Uzêda et al. 2007). Sensitivity and specificity results of the ELISA assay for field serum samples when compared with the results of the IFAT assay were 91.3% sensitivity and 97.7% specificity.

Serological surveys conducted around the globe have reported prevalences of antibodies against *N. caninum* in goats ranging between 2 and 23% using the IFAT, the ELISA and the NAT with different cut-off points (Dubey & Schares 2011). In the present study, field samples were tested with the three techniques routinely used for the detection of anti-*N. caninum* IgG antibodies, i.e. ELISA, NAT, and IFAT. We found a higher seroprevalence of neosposis in goats (6.4%) by using the ELISA assay. Wide variation in the seroprevalence of caprine neosporosis have been reported in the southeastern region of the country. In the State of São Paulo, southeast Brazil, a seroprevalence of 2.7% of caprine neosporosis was reported in the IFAT assay with a cut-off point of 1:50 (Santos et al. 2013) in contrast with a seroprevalence of 19.7% obtained by Modolo et al. (2008) and a seroprevalence of 17.23% found by Costa et al. (2012) when using the NAT with a cut-off point of 1:25. These variations in seropositivity may be due to a number of differences in each farm including herd management, herd hygiene, presence of definitive hosts in the area, climate variations, and environmental contamination (Dubey & Schares 2011).

The IFAT has been the assay of choice for the serological diagnosis of neosporosis in goats and sheep over the years mainly because it was the first serological test developed for such purpose (Dubey et al. 1988), and also due to the fact that cross-reactivity with other coccidian parasites is low (Dubey & Schares 2011). Therefore, the IFAT was adopted as a reference test (gold standard) in our study in order to compare its diagnostic performance with the diagnostic performance of other assays (NAT and ELISA). High background values in absorbance reading and cross-reactivity between related parasites have been reported for the ELISA assay depending on the method of antigen preparation (production) and polyclonal antibody used in the assay (Björkman et al. 1999).

In the present study, since a commercially available test and a species-specific antibody were both lacking in the market, an in-house ELISA protocol using a G protein as the conjugate, which has an affinity for both caprine and ovine immunoglobulins, was followed (Porto et al. 2017). Our findings show that the lyophilized tachyzoite-based ELISA was a highly sensitive assay which is able to detect true negative serum samples with a negative predictive value of 99.6%. Antigens based on soluble extracts contain large amounts of molecules with antigenic properties which are mainly intracellular. There are also the antigens from the membrane surface of the parasite which are preferentially recognized by the IFAT assay (Lasri et al. 2004, Silva et al. 2007). However, in the case of *Toxoplasma gondii*, the specificity and sensitivity of a technique based on the recognition of intracellular antigens have been challenged (Lasri et al. 2004). We do not know whether the variation in the preparation/production of the antigen used in the in-house ELISA has influenced the sensitivity and specificity values in the field samples. Thus, it is important to compare this assay with another assay in which soluble antigens are also used.

The low agreement between the NAT for field samples (k=0.281) and the reference technique (k=1.0) may have occurred due to a difference in the time course of infection and also because of variations in the amount of specific circulating antibodies, especially because these are samples from experimental inoculations. There are fluctuations in the levels of antibodies in chronic natural *N. caninum* infections (Packham et al. 1998). However, these fluctuations did not interfere in the identification of negative animals due to the high values of specificity that were obtained with this technique (Sp=97.9%).

The sensitivity and specificity of a particular serological test can vary according to the different cut-off points that are chosen (Dubey 2003, Lasri et al. 2004, Silva et al. 2007). Divergences in prevalences using different techniques and different cut-off points have also been reported in seroepidemiological studies about *T. gondii* infections in horses. Aroussi et al. (2015) reported that when the MAT (modified agglutination test) and the ELISA were both used, the seroprevalence had significant variations ranging between 13% and 90%. Dubey et al. (1990) concluded that the assessment of the prevalence would not be possible until additional studies were conducted to determine the sensitivity and specificity of serological tests for equine toxoplasmosis. Few studies on the seroprevalence of *N. caninum* infections in goats have used the NAT assay. This assay should be better evaluated in this animal species in additional studies to be conducted in the future.

CONCLUSIONS

The need for a careful interpretation of the serological test chosen by the investigator is essential during the diagnostic assessment of a herd and should include the analysis of individual serum samples.

We suggest that the IFAT should remain the assay of choice in the study of caprine neosporosis in individual serum samples. The cut-off points and guidelines provided in the present study should be followed.

We also recommend that, whenever possible, a combination of serological assays with high sensitivity and specificity is used in seroepidemiological surveys of caprine neosporosis.

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Original Article
Livestock Diseases

Clinical and pathological aspects of bovine lymphoma affecting the spinal cord[1]

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ABSTRACT.- Mello L.S., Panziera W., Bandinelli M.B., Sonne L., Driemeier D. & Pavarini S.P. 2019. **Clinical and pathological aspects of bovine lymphoma affecting the spinal cord.** *Pesquisa Veterinária Brasileira 39(1):32-39*. Setor de Patologia Veterinária, Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves 9090, Prédio 42505, Porto Alegre, RS 91540-000, Brazil. E-mail: sauloppvet@yahoo.com.br

Clinical and pathological features of bovine lymphoma involving the spinal cord were evaluated through a retrospective study of the necropsy database from 2005 to 2017. Thirty-four cases of bovine lymphoma were found, 24 of which had central nervous system involvement restricted to the spinal cord. All cattle were Holstein cows 2.5-12 years-old (median age, six years-old). The clinical course was 7-21 days, and the main neurological sign was pelvic limb paresis (81.8%). The lymphoma often affected the spinal cord in a multifocal manner. Lumbar segments were the mostly affected sites (23/24), followed by the sacral segments and cauda equina (20/24), cervical (5/24) and thoracic (5/24) segments. Tumors were in the epidural space, peripheral to the pachymeninges (extradural) and between layers of adipose tissue. In addition, two cases had progressive hemorrhagic myelomalacia. Further organs affected included the lymph nodes (100%), abomasum (79.2%), heart (75%) and kidneys (45.8%). Microscopically, all lymphomas had a diffuse pattern, with no meningeal or medullar infiltration. According to the REAL/WHO classification, all these neoplasms were mature B-cell lymphomas. Diffuse large B-cell lymphoma (DLBCL) was observed in 95.8% (23/24) of the cases. The following subtypes were observed in the DLBCL group in descending order: immunoblastic (60.9%, 14/23), centroblastic (26.1%, 6/23), anaplastic (8.7%, 2/23) and T-cell rich (4.3%, 1/23).

INDEX TERMS: Clinics, pathology, bovine lymphoma, spinal cord, veterinary neuropathology, paresis, lymphosarcoma, dairy cattle, cattle.

RESUMO.- [**Aspectos clínicos e patológicos de linfoma bovino afetando a medula espinhal.**] Os aspectos clínicos e patológicos do linfoma bovino afetando a medula espinhal foram avaliados através de um estudo retrospectivo dos protocolos de necropsia durante o período de 2005-2017. De um total de 34 bovinos com linfoma, 24 apresentaram afecção do sistema nervoso central (SNC) restrito a medula espinhal. Todos os bovinos afetados eram fêmeas, da raça Holandesa, com 2,5 a 12 anos de idade (idade mediana de seis anos). Clinicamente, os casos tiveram uma evolução de sete a 21 dias, com a principal alteração neurológica caracterizada por paresia de membros pélvicos, a qual foi observada em 81,8% dos casos. O linfoma afetou frequentemente a medula espinhal de maneira multifocal. Os segmentos lombares foram os mais envolvidos (23/24), seguidos pelos sacrais e cauda equina (20/24), cervicais (5/24) e torácicos (5/24). Os tumores estavam localizados no espaço epidural, periférica à paquimeninge (extradural) e associada ao tecido adiposo. Em dois casos foi também observada mielomalacia hemorrágica progressiva. Os órgãos acometidos com maior frequência, concomitantemente ao espaço epidural, foram os linfonodos (100%), abomaso (79,2%), coração (75%) e rins (45,8%). Microscopicamente, todos os linfomas exibiam um padrão difuso, sem infiltração em meninges e medula espinhal (extradural). De acordo com a classificação da REAL/WHO, todos esses neoplasmas foram incluídos como linfomas de células B maduras. O linfoma difuso de grandes células B (LDGCB) foi observado em 95,8% (23/24) dos casos. Os subtipos classificados dentro do grupo

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dos LDGCB's foram em ordem decrescente: imunoblástico (60,9%; 14/23), centroblástico (26,1%; 6/23), anaplásico (8,7%; 2/23), e rico em células T (4,3%; 1/23).

TERMOS DE INDEXAÇÃO: Clínica, patologia, linfoma bovino, medula espinhal, neuropatologia veterinária, paresia, linfossarcoma, bovino de leite, bovinos.

INTRODUCTION

Lymphoma encompasses a heterogeneous group of neoplasias originating from lymphoid tissues that have variable clinical, morphological and prognostic presentations (Parodi 2001, Boes & Durham 2017). Bovine lymphoma has a particular anatomical classification that differs from that of other species of domestic mammals. In general, bovine lymphoma can be divided into two groups: enzootic bovine leukosis (EBL) and sporadic bovine lymphoma (SBL) (Angelos & Thurmond 2015). The enzootic form is the most common and is an infectious contagious disease of mature cattle associated with bovine leukaemia virus (BLV) (Aida et al. 2013). EBL is predominantly observed in dairy cattle, with a peak incidence between five and eight years of age (Marshak et al. 1962, Vernau et al. 1992). The distribution of EBL is multicentric, since any organ can be involved, and, consequently, clinical signs vary according to tumor site (Burton et al. 2010, Valli et al. 2016). Morphologically, EBL is characterized by monoclonal proliferation of B lymphocytes (Vernau et al. 1997, Yin et al. 2003). There is increasing demand for immunophenotyping of neoplastic lymphocytes to better understand the pathogenesis of the disease (Valli et al. 2017). Lymphoma in animals has numerous similarities with human non-Hodgkin's lymphoma (NHL), and, consequently, most of the classifications used in veterinary medicine were adapted from the human schemes (Sueiro et al. 2004, Ponce et al. 2010, Valli et al. 2011, Vezzali et al. 2010). Among the adapted classifications, the most important and frequently used system for animals is the Revised European-American Classification of Lymphoid Neoplasms (REAL), which was later incorporated into the World Health Organization (WHO) classification (Valli et al. 2016). Although spinal cord involvement is a common presentation of enzootic lymphoma in cattle, there are limited data related to the epidemiological and clinicopathological characterization of this neoplasm in the spinal cord with simultaneous approaches in histological and immunohistochemical (IHC) identification (Valli et al. 2016, 2017). Thus, the aim of this study was to describe the epidemiological, clinical and pathological features of lymphomas with CNS (spinal cord) involvement, in addition to performing a phenotypic and immunophenotypic evaluation of these neoplasms based on the REAL/WHO classification.

MATERIALS AND METHODS

From January 2005 to January 2017, the necropsy files of cattle with a lymphoma diagnosis were reviewed, and cases with CNS involvement were selected. All cattle studied were from Rio Grande do Sul, Brazil, mainly from Metropolitan Porto Alegre. The protocol information was grouped, registered, and categorized according to age, breed, sex, clinical signs, affected organs and neoplastic distribution in epidural space involving the spinal cord.

Microscopic analysis of lymphomas was performed on histological slides stained with haematoxylin and eosin (HE). This evaluation recorded the distribution pattern of lymphocytes (diffuse and follicular), cell size and mitotic rate. The cell type was characterized based on cell size, nuclei aspect, and characteristics relating to chromatin and to the nucleoli. The mitotic rate was calculated as the mean number of mitoses in 10 high power fields (HPF, 400x). Cell size was based in the average of nuclear diameter and defined as small when the nuclear diameter was equivalent to at most 10μm, intermediate when the nuclear diameter was between 10 and 14μm and large when the diameter corresponded to more than 14μm (NCI 1982, Vernau et al. 1992, Valli et al. 2016).

Immunophenotypic analysis of neoplastic lymphocytes was carried out using the REAL/WHO classification adapted for animal use, which is based on morphological and immunophenotypic features (Valli et al. 2016). The immunohistochemical (IHC) analysis was performed using the MACH 4™ Universal AP Polymer Kit (Biocare Medical). Primary antibodies anti-CD79α (M7051, clone HM57; Dako; 1:100) for B lymphocytes and anti-CD3 (A452; Dako; 1:500) for T lymphocytes were applied and incubated overnight at 25°C. To block endogenous peroxidase activity, a 10% hydrogen peroxide solution was used. Retrieval of the CD79α antigen was performed in a pressure cooker (96°C, 20 min) with citrate buffer (pH 6.0), and Protein Block (Dako) was applied for 7 min to prevent nonspecific binding. For CD3 IHC, the antigen retrieval step was performed with Protease type XIV (Sigma), and milk (15 min) was used to prevent nonspecific binding. Immunoreactivity was visualized by using the chromogens DAB (3,3′-diaminobenzidine) for CD3 IHC and AEC (3-amino-9-ethylcarbazole) for CD79α. Immunohistochemistry sections were counterstained with Harris haematoxylin. Negative control sections were incubated with Tris-buffered saline (TBS) in place of specific antibodies. Tissue sections of bovine tonsils were used as positive controls.

RESULTS

Thirty-four necropsies of cattle diagnosed with lymphoma were performed during the analysis period, of which 24 (70.6%) had spinal cord involvement. All cases with CNS involvement corresponded to Holstein females ranging from 2.5 to 12 years-old, with mean and median ages of 5.9 and 6.0 years, respectively.

The most common neurological manifestation was paresis of the pelvic limbs (PL) (81.8%) (Fig.1A,B), evolving to PL paralysis in 9.1% of these cases. Tetraparesis was observed in 18.2% of the cases, with a previous paresis of the thoracic limbs in one of these cases. The reported clinical course of gait alterations ranged from seven to 21 days. The CNS involvement by lymphoma were restricted to the spinal cord. Concerning the anatomic distribution on the spinal cord, tumors proliferation was found in one (16.7%, 4/24) or more (83.3%, 20/24) regions. The lumbar region was the most involved (23/24), followed by the sacral/cauda equina (20/24) (Fig.1C), cervical (5/24) and thoracic regions (5/24). Lesions involving more than one region were characterized as multifocal (Fig.1D) or focally extensive lesions, extending from one anatomical location to another. Grossly, the neoplastic masses varied in size, were irregular, soft, and with white or yellow coloration. The masses were in the epidural space, peripheral to the pachymeninges (extradural) and associated with the adipose tissue (Fig.1E). Progressive hemorrhagic myelomalacia was observed in two cases (both affecting the lumbosacral segment) (Fig.1F), of which the grey matter was mainly affected, involving the dorsal and ventral horns. Gross lesions in these cases were characterized by bleeding and soft

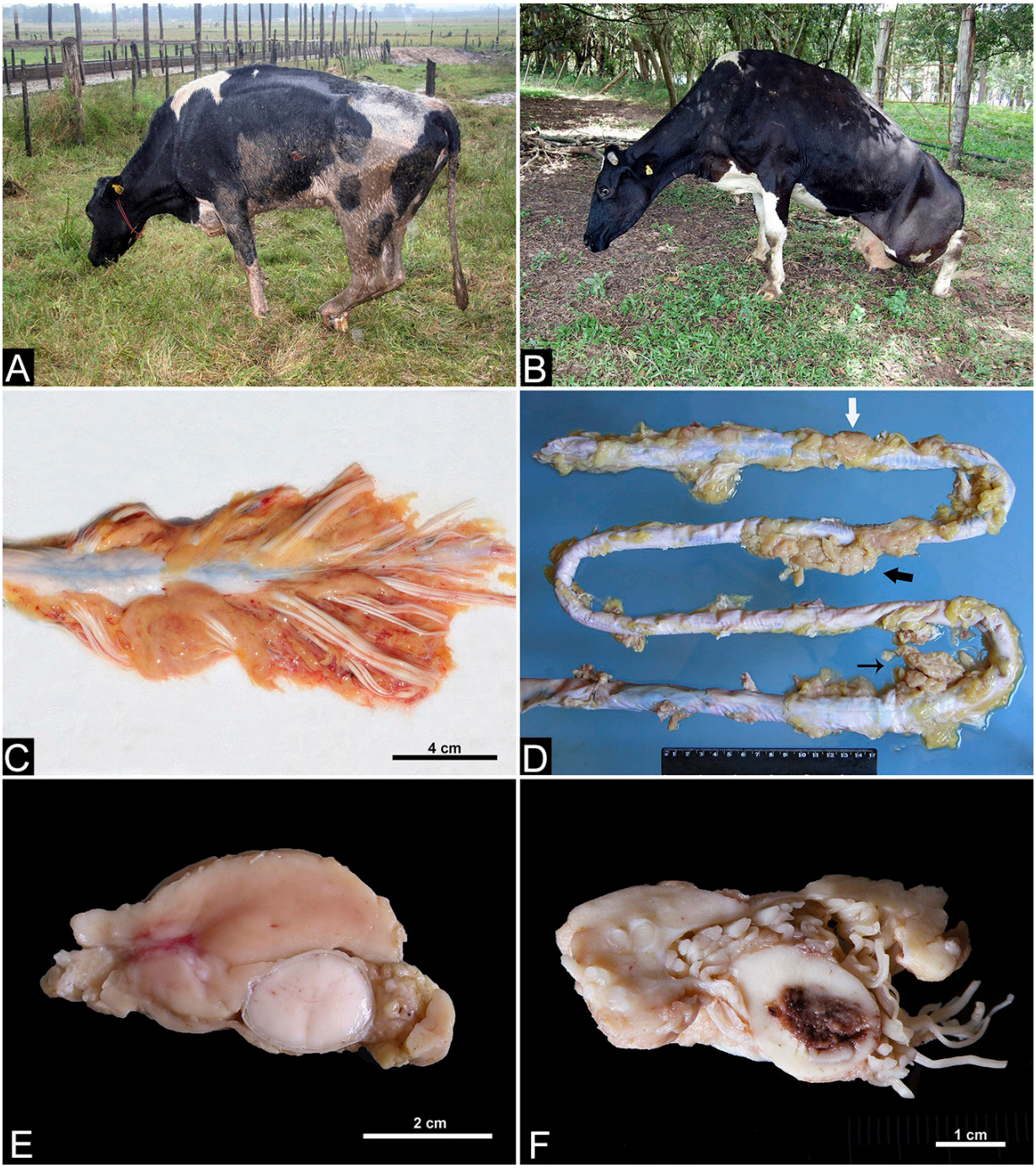

Fig.1. Clinical and gross features of bovine lymphoma in the spinal cord. (**A**) Thoracolumbar spinal cord compression by lymphoma in a cow causing abnormal posture characterized by arched back, low head carriage and knuckling at the fetlock. (**B**) Epidural Lumbosacral compression by lymphoma in a cow causing severe pelvic limb paresis. (**C**) Focally extensive pattern of lymphoma in cervical segments of the spinal cord and cauda equina. (**D**) Multifocal pattern of lymphoma in the cervical (thin arrow), thoracic (black arrow) and lumbar (white arrow) segments of the spinal cord. (**E**) Lumbar spinal cord. Extradural pattern of bovine lymphoma. (**F**) Sacral spinal cord. Hemorrhagic myelomalacia due to extradural lymphoma.

areas, in addition to occasional cavitation. Other organs were simultaneously affected in all cases of bovine lymphoma with spinal cord involvement. Among the main sites of neoplastic localization, the most common were the lymph nodes (100%, 24/24), abomasum (79.2%, 19/24), heart (75%, 18/24) and kidneys (45.8%, 11/24).

Histologic evaluation revealed a diffuse pattern of neoplastic cells distribution with an extradural predominance, without infiltration in the meninges and spinal cord. All the lymphoma cases in this study demonstrated cytoplasmic immunostaining for the CD79α antibody, identifying lymphocytes as the B immunophenotype. According to the REAL/WHO

classification criteria, all these neoplasms were mature B-cell lymphomas (Fig.2A). Within this large group, 95.8% (23/24) of cases were classified as diffuse large B-cell lymphoma (DLBCL). The subtypes within the DLBCL group included immunoblastic (60.9%, 14/23), centroblastic (26.1%, 6/23), anaplastic (8.7%, 2/23), and T-cell rich (4.3%, 1/23).

Immunoblastic DLBCLs were predominantly composed of large cells with round euchromatic nuclei (approximately 14.0-17.5 µm), vesicular or coarsely granular chromatin, a single central nucleolus and scant cytoplasm (Fig.2B). The mitotic rate per 400x HPF ranged from two to eight figures, and the mean mitotic rate was 3.4. Centroblastic DLBCLs were characterized by large oval cells with scant cytoplasm. The nuclei size (10.5-17.5 µm) and shape were highly variable, ranging from round to indented (cleaved) and exhibiting vesicular or coarsely granular chromatin and multiple nucleoli that appeared associated with the nucleolemma (Fig.2C). The mean mitotic rate (HPF, 400x) was 6.3 (range, two to 12). Anaplastic DLBCLs were composed of pleomorphic cells of varying sizes, occasionally with bizarre nuclei, and frequent multinucleation (Fig.2D). The mitotic figures were higher, with a mean mitotic rate of 6 (HPF, 400x). Generally, most of the cells were large, with a nuclear size of approximately 21 to 28 µm. T-cell rich DLBCL was characterized by heterogeneous cellular patterns and the presence of small and large lymphocytes. Most of the cells (approximately 80%) were small non-neoplastic T cells that showed positive immunostaining for CD3. The large neoplastic cells (approximately 20%) were of the B immunophenotype and had approximately two mitotic figures (HPF). The nuclei had one or more evident nucleoli, dispersed chromatin and moderate basophilic cytoplasm. In high mitotic rate lymphomas (20.8%), interspersed macrophages filled by apoptotic lymphocytes (tingible body macrophages) resembling a "starry-sky" were observed.

Small lymphocytic B-cell lymphoma, also termed mature (peripheral) B-cell neoplasia, was observed in one case (4.2%). This lymphoma was composed of small lymphocytes with scant cytoplasm, round nuclei (approximately 7µm), dense chromatin and inconspicuous nucleoli. One mitotic figure (HPF) was observed. Spinal cord injuries secondary to enzootic lymphoma involvement in the epidural space were observed in 54.2% of the cases. These lesions resulted in compression by the extradural tumor masses and consisted mainly of Wallerian degeneration with the formation of

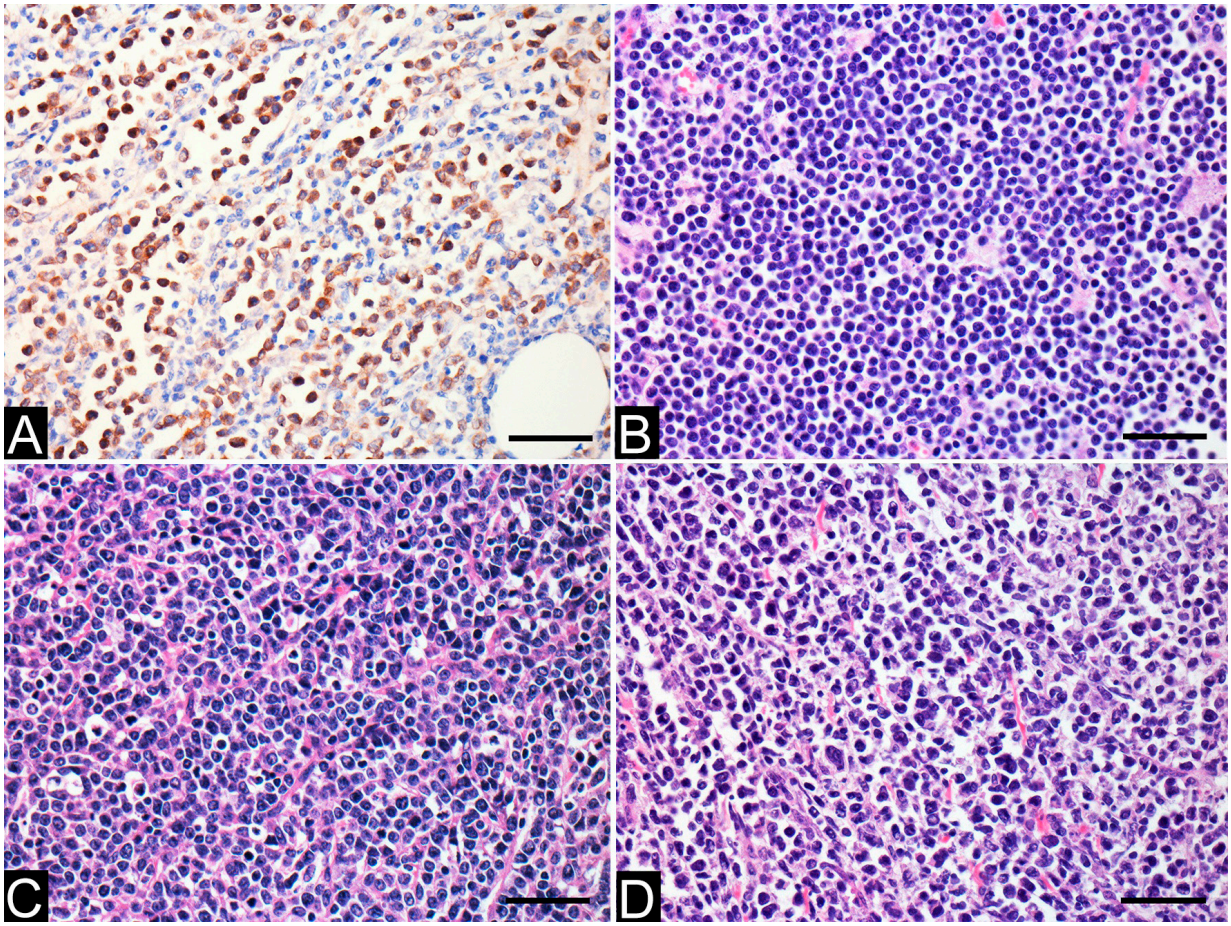

Fig.2. Microscopic features of bovine lymphoma in the spinal cord. (**A**) Epidural mass, positive cytoplasmic staining of neoplastic lymphocytes. IHC for CD79α, bar = 100µm. (**B**) Diffuse large B-cell lymphoma, immunoblastic variant. Sheets of large cells with round, euchromatic nuclei. HE, bar = 100µm. (**C**) Diffuse large B-cell lymphoma, centroblastic variant. Sheets of large cells with highly variable nuclei ranging from round to indented (cleaved) and frequently with multiple nucleoli. HE, bar = 100µm. (**D**) Diffuse large B-cell lymphoma, anaplastic variant. Sheets of pleomorphic large cells with bizarre nuclear features. HE, bar = 100µm.

axonal spheroids. Hemorrhagic myelomalacia (8.3%, 2/24) was observed in the lumbosacral portion. This lesion was found predominantly in grey matter and was characterized by extensive hemorrhagic areas, neutrophilic vasculitis, gitter cell infiltrate and malacia. Necrotic neurons showed retraction, a hypereosinophilic cytoplasm and pyknotic nuclei. In the remaining white matter, axonal spheroids, Wallerian degeneration and neutrophil infiltration were observed.

DISCUSSION

Bovine lymphoma is the most common neoplasm in milk-producing regions and is an important cause of economic loss in this industry in several parts of the world (Jacobs et al. 2002, Trainin & Brenner 2005). This neoplasm was appointed as one of the main causes of death in dairy cows in southern Brazil, in addition to being the most frequent neoplastic disease (Mello et al. 2017). All the cattle studied were adult and presented a multicentric distribution of lymphoma. Although were not performed tests for BLV detection, according to the epidemiological data, clinical and anatomopathological presentation in this study all cases were consistent with the adult, or enzootic form of lymphoma (Angelos & Thurmond 2015, Valli et al. 2016). This form is known to be associated with BLV infection and the frequent presentation occur in age groups older than two years, with a peak incidence between four and eight years as seem in the cattle studied (Vernau et al. 1992, Schwartz & Levy 1994, Kabeya et al. 2001). In this study, female dairy cattle were the most likely to be affected by lymphoma. The high prevalence in dairy cows was mainly associated with longevity and intensive practices that favor the spread of BLV (Hopkins & Digiacomo 1997, Kobayashi et al. 2010). The predominance among females is an expected feature due to a lower proportion of males in dairy herds (Marshak et al. 1962). Furthermore, the higher susceptibility in females may also be associated with the immunosuppressive role of physiological stress generated by the high demand for milk production (Wu et al. 1989). Studies have shown potential genetic susceptibility as a predisposing factor in the development of lymphoma in some herds (Jacobs et al. 2002, Aida et al. 2013, Angelos & Thurmond 2015). Beef cattle, which were not affected in this study, are usually less affected due to the long course of the illness and the less intensive handling techniques employed (Jacobs et al. 2002, Radostits et al. 2007, Angelos & Thurmond 2015).

Extradural lymphoma in cattle is a common presentation of this multicentric illness in addition to being the main compressive neoplasm in this species (Ferrer 1980, Rebhun et al. 1984, Sherman 1987, Jacobs et al. 2002, Lahunta & Divers 2008, Burton et al. 2010). As has been frequently reported in studied cattle, locomotor disorders of the hind limbs (paresis and paralysis) are a common neurologic manifestation resulting from tumor compression in the lumbosacral region, which is the most common site of neoplastic infiltration (Lahunta & Divers 2008, Lahunta & Glass 2009, Angelos & Thurmond 2015, Washburn 2017). The clinical signs of tetraparesis and paresis of thoracic limbs related to medullary compression in the craniocervical and caudal cervical regions were less common due to the low frequency of tumor masses in these regions (Lahunta & Glass 2009). Vertebral bone infiltration by lymphoma was not observed in any of the cases; such infiltration is rare and is observed mainly in young cattle with the sporadic form (Theilen & Dungworth 1965, Bundza et al. 1980, Oliver-Espinosa et al. 1994). Brain involvement in cases of bovine lymphoma may occur; however, it is considered uncommon (Sweeney et al. 1986, Sherman 1987, Fighera & Barros 2004, Braun et al. 2005, Tawfeeq et al. 2012), and it was not observed in the present study. The pathogenesis of extradural lymphoma remains uncertain. Despite the controversial presence of lymphoid tissue around the epidural venous plexus, lymphoma on the spinal canal may originate primarily from this location (Love et al. 1954). However, lymphoma that establishes in this area may originate elsewhere in a systemic disease. It has been previously observed that the propagation may occur through the paravertebral region to the inside of the canal by the intervertebral foramen (Cugati et al. 2011). Therefore, tumor masses may be observed around paravertebral structures, such as spinal vessels and nerves (Mullins et al. 1971, Valli et al. 2016). Another suggested origin is neoplastic lymphocyte infiltration through hematogenous spread through the epidural venous plexus, which is an important route of neoplasm establishment in this region (Harrington 1986, Maccauro et al. 2011). The epidural venous plexus, which contains multiple epidural vessels, is characterized by a venous net with thin walls lacking valves and muscular fibers (Batson 1957, Dyce et al. 2010). The connections of this system with thoracic, abdominal and pelvic veins allow for retrograde flow to the interior of the vertebral spine when the pressure increases in these cavities (Batson 1940). In an experiment in rats and rabbits, 70% of cases showed tumor development in the lumbar region after tumor cell injection in the femoral vein and abdominal pressure elevation (Coman & Delong 1951).

The hemorrhagic myelomalacia observed in two cattle in this study represents a neurovascular disorder secondary to compressive medullar injury (Lahunta & Glass 2009). This alteration occurs frequently in dogs and is usually associated with extramedullary pressure, as in intervertebral disc disease (Lahunta & Glass 2009). Lymphoma as a cause of hemorrhagic myelomalacia was previously described in horses (Rousseaux et al. 1989), cats (Laisse et al. 2017) and dogs (Zilio & Arias 2013), but to the best of our knowledge, there have been no reports in cattle. The hemodynamic disturbances caused by epidural compression generate vascular stasis, blood perfusion failure, plasma protein extravasation, increased colloid osmotic pressure and consequent edema (Vandevelde et al. 2012). Spinal cord lesions resulted from the cumulative effect of these pathological processes associated with inflammatory mediators that provoke vasospasm (Mautes et al. 2000). Moreover, the grey matter tends to be severely affected compared to the white matter due to the high metabolic rate (Risio & Platt 2010), as observed in the cases studied here.

According to the REAL/WHO classification criteria, all lymphomas in this study were mature B-cell lymphomas. Within this wide group, DLBCL was the more common sub-classification, accounting for approximately 95% of tumors. This subtype is the main type of lymphoma in cattle and usually presents as the enzootic form (Vernau et al. 1992, 1997, Panziera et al. 2014, 2016). This type of lymphoma is an aggressive and rapidly growing tumor. In addition to being more commonly observed affecting the lymph nodes, it can

be found in any tissue (Valli et al. 2016). The pathogenesis of BLV regarding the specific development of this type of lymphoma has not been completely elucidated. It is known that this virus provokes a clonal proliferation of B-cells, and disease severity increases according to the number of pro-viral copies (Aida et al. 2013). High proliferative rates induced by BLV may be responsible for the loss of differentiation, thus explaining the low occurrence of follicular lymphoma in cattle (Vernau et al. 1992). Although viral involvement is not a premise for the development of DLBCL, the occurrence of this disease has been related to viral agents in other species. Lymphomas can be associated with feline leukemia virus (FeLV) and feline immunodeficiency virus (FIV) in cats and with Epstein-Barr virus (EBV) and hepatitis C virus (HCV) in humans (Callanan et al. 1996, Hoeller et al. 2010, Visco & Finotto 2014, Valli et al. 2017). In dogs, DLBCL represents approximately 50% of lymphomas, mainly constituted by the centroblastic subtype and less frequently by the immunoblastic type (Valli et al. 2011, Vezzali et al. 2010). In contrast to what is seen in dogs, the immunoblastic type was more frequently observed than the centroblastic type in cattle in this study. However, these two subtypes show no clinical or therapeutic differences because they are morphological variants that can coexist inside the same neoplasm (Valli et al. 2016). Anaplastic lymphoma is one of the DLBCL subtypes that is less frequently observed in different species (Valli et al. 2017). In humans, this subtype is more aggressive than anaplastic T-cell lymphoma (Weisenburger et al. 2001). In the studied cattle, the T-cell rich subtype of DLBCL was the least frequent; however, this subtype is the most common in horses, accounting for approximately half of the lymphomas (Durham et al. 2013).

CONCLUSIONS

Compression of the spinal cord is the main site of compromise in cases of central nervous system lymphoma. Adult dairy cows are mainly affected and hind limb paresis is the main clinical sign.

Epidural sites are the main location of tumors which are associated with the extradural adipose tissue. Distribution of tumors is mainly multifocal and lumbar and sacral regions were most affected.

According to the WHO/REAL criteria, all extradural lymphomas were classified as mature B-cell lymphomas, and diffuse large B-cell lymphomas were the most common.

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Original Article
Small Animal Diseases

Association between decreased expression of estrogen receptor alpha, androgen receptor and phosphatase and tensin homolog immunoexpression in the canine prostate[1]

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ABSTRACT.- Kobayashi P.E., Rodrigues M.M.P., Gartner F., Rema A., Fonseca-Alves C.E. & Laufer-Amorim R. 2019. **Association between decreased expression of estrogen receptor alpha, androgen receptor and phosphatase and tensin homolog immunoexpression in the canine prostate.** *Pesquisa Veterinária Brasileira 39(1):40-46.* Departamento de Clínica Veterinária, Faculdade de Medicina Veterinária e Zootecnia, Universidade Estadual Paulista, Rua Prof. Dr. Walter Mauricio Correra s/n, Rubião Junior, Botucatu, SP 18618-970, Brazil. E-mail: renee.laufer-amorim@unesp.br

Canine prostate gland is a hormonal dependent organ and its imbalance of estrogen and androgen receptor expressions are directly associated with the development of different diseases. Due to the lack of information regarding the behavior of the aforementioned receptors in canine prostate cancer (PC), this study aimed to identify estrogen receptor alpha (ERα), androgen receptor (AR), Ki67 and phosphatase and tensin homolog (PTEN) protein expressions in canine PC by immunohistochemistry. We found nuclear expression of ERα and AR in the epithelial cells of normal canine samples and a loss of protein expression in PC samples. Normal samples showed Ki67 expression in a few basal cells and the PC samples showed the highest mean of positive cells (253.1). Canine prostate cancer showed a high proliferative index, which was associated with independence of hormonal actuation. PTEN showed positive nuclear and cytoplasmic expression in normal canine samples and a loss in PC. Loss of ERα, AR and PTEN indicated that canine PC exhibits the same immunohistochemical phenotype as in human patients with PC resistant to hormonal therapy. Therefore, canine PC should be considered as a model to study human PC resistant to hormonal therapy.

INDEX TERMS: Estrogen receptor alpha, androgen receptor, phosphatase, tensin homolog, immunoexpression, canine prostate, dogs, prostate lesions, immunohistochemistry, hormonal receptors, PTEN.

RESUMO.- [**Associação entre diminuição da expressão dos receptores de estrógeno alfa e andrógeno, e imunoexpressão de fosfatase e tensina homóloga na próstata canina.**] A glândula prostática canina é um órgão dependente de hormônio, e o desequilíbrio na expressão dos receptores de estrógeno e andrógeno estão diretamente associados com o desenvolvimento de diferentes doenças. Devido à falta de informação sobre o comportamento desses receptores no câncer prostático canino (PC), este estudo tem por objetivo identificar a expressão proteica através da técnica de imuno-histoquímica do receptor de estrógeno alfa (REα), receptor de andrógeno (RA), Ki67 e fosfatase e tensina homóloga (PTEN). Foi encontrado nas células epiteliais prostáticas normais caninas a expressão nuclear de REα e RA, e perda de expressão proteica nas amostras de PC. As amostras normais apresentaram expressão de Ki67 em poucas células basais e as amostras de PC apresentaram a maior média de células positivas (253,1). O câncer de próstata canino apresentou uma taxa alta de proliferação, o qual foi associado com a atuação independente de hormônio. As amostras de próstatas caninas normais revelaram marcação nuclear e citoplasmática da proteína PTEN e perda nas amostras de PC. A perda de REα,

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RA e PTEN indicam que as amostras de PC exibem o mesmo fenótipo imuno-histoquímico de pacientes humanos com câncer prostático resistente a terapia hormonal. Sendo assim, o PC canino deve ser considerado um modelo para estudos de câncer prostático humano resistente a terapia hormonal.

TERMOS DE INDEXAÇÃO: Estrógeno alfa, receptor andrógeno, imunoexpressão, fosfatase, tensina homóloga, próstata canina, caninos, lesões prostáticas, imuno-histoquímica, receptores hormonais, PTEN.

INTRODUCTION

Cancer is one of the most important public health concerns in the world and is the second leading cause of death (Gregory et al. 2001, Linja et al. 2001). Prostate cancer (PC) is the most prevalent cancer among men in the western world and there was a significant increase in the prevalence in the past years (Siegel et al. 2017). Hormonal receptors play a crucial role in normal prostate development and are strongly associated with the pathogenesis of human PC (Taplin et al. 1995). The most relevant hormonal receptors include the estrogen receptor (ER), and the androgen receptor (AR). The ER belongs to the nuclear receptor superfamily and is a transcriptional factor (Omoto & Iwase 2015). There are two ER subtypes: estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ) (Christoforou et al. 2014). In human PC there is no consensus whether the ERα is expressed in epithelial or stromal cells and neither if its expression is directly associated to pro tumorigenic actions during cancer development (Takizawa et al. 2015). Besides, protein expression of ERα in human PC epithelial cells is still controversial, varying to positive in 81% cases to negative (Hobisch et al. 1997, Bonkhoff et al. 1999, Linja et al. 2003). Meanwhile, studies involving androgen independent signaling in human PC, reveal more aggressive behavior, associated with higher metastatic rates and increased resistance to treatment (Mohler et al. 2004). One of the treatments option for advanced human PC is the combination of androgen ablation and radiotherapy (Lee & Cho 2017). This procedure has been associated with decreased morbidity, metastasis growth and increased survival time (Gregory et al. 2001).

In the PC development mutation or amplification of androgen receptor genes induce higher sensitivity of neoplastic cells to androgen and steroids hormones, produced by adrenal gland (Grossmann et al. 2001). In a late stage, tumors can become refractory to androgen deprivation and different pathways, related to cell growth and resistance to apoptosis, can be activated (Mohler et al. 2004). PI3K is one of these pathways associated with resistance to androgen deprivation (Zhang et al. 2009, Carver et al. 2011). Activation of PI3K pathway is associated with low expression of AR and PTEN (Tran et al. 2009, Carver et al. 2011).

Comparative oncology studies using dogs are a unique opportunity to evaluate spontaneous tumors in animal models beneficiating both species: humans and dogs. Dogs are the only mammal besides humans in which spontaneous PC develops with higher frequency (Palmieri et al. 2014, Fonseca-Alves et al. 2017). An important difference between both species is that dogs show higher incidence of androgen independent tumors than humans (Bryan et al. 2007). Neutered dogs have an increased risk of developing PC and they usually develop tumors from androgen independent cell (Lai et al. 2008).

In Veterinary Medicine, there are few studies describing the expression of hormonal receptors: ERα and AR. Additionally only two previous studies performed immunohistochemical analysis of PTEN in canine PC (Lin & Palmieri 2016, Rivera-Calderón et al. 2016). Due to the importance of the dog as a model for human prostate cancer, this research aimed to evaluate ER, ARα, Ki67 and PTEN expression in canine prostate cancer.

MATERIALS AND METHODS

Patients. PC samples were collected from a total of 26 adult dogs, age range from 1 to15 years-old, with no breed prediction. All included animals were intact. Inclusion criteria were based on the performance of necropsy within 30 minutes after death.

Histological analysis. Twenty-six paraffin-embedded blocks (FFPE) of prostate samples were selected from the archive of Veterinary Pathology Service, School of Veterinary Medicine and Animal Science, São Paulo State University (Unesp) and Institute of Biological Sciences Abel Salazar at the University of Porto, Portugal (ICBAS-UP). Hematoxylin and eosin (HE) stain slides were examined and histological diagnosis was established by three independent pathologists simultaneously, using a multi-head microscope. The diagnoses were made according to Fonseca-Alves et al. (2010) for benign prostatic hyperplasia (BPH) and prostatic intraepithelial neoplasia (PIN), proliferative inflammatory atrophy (PIA). Prostatic carcinomas (PC) were diagnosed according to Palmieri et al. (2014).

Five normal prostates, five BPH, three PINs, seven PIAs and six PCs were included in this study. The histopathological analysis of the six PCs revealed three tumors with acinar pattern, one tumor with solid pattern and one tumor with cribriform pattern.

Immunohistochemistry (IHC). Tissue sections (3μm) were transferred to glass slides positively charged (Fisherbrand-Color Frost™, Fisher Scientific, Pittsburgh, Pennsylvania). Tissue samples were subjected to immunohistochemical stain using peroxidase method and DAB. Immunohistochemistry for ER and AR and Ki67 were performed at the Veterinary Pathology Service at São Paulo State University (Unesp). PTEN immunohistochemistry was performed in the Institute of Biological Sciences Abel Salazar (ICBAS), University of Porto (Portugal). Slides were dewaxed in xylol and rehydrated in graded ethanol. For the antigen retrieval, slides were incubated in a citrate buffer (pH 6.0) for 30 minutes in a pressure cooker (Pascal®; Dako, Carpinteria, CA). Endogenous peroxidase was neutralized by hydrogen peroxide 3% in methanol for 30 minutes. Protein block and secondary antibody incubation was performed using the Novolink Polymer (Novocastra, RE7260), according to manufacture instructions. Appropriate positive and negative controls were included for all reactions.

ER, AR and PTEN immunostaining were assessed by percentage scores of labeled cells (0 = absence of staining, score 1 = 1 to 25% of positive cells, score 2 = 26 to 50% of positive cells, score 3 = 51 to 75% of positive cells, score 4 = more than 76% of positive cells). The percentages of stained cells were evaluated in five fields at high power field (HPF) to determine the proportion of positive cells. For the evaluation of Ki67 antibody, negative and positive cells were counted in a total of 1,000 cells, from each lesion, in a 40X HPF.

Statistical analysis. The Chi-square or Fisher exact test was used to determine the association between the categorical variables for immunochemistry. Statistical analysis was performed using GraphPad Prism 5 v.5.0 (GraphPad Software Inc., La Jolla, CA). Statistical significance was defined as $p<0.05$.

RESULTS

Immunohistochemical results are summarized in Table 1. For immunohistochemical score, it was considered only expression in epithelial cells. ER presented strong nuclear expression in the epithelial, basal and some stromal cells (Fig.1A). Normal prostate and BPH tissues had greater positive ER expression when compared with PIN, PIA and PC lesions. We observed a gradual reduction of ER expression among different lesions (Table 1). PIN and PIA lesions showed decreased ER expression when compared with normal prostate (p=0.01) and PC showed lower levels of ER expression (Fig.1B) (p= 0.02).

AR expression was observed in the nuclei of epithelial cells and in a few basal and stromal cells. For immunohistochemical analysis only the expression in the nuclei of epithelial cells was approached in this study. Epithelial cells showed higher expression scores in nuclei of normal epithelial cells while tumor cells had fewer cells with nuclear expression. Normal tissue and BPH showed high expression of AR (over 75% of positive cells for all samples, Fig.1C). All pre-neoplastic lesions showed AR expression score, between 50 and 75% of positive cells. Moreover, we detected loss of AR expression in PC samples (3 samples with less than 25% of positive cells and three samples negative, Fig.1D) compared to normal, BPH and pre-neoplastic lesions.

Ki67 expression in normal prostates was found in basal cells (Fig.1E). The mean average of positive cells in normal samples was 4.6 (range of 1-7). BPH samples revealed Ki67 expression in luminal epithelial and basal cells. The mean average of positive cells was 11.8 (range of 0-29). PIN, PIA and PC samples showed positive expression in epithelial and basal cells. The PC samples (Fig.1F) presented the highest mean average (253.1 to 751) of positive cells. The mean average of positive cells in PIN and PIA samples were 91.3 and 70.4, respectively.

Immunohistochemical analysis of PTEN revealed nuclear and cytoplasmic expression in normal samples. Normal prostatic tissue and BPH showed high expression of PTEN (over 75% of positive cells, Fig.1G), while pre-neoplastic lesions (PIN and PIA) had decreased protein expression and PC presented loss of expression (Fig.1H).

DISCUSSION

In this study, we used canine FFPE samples and immunohistochemical technique to determine ERα, AR, Ki67 and PTEN expression in normal prostate, BPH, pre-neoplastic lesions and PC. Differently from our findings, ERα expression in humans mainly occurs in stroma of normal prostate and less frequently in androgen-independent basal cell layer (Takizawa et al. 2015, Bonkhoff 2018). In our canine samples, normal prostatic samples and BPH had high number of ERα positive in epithelial cells (over 50% of positive cells). Moreover, normal prostatic tissue and BPH showed higher number of ERα positive cells (>50% of positive cells) than PIN, PIA and PC. Pre-neoplastic lesions showed lower number of

Table 1. Immunohistochemistry scoring of ER, AR, Ki67 and PTEN protein expression on canine prostatic lesions

Identification	Diagnosis	Estrogen receptor	Androgen receptor	PTEN	Ki67
Case 1	Normal	3	4	3	1
Case 2	Normal	3	4	4	4
Case 3	Normal	4	4	3	5
Case 4	Normal	3	4	4	6
Case 5	Normal	4	4	3	7
Case 6	BPH	3	4	3	9
Case 7	BPH	3	4	4	0
Case 8	BPH	3	4	3	29
Case 9	BPH	4	4	3	8
Case 10	BPH	4	4	4	13
Case 11	PIN	2	3	2	29
Case 12	PIN	2	3	3	43
Case 13	PIN	2	3	2	202
Case 14	PIA	0	3	2	104
Case 15	PIA	1	3	3	18
Case 16	PIA	1	3	2	36
Case 17	PIA	2	3	1	68
Case 18	PIA	2	3	2	59
Case 19	PIA	2	3	2	6
Case 20	PIA	2	3	3	77
Case 21	PC	1	1	1	0
Case 22	PC	1	1	0	53
Case 23	PC	0	0	1	647
Case 24	PC	1	0	0	751
Case 25	PC	0	0	0	68
Case 26	PC	0	0	0	0

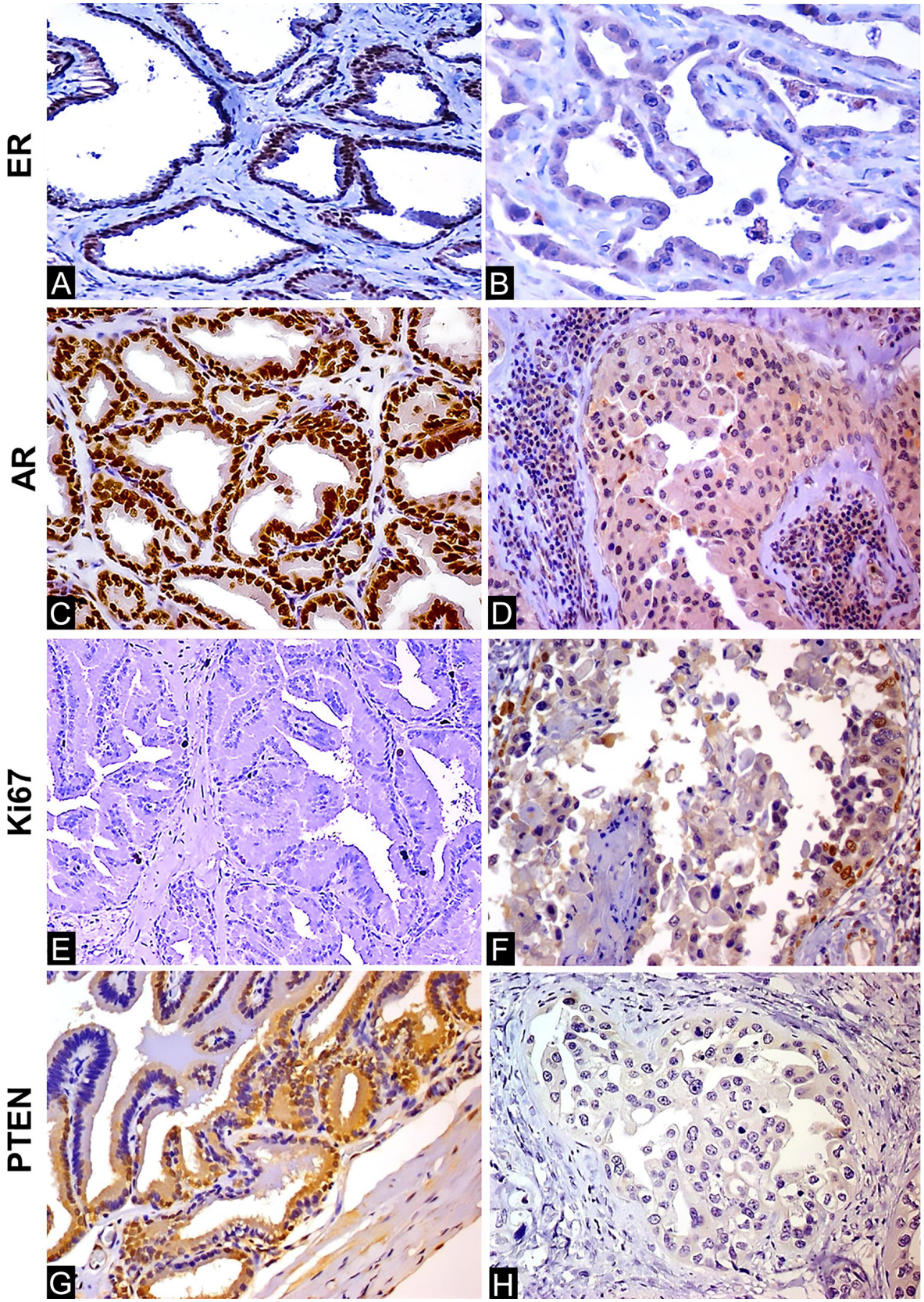

Fig.1. (**A**) ERα positive cells in BPH tissue. IHC anti-ERα, obj.10x. (**B**) Loss in prostate carcinoma samples. IHC anti-ERα, obj.40x. (**C**) High expression of nuclear AR staining in normal prostate. IHC anti-AR, obj.20x. (**D**) Loss of nuclear AR staining in prostate cancer areas (right). IHC anti-AR, obj.40x. (**E**) Ki67 immunostaining in basal cells of normal sample. IHC anti-Ki67, obj.10x. (**F**) Ki67 immunostaining increased expression in carcinoma. IHC anti-Ki67, obj.40x. (**G**) Normal prostatic tissue showing high expression of PTEN. IHC anti-PTEN, obj.20x. (**H**) Normal prostatic tissue showing loss of PTEN in PC samples. IHC anti-PTEN, obj.40x.

positive cells (<50% of positive cells) and PC samples were negative or lower expression (less than 25% of positive cells). In humans there are contradictory results involving the presence or absence of ERα in PC. However, the absence of ERα in human PC cell lines and PC metastases is usually described in patients with distant metastasis who have failed endocrine therapy (Hobisch et al. 1997, Takizawa et al. 2015). The ER methylation in human PC cell lines and prostate cancer tissues were observed and associated with tumor pathological grades, suggesting a mechanism of ER gene inactivation and consequently downregulation of ER protein in PC (Li et al. 2000). These results indicate that PC becomes independent of ERα expression, suggesting a more aggressive and unresponsive behavior towards anti-estrogens treatments.

Previous researches evaluating AR expression in canine prostate cancer showed cytoplasmic localization (Lai et al. 2009, Akter et al. 2015). However, as a nuclear receptor, its role is mainly nuclear to control transcription of specific genes (Tan et al. 2015). We found AR nuclear expression in all prostatic lesions and occasional cytoplasmic expression, but we only considered the nuclear staining. Normal prostatic tissue and BPH showed higher levels of AR expression (100% of positive cells) and PIA and PIN lesions had moderate staining of AR (50 to 75% of positive cells). Four cases of PC were negative for AR and two tumors showed lower expression (less than 25% of positive cells).

In Veterinary Medicine, there are few studies evaluating the expression of hormonal receptors in canine prostate samples using different methodologies and analysis (Jiang et al. 2005, Grieco et al. 2006, Gallardo et al. 2007). Additionally, comparative oncology studies between human and canine methodology standardization is required. We believe that canine carcinogenic process is similar to humans, although dogs show some particularities related with genetic alterations and protein localization (Alves et al. 2014).

Human PC shows multifactorial etiology such as diet, race, gene alterations and hormones. Estrogens and androgens hormones play essential role in cellular growth and differentiation control of normal prostatic tissue (Cooney 2017). Previous epidemiological and experimental results suggested a relationship among estrogen levels, PC carcinogenesis and cancer progression (Asgari & Morakabati 2011). Neutered dogs show higher risk of PC development and more aggressive histological subtypes when compared to humans (Teske et al. 2002, Bryan et al. 2007). Thus, is a hypothesis that canine PC shows local aggressive behavior, high metastatic rate and unresponsive to chemotherapy and radiotherapy (Gallardo et al. 2007). Our results of loss of ERα and AR immunohistochemical expression in canine PC indicate that hormone-based therapies (such as tamoxifen or androgen ablation) is not an optimal therapy for dogs. Therefore, dogs are a unique model to study drug response associated to hormonal independent PCs.

We found none immunohistochemical staining of Ki67 in three prostatic samples (one BPH and two PC). In these samples, we did not detect positive internal control, so these cases were not included for Ki67. One PC sample negative to Ki67 was positive to AR and PTEN and the other one was positive to ERα, AR and PTEN indicating that the Ki67 protein is most sensitive to fixation time. PC showed higher proliferative index when compared to normal, BPH and preneoplastic lesions.

Ki67, a nuclear based protein and proliferation marker, is reported as predictive factor of human PC progression (Tollefson et al. 2014, Ma et al. 2018). Our study confirmed previous findings demonstrating association between high Ki67 index and canine PC (Rodrigues et al. 2013, Fonseca-Alves et al. 2017). However, further studies are necessary to determine the role of Ki67 as a predictive marker in canine PC.

Our previous results evaluating copy number abnormalities (CGH) in canine prostatic lesions showed loss of *AR* and *PTEN* genes in 21,5% of canine PC (unpublished data). This previous result associated with our immunohistochemical data show a strong indication that AR loss play important role in PC development.

PTEN is a tumor suppressor gene and controls PI3K signaling pathway (Carver et al. 2011). PI3K pathway is associated with AR nuclear signaling and human studies had shown loss of AR and PTEN in PC (Scher & Sawyers 2005). These findings demonstrate the independence of androgen signaling for cell proliferation associated with activation of PI3K pathway in human PC subject to androgen deprivation therapy. No previous research evaluated PTEN immunohistochemical expression in canine prostate cancer. We found strong nuclear and low cytoplasmic staining of PTEN in normal canine and BPH samples (>50% of positive cells) and loss of nuclear staining in all PC samples in which only two PC had also shown weak cytoplasmic expression.

We believe that high proliferative index of canine PC may be associated with ERα, AR and PTEN losses, which are important proteins related to cell proliferation and differentiation control in canine PC and mediate the independence of hormonal stimulation for tumor initiation (Amorim et al. 2014).

CONCLUSIONS

Canine prostate cancer shows high proliferative index and is associated with independence of hormonal action.

Loss of ERα, AR and PTEN in canine PC exhibits the same immunohistochemical phenotype of human patients with PC resistant to hormonal therapy.

Expression of Ki67 was significantly up regulated in malignant lesions compared to normal, BPH and pre neoplastic lesions associated with prostate cancer progression.

Canine PC should be considered as an interesting model to study human prostate cancer resistant to hormonal therapy.

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Original Article
Small Animals Diseases

Neuron-specific enolase as biomarker for possible neuronal damage in dogs with distemper virus[1]

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ABSTRACT.- Elias B.C., Alfieri A.F., Navarro I.T. & Gomes L.A. 2019. **Neuron-specific enolase as biomarker for possible neuronal damage in dogs with distemper virus.** *Pesquisa Veterinária Brasileira 39(1):47-51*. Departmento de Clínicas Veterinárias, Faculdade de Medicina Veterinária, Universidade Estadual de Londrina, Rodovia Celso Garcia Cid PR-445 Km 380, Cx. Postal 10.011, Campus Universitário, Londrina, PR 86057-970, Brazil. E-mail: lagomes@uel.br

Neuron-specific enolase (NSE) is a biomarker of neuronal cell lysis, which demonstrates stability in extracellular fluids such as blood and cerebrospinal fluid. To the authors knowledge there is no research information comparing the use of NSE in dogs with and without encephalitis, putting in evidence the importance of that biomarker to detect neuronal damage in dogs. The objective was to compare the serum NSE levels in dogs with and without encephalitis, and to determine the serum NSE levels in normal dogs. Thirty eight dogs were evaluated, 19 dogs with encephalitis (EG Group) and 19 dogs without encephalitis (CG Group). The criteria for inclusion in the EG Group were presence of neurological signs in more than one part of the CNS (multifocal syndrome) and positive molecular diagnosis for canine distemper virus; for the CG Group were an age between 1 to 7 years and be clinically normal; NSE were measured in serum using an ELISA assay, and the results were compared. In the EG Group the NSE values were higher with significant difference (P=0.0053) when compared with the CG Group. NSE is a biomarker that can be measured in serum samples of dogs to monitor neuronal lesions in encephalitis.

INDEX TERMS: Neuron-specific enolase, neuronal damage, dogs, distemper virus, brain, biomarkers, neurology, inflammation, central nervous system.

RESUMO.- [**Enolase neuronal específica como um possível biomarcador para lesão neuronal em cães com cinomose.**] Enolase neuronal específica (NSE) é um biomarcador de lise de neurônios, que demonstra estabilidade em fluidos extracelulares como sangue e líquido cerebrospinal. Para o conhecimento dos autores, não há informações de pesquisa que comparem o uso de NSE em cães com e sem encefalite, evidenciando a importância desse biomarcador para detectar danos neuronais em cães. O objetivo foi comparar os níveis séricos de NSE em cães com e sem encefalites, e determinar os níveis séricos de NSE em cães saudáveis. Trinta e oito cães foram avaliados, 19 cães com encefalites (Grupo EG) e 19 cães sem encefalite (Grupo CG). O critério para inclusão no Grupo EG foi presença de sinais neurológicos em mais de uma estrutura do SNC (síndrome multifocal) e positividade no diagnóstico molecular para o vírus da cinomose canina; para o Grupo CG foi idade entre 1 e 7 anos e ser clinicamente normal; NSE foram mensuradas em amostras séricas usando o método de ELISA, e os resultados comparados. No Grupo EG os valores de NSE foram altos com diferença significativa (P=0.0053) quando comparado com o Grupo CG. NSE é um biomarcador que pode ser mensurado em amostras séricas de cães para monitorar lesões neuronais em encefalites.

TERMOS DE INDEXAÇÃO: Enolase neuronal, lesão neuronal, caninos, cinomose, cérebro, biomarcadores, neurologia, inflamação, sistema nervoso central.

INTRODUCTION

Neuron-specific enolase (NSE) is an intracytoplasmic glycolytic enzyme found in neurons and in neuroendocrine cells (Marangos & Schmechel 1987) and is considered a major neuronal injury biomarker in human medicine (Yokobori et al. 2013). However,

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there are few studies on NSE in veterinary medicine (Nishida 2014). This enzyme can be found in cerebrospinal fluid (CSF) and serum, and several studies are currently investigating whether it is a predictive prognosis factor in human medicine (Chabok et al. 2012, Fendler et al. 2015, Xue et al. 2015). In cases of CNS inflammation in human beings, NSE were elevated either in serum as in CSF, but the values were superior for CSF in comparison (Lima et al. 2004). Erythrocytes and platelets also express NSE; therefore, hemolysis can cause alterations in NSE levels in the serum and this can be a condition to false positive results when NSE is elevated in serum samples (Day & Thompson 1984, Planche et al. 2010).

In a study comparing the results of proteomic analysis in CSF from healthy dogs and dogs with meningoencephalitis of unknown origin, NSE was present in both groups but the meningoencephalitis group demonstrated increased levels (Nakamura et al. 2012). However, these results do not suggest that the biomarker can be used as a diagnostic method since NSE is related to encephalitis and not specifically to etiology. A study assessing biomarkers in dogs with gangliosidosis, a neurodegenerative disease with a progressive chronic nature, showed higher NSE values in CSF of the disease group compared to controls, suggesting that the biomarker was efficient to monitor brain lesions (Satoh et al. 2007). Although most studies evaluating NSE levels in dogs use CSF samples, one study used the presence of biomarkers in serum and CSF samples to assess brain damage in dogs undergoing cardiac arrest. They observed that NSE values in CSF and serum are elevated one and two hours after the reestablishment of circulation, respectively (Usui et al. 1994).

In Brazil, the prevalence of canine distemper in dogs with encephalitis is high (Fighera et al. 2008, Sonne et al. 2009). The CNS disease manifestations can be variable and are named as young dog encephalitis, adult dog encephalitis, old dog encephalitis, post-vaccination encephalitis, and poliencefalite with corpuscle inclusion of distemper (Headley & Graça 2000, Headley et al. 2009, 2012). The neurological symptoms caused by the virus may be variable according to the location of the lesion (Summers et al. 1984) and the immune response (Beineke et al. 2009).

In this study, it was hypothesized two points: the first one is that dogs with distemper encephalitis express NSE in the blood; the second one is that dogs with encephalitis have increased serum levels of NSE when compared to normal dogs, indicating that NSE may be a potential biomarker that can be used to identify and monitor encephalitis. Thus, the aims of this study were to evaluate the levels of NSE in serum samples of dogs with and without encephalitis and to compare the values obtained.

MATERIALS AND METHODS

Ethics statement. In this study were included a total of 38 dogs from a Veterinary Teaching Hospital in South Brazil (*Canis familiaris* Linnaeus, 1758) and were separated into two groups: dogs without encephalitis denominated as control group (CG), and dogs with encephalitis denominated as encephalitis group (EG), each one with 19 dogs. The study was approved by the Ethics Committee from the University.

Study design. The inclusion criteria for dogs in the CG Group were age between 1 and 7 years, no restrictions about sex or breed, normal clinical examination and laboratory tests (blood count, creatinine, BUN, glucose, alanine aminotransferase, alkaline phosphatase). For all dogs in this group, serum samples were obtained for NSE measurement. For the EG Group, the inclusion criteria were no restriction about sex or breed, presence of neurological signs compatible, as well as be positive for canine distemper virus using molecular diagnostic (RT-PCR for the N gene of canine distemper virus). For EG Group was obtained venous blood sample, urine when available, and a CSF tap from the cisterna magna only for dogs that were euthanized by the owner's request. The time taken for all procedures for material storage and processing did not exceed 60 min. No serum samples that presented hemolysis were included.

Measurement of serum NSE. To determine the serum values of NSE by ELISA, the samples were stored at -80°C. Processing of samples, as well as standards and control samples of the kit, was performed in duplicate in a single step following the guidelines of the human Neuron-Specific Enolase commercial test (ALPCO®, NSEHU-43-E01).

Distemper virus encephalitis characterization and diagnosis. For diagnosis of encephalitis, a neurological examination was performed to identify neurological signs compatible with multifocal syndrome. The dogs with neurological signs should be positive to distemper virus PCR test (Fig.2). The presence of the virus was confirmed by RT-PCR (primer designed to amplify the N gene for canine distemper) using urine or blood samples in EDTA. All samples were stored at -80ºC (Boom et al. 1990, Frisk et al. 1999, Amude et al. 2006).

Statistics. The statistical analysis was performed using the *Kruskal-Wallis* test to evaluate the differences between the NSE mean for the CG and EG Groups, with the significance level set at 5% ($p<0.05$). A t-test was used to analyze the other comparisons with the same significance level.

RESULTS

NSE values of serum samples

The ELISA test developed to be used for human samples demonstrated to be efficient to detect NSE in dog's serum samples.

Comparison of NSE values between the groups showed a significant difference (P=0.0053), as shown in Figure 1. For the CG Group, NSE results were as follows: mean ± SD = 10.43±5.94ng/ml, median 8.53ng/ml. For the EG Group, the values were as follows: mean ± SD = 71.73±104.2ng/ml, median = 26ng/ml (Table 1). When comparing the NSE values between individuals of the same group, there was no significant difference between males (CG: mean ± SD = 11.84±7.39ng/ml, median = 7.39ng/ml; EG: mean ± SD = 44.62±64.46ng/ml, median = 20.3ng/ml) and females (CG: mean ± SD = 9.62±5.09ng/ml, median 8.88ng/ml; EG: mean ± SD = 87.54±121.49ng/mL, mean = 34.29ng/ml; CG: P = 00:50; EG: P = 0.33).

Neurological findings

All dogs with encephalitis exhibited more than one neurological signs corresponding to more than one affected neuroanatomical structure and was therefore classified as a multifocal syndrome. From the dogs in EG Group 6/19 (32%) had lesions in the forebrain and brainstem; 4/19 (21%) had lesions in the forebrain, brainstem and spinal cord; 3/19 (16%) had lesions in the brain stem and spinal cord; 2/19 dogs (11%) had lesions in the forebrain and spinal cord; 2/19 (11%) had lesions in the forebrain, cerebellum and spinal cord; 1/19 (5%) had lesions in the forebrain, brain stem, and cerebellum; and 1/19 (5%) had lesions in the forebrain and

cerebellum (Table 2). Urine samples revealed that all dogs were positive for canine distemper.

CSF taps were obtained from 7/19 (37%) dogs whose owners requested euthanasia, and results demonstrated elevated protein levels in all cases and lymphocytic pleocytosis in 5/7 (71%) dogs (Table 2).

DISCUSSION

Dogs with encephalitis had significantly higher NSE values compared to dogs without encephalitis (p=0.0053), demonstrating that NSE is a potential biomarker that can be used to detect and monitor encephalitis in dogs like the same used for humans with encephalitis. The results in this work indicated that the method used to detect NSE for humans was also effective in the measurement of NSE in dog's serum samples. Regarding to this it is important to emphasize that serum NSE can be used as an indicator of brain damage in dogs with encephalitis. This result is in agreement with a study conducted by Lima et al. (2004) when the authors tested the expression of NSE in serum samples of human beings with encephalitis and detected that this biomarker was present in high levels when compared to patients without this problem. Besides that, the ELISA test developed to be use for human samples demonstrated to be efficient to detect NSE in serum samples of dogs, which represents a cheap method to be used in veterinary practice.

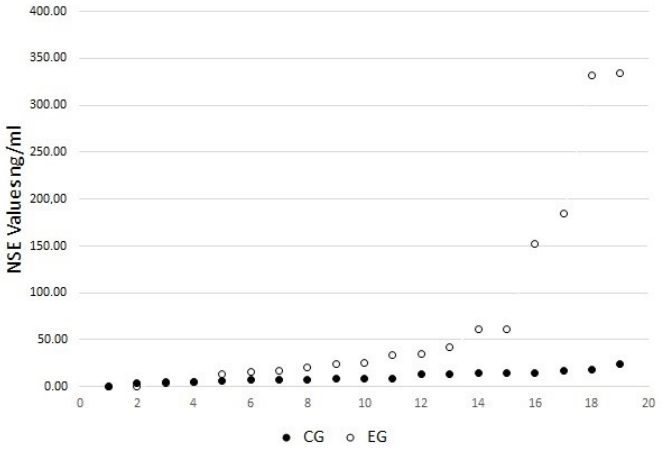

Fig.1. Scatter chart of the neuron-specific enolase (NSE) values of the groups with and whithout encephitis. Control group (CG), encephalitis group (EG).

Table 1. Summary of the statistical analysis of the serum samples of CG and EG dogs

Group	NSE (ng/ml)		P
	Mean +- SD	Median	
GS	10.43 +- 5.94	8.53	0.005299
GE	71.73 +- 104.2	26	

CG = Group without encephalitis, EG = encephalitis group, +- SD = standard deviation, P = value of p usually *Kruskal-Wallis* test

In the herein study, the elevation in NSE serum levels was resultant of the brain inflammation (encephalitis) which occurred naturally by distemper virus infection. Considering this, it seems to be clear that the detection of NSE in serum is an effective tool for identification of brain inflammation in dogs in the majority of the cases.

Another important advantage in measuring NSE in the serum is the noninvasive characteristic of this procedure, which only requests a simple blood sample when compared to CSF collection. Regarding to this, measurement of NSE in serum can be a safe method to evaluate patients with encephalitis progressively, similar to what Satoh et al. (2007) and Nakamura et al. (2012) did using CSF samples, thus, representing to be a good alternative.

In a study with human, it was found that NSE was only detected in cases of injury to gray matter, but not in cases of injury to white matter (Marangos & Schmechel 1987). Based on this, it is possible to suggest that herein in this study; the elevation of NSE in serum was associated to damage to the gray matter due to the presence of distemper virus. In addition, NSE release in the blood is associated with a neuronal death process (Marangos & Schmechel 1987), and subsequent overflow to the CSF and later in the blood (Usui et al. 1994). While biokinetic concentration is associated with the time required for clearance of blood biomarkers (Usui et al. 1994), thus, acute cases may be associated with higher and increasing concentrations of NSE (Choi et al. 2016), once chronic diseases may not result in changes due to the amount of protein released (Satoh et al. 2007). Regarding to this, the results herein for dogs with high serum levels of NSE might be suggest that the patients were in the acute phase of the disease.

In EG, there was a high standard deviation, the highest NSE value was 334.16ng/ml and the lowest was 0ng/ml.

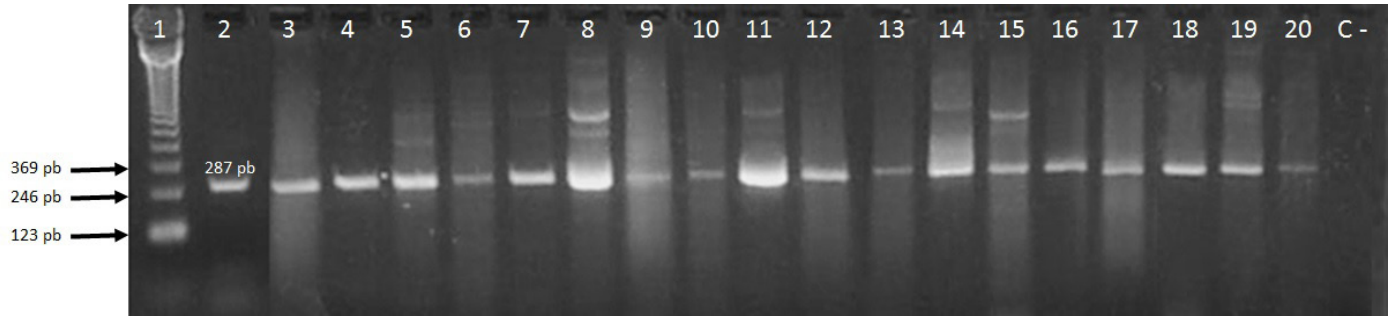

Fig.2. RT-PCR assay designed to amplify a 287 bp fragment of CDV N gene. Ladder 123bp, Invitrogen™ Life Technologies®, USA (lane 1). Urine samples (lanes 2-12). Whole blood samples (lanes 13-20). Negative control using ultrapure water treated with diethylpyrocarbonate (DEPC), Invitrogen™ Life Technologies®, USA (line C-).

Table 2. Signalment, neuroanatomical diagnosis, CSF analysis and NSE values of the EG Group

Signalment	Neuroanatomical Diagnosis	CSF	NSE
1,8 year, F, SRD	Multifocal - Prosencephalon, brainstem and spinal cord	Lymphocitic Pleocytosis + Hyperproteinorrachia	0.00
1,8 year, F, SRD	Multifocal – Prosencephalon and cerebellum	Hyperproteinorrachia	0.00
6 months, M, CBD	Multifocal – Prosencephalon and spinal cord		3.78
2 years, M, Fila Brasileiro	Multifocal - Prosencephalon, brainstem and spinal cord		4.99
6 years, M, CBD	Multifocal - Prosencephalon, spinal cord and cerebellum	Lymphocitic Pleocytosis + Hyperproteinorrachia	13.53
2,5 years, F, CBD	Multifocal - Brainstem and spinal cord		16.44
1,1 year, F, CBD	Multifocal - Prosencephalon, brainstem and spinal cord		16.63
10 years, M, CBD	Multifocal - Brainstem and spinal cord	Lymphocitic Pleocytosis + Hyperproteinorrachia	20.30
7 years, M, Boxer	Multifocal – Prosencephalon and brainstem		24.61
10 years, F, CBD	Multifocal - Prosencephalon and brainstem		26.00
7 years, F, CBD	Multifocal - Prosencephalon and brainstem		33.47
2,5 months, F, CBD	Multifocal - Prosencephalon, brainstem and Cerebellum	Lymphocitic Pleocytosis + Hyperproteinorrachia	35.11
1, 4 years, F, CBD	Multifocal - Brainstem and spinal cord	Hyperproteinorrachia	42.46
1, 7 years, M, Labrador	Multifocal - Prosencephalon and brainstem		61.00
2 year, F, CBD	Multifocal - Prosencephalon, brainstem and spinal cord		61.51
1 year, F, CBD	Multifocal - Prosencephalon, spinal cord and cerebellum		152.84
2,5 years, M, CBD	Multifocal - Prosencephalon and spinal cord		184.16
1 year, F, Lhasa Apso	Multifocal - Prosencephalon and brainstem		331.89
5 months, F, CBD	Multifocal - Prosencephalon and brainstem	Lymphocitic Pleocytosis + Hyperproteinorrachia	334.16
			X = 71.73

EG = Encephalitis group, CSF = cerebral spinal fluid, NSE = neuronal específic enolase, CBD = crossbreed, F = female, M = male.

An explanation for this lower value would be the possible chronicity in the evolution of brain inflammation, reinforcing the hypothesis that false negative results in serum measurement of serum NSE may occur in dogs with neurological signs characterized by encephalitis, thus characterizing one of the limitations of the test.

In this study, CSF samples were obtained only from dogs that the owner requested euthanasia because of the poor prognosis related to the neurological signs. The CSF samples would be a good form to compare the results obtained by the NSE samples, however, there are at least two limitations regarding to this: the first one is related to ethical aspects, once would not be possible to obtain a CSF tap from the dogs without encephalitis (considered normal) in the CG; the second one, which is a consequence of the first one and is related to a lack of results to compare the NSE values in the CSF tap from the dogs with encephalitis in the EG.

CONCLUSION

The test used was able to detect neuron-specific enolase (NSE) in serum samples of dogs and that the values were increased in most of the EG Group animals when compared to CG Group. False-negative results may also occur.

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

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Original Article
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Use of different fixation times and application of two immunohistochemical methods for detection of KIT and Ki67 proteins in canine cutaneous mast cell tumors[1]

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ABSTRACT.- Santos A., Nascimento H.H.L., Flores M.M. & Kommers G.D. 2019. **Use of different fixation times and application of two immunohistochemical methods for detection of KIT and Ki67 proteins in canine cutaneous mast cell tumors.** *Pesquisa Veterinária Brasileira 39(1):52-60*. Laboratório de Patologia Veterinária, Departamento de Patologia, Universidade Federal de Santa Maria, Camobi, Santa Maria, RS 97105-900, Brazil. E-mail: glaukommers@yahoo.com

Due to the high prevalence of mast cell tumors (MCTs) in the diagnostic routine, several factors, especially prognostic, have been sought to determine the biological behavior of these neoplasms. Immunohistochemistry (IHC) is one of the main tools utilized to biologically differentiate more aggressive tumors from less aggressive ones. However, some immunostainings are influenced by formalin fixation, interfering with the results. This is both a retrospective and prospective study of MCTs diagnosed in laboratory routine. A total of 25 samples, without knowledge about fixation time, were analyzed in the retrospective study, whereas 12 samples, with known fixation times, were assessed in the prospective study. Two histologic grading systems (Patnaik and Kiupel), special staining of toluidine blue, and IHC for KIT and Ki67 proteins were applied in both studies. Additionally, two amplification systems (biotinylated and non-biotinylated) for Ki67 protein and counting of the argyrophilic nucleolar organizing regions (AgNOR method) were tested in the prospective study. In the retrospective study, greater agreement between the evaluating pathologists was observed when the Kiupel system was used. IHC staining for KIT protein was effective in both studies, regardless of fixation time. IHC staining for Ki67 protein was highly sensitive to formaldehyde, and staining failure was observed in 56% of the cases in the retrospective study. In the prospective study, samples fixed for longer than 24 hours showed a reduction in the number of stained cells (altering the determination of the cell growth fraction) or showed absence of IHC staining in both amplification systems. The use of the AgNOR method to evaluate the rate of cell proliferation may be an alternative when the fixation time of the neoplasm is unknown or longer than 24 hours.

INDEX TERMS: Immunohistochemistry, KIT protein, Ki67 protein, cutaneous mast cell tumors, histologic grading, dogs.

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RESUMO.- [**Utilização de diferentes tempos de fixação e métodos imuno-histoquímicos na detecção das proteínas KIT e Ki67 em mastocitomas cutâneos caninos.**] Devido a alta prevalência dos mastocitomas cutâneos caninos (MCCs) na rotina diagnóstica, vários fatores, especialmente fatores prognósticos, têm sido buscados para auxiliar na determinação do comportamento biológico desse neoplasma. A imuno-histoquímica é uma das principais ferramentas

empregadas para diferenciar tumores biologicamente mais agressivos de tumores menos agressivos. Entretanto, algumas imunomarcações sofrem influência pela fixação em formol, interferindo nos resultados. Este estudo compreendeu avaliar através de uma etapa retrospectiva e uma etapa prospectiva casos de MCCs diagnosticados na rotina laboratorial. Um total de 25 amostras, sem conhecimento do tempo de fixação, foi analisado no estudo retrospectivo e 12 amostras, com tempos de fixação conhecidos, no estudo prospectivo. Foram aplicados nos dois estudos, dois sistemas de graduação histológica (Patnaik e Kiupel), a coloração especial de azul de toluidina e a imuno-histoquímica para as proteínas KIT e Ki67. Adicionalmente, no estudo prospectivo, foram testados dois sistemas de amplificação (biotinilado e não biotinilado) para a proteína Ki67 e a técnica de AgNOR (contagem das regiões organizadoras nucleolares argirofílicas). Na etapa retrospectiva, observou-se uma maior concordância entre os patologistas avaliadores quando o sistema Kiupel foi utilizado. A imunomarcação para KIT se manteve eficaz em ambos os estudos, independentemente do tempo de fixação. A imunomarcação para o Ki67 mostrou-se altamente sensível ao tempo de fixação em formol, sendo observada falha na imunomarcação em 56% dos casos do estudo retrospectivo. No estudo prospectivo, constatou-se que amostras fixadas por mais de 24 horas em formol apresentaram redução na quantidade de células imunomarcadas (alterando a determinação da fração de crescimento celular) ou apresentaram ausência de imunomarcação em ambos os sistemas de amplificação. A utilização do método AgNOR, para avaliar a taxa de proliferação celular, pode ser uma alternativa quando o tempo de fixação do neoplasma for desconhecido ou superior a 24 horas.

TERMOS DE INDEXAÇÃO: Imuno-histoquímica, proteína KIT, proteína Ki67, mastocitoma cutâneo, graduação histológica, cães.

INTRODUCTION

Cutaneous mast cell tumors (MCTs) are among the most prevalent malignant neoplasms affecting the skin of dogs (Patnaik et al. 1984, Goldschmidt & Hendrick 2002, Romansik et al. 2007, Kiupel 2017). Despite the differences in epidemiology, it is worth noting that the biological behavior of MCTs is highly variable (Webster et al. 2007, Gross et al. 2009).

Histologic grading has been used in an attempt to predict the biological behavior of MCTs for decades (Bostock 1973, Patnaik et al. 1984, Kiupel et al. 2011). Patnaik et al. (1984) suggested the grading classification most commonly used and applied in veterinary pathology laboratories until recently. According to this system, MCTs are divided into three grades: grade I (well-differentiated), grade II (intermediately differentiated), and grade III (poorly differentiated). Although widely used, this method is considered as of difficult application by some authors (Northrup et al. 2005a, 2005b), mainly because it includes a large number of subjective histologic criteria.

Owing to criticisms of the Patnaik system (PS), a new grading method was proposed in 2011 (Kiupel et al. 2011). This new method is considered easier to apply due to: (1) use of fewer histologic criteria; (2) lower subjectivity of the criteria used; (3) classification of tumors into only two groups: high and low grade. Despite the advantages of this method, ancillary techniques must be used together with histologic grading in an attempt to predict the biological behavior of MCTs (Kiupel et al. 2011).

In parallel, immunohistochemistry (IHC) and polymerase chain reaction (PCR) have been used to assist with determining the prognosis of MCTs (Kiupel et al. 2004, Webster et al. 2006, 2007, Kandefer-Gola et al. 2015). Using IHC, especially with antibodies that detect KIT (tyrosine kinase membrane receptor) and Ki67 (marker of cell proliferation) proteins, it is possible to identify the tumors that are most likely to be biologically more aggressive (Webster et al. 2007, Strefezzi et al. 2010, Flores et al. 2016, Sledge et al. 2016). PCR has been used in the search for mutations in the c-KIT gene, which present prognostic value and influence the therapeutic approach to each patient (Webster et al. 2006, Kiupel 2017). In addition to histologic grading, with application of IHC as well as PCR, it is possible to obtain a more reliable assessment of the biological behavior of MCTs, which directly influences the choice of therapy (Kiupel et al. 2004, Webster et al. 2006, Sledge et al. 2016, Kiupel 2017).

Despite the existence and relevance of the complementary prognostic tools previously discussed, the influence of tissue fixation time on formaldehyde is an important concern, and may hinder the prognostic evaluation of MCTs in a diagnostic routine. The most widely used tissue fixative in histopathology is formaldehyde, which is usually used as 10% neutral buffered formalin. This cross-linking fixative preserves the architecture of tissue, especially the peptides and cellular organelles, avoiding its degradation. In contrast, prolonged fixation causes changes in the conformation of macromolecules, which may hinder or even prevent the recognition of proteins (antigens). Antigen retrieval (enzymatic or heat-induced), a method applied for IHC, may partially or fully reverse the deleterious effects of prolonged formalin fixation (Ramos-Vara & Miller 2014). Moreover, the use of different amplification systems in IHC can lead to an optimized immunostaining method for certain antigens (Galiza et al. 2014).

In this context, this study aimed mainly at (1) assessing the efficacy of immunostaining for KIT and Ki67 proteins in MCTs classified according to the Patnaik and Kiupel systems and relating it to formalin fixation time; (2) testing two different IHC protocols (with variations in the amplification system) in an attempt to determine possible differences for each of the two antibodies used. The purpose of this study is to generate knowledge about the technical aspects of IHC for KIT and Ki67 proteins, providing subsidies for the application of this technique in diagnostic routine, particularly in Brazilian laboratories, which present difficulties regarding variation in the fixation times of the MCT samples received.

MATERIALS AND METHODS

Retrospective study

Histologic grading and histochemical evaluation. The retrospective study sample was composed of 25 cases of cutaneous mast cell tumors (MCTs) diagnosed in dogs at the Veterinary Pathology Laboratory of the Federal University of Santa Maria from January 2011 to January 2016. In addition to the diagnosis, the inclusion criteria comprised availability of paraffin blocks for each selected case. Two slides of each case (3µm-thick histologic section) were prepared: one slide was stained using the hematoxylin and eosin (HE) technique and the other was stained using the toluidine blue (TB) method, for detection of metachromatic granules in the cytoplasm. Measurement

classified cytoplasmic granules into accentuated, moderate, and discrete. Subsequently, the HE slides were submitted to the Patnaik (grades I, II, and III; Patnaik et al. 1984) and Kiupel (high and low grade; Kiupel et al. 2011) histologic grading methods independently by three pathologists (*AS*, *MMF*, and *GDK*). Aiming to reduce analysis subjectivity, the evaluators received a table with all the histologic criteria to be assessed for each system according to the respective literature. Subsequently, the cases with divergence were discussed, and a consensus table was compiled with a single grading of each method, Patnaik (PS) and Kiupel (KS) systems, for each neoplasm.

Immunohistochemistry (IHC). IHC was performed on the 25 MCTs using the primary rabbit polyclonal anti-human KIT antibody (CD117, Dako Cytomation, A4502) and the primary mouse monoclonal anti-human Ki67 antibody (clone MIB-1, Dako Cytomation, M7240). The methods used for KIT and Ki67 proteins were adapted from Kiupel et al. (2004) and Webster et al. (2007), respectively. Tissue sections were submitted to the IHC protocols described in Table 1. In all protocols, endogenous peroxidase activity was blocked with 3% hydrogen peroxide 2x for 10 min, and blocking of non-specific reactions was performed with protein blocker (EP-12-20532, EasyPath) at room temperature (25°C) for 10 min. After quick washing with distilled water, the one-stage non-biotinylated amplification system (HRP-polymer, EasyLinkOne, EP-12-20502, EasyPath) was incubated at room temperature for 20 minutes. Counterstain was performed with Harris hematoxylin. Cases of MCT previously submitted to IHC for KIT protein by Flores et al. (2016) were used as positive controls. A section of the analyzed tissue incubated only with the antibody diluent (phosphate buffered saline with Tween® 20-Sigma - PBST) was used as negative control of each case.

Classification of KIT antigen was performed as in Kiupel et al. (2004), according to localization of immunostaining in the tumor mast cells, with tumors divided into three patterns: pattern I, membrane-associated, with little to no cytoplasmic staining; pattern II, intense cytoplasmic staining with focal or stippled distribution; pattern III, diffuse cytoplasmic staining of neoplastic mast cells. Intensity, number, and distribution of the immunostained cells were also analyzed for this immunomarker. Classification of Ki67 protein was performed according to Webster et al. (2007), that is, after identification of the grid area with the largest number of immunostained neoplastic cells (nuclear dotted black), the number of immunopositive cells present was counted over five distinct high-power fields (HPF; 40x) by two examiners and subsequently averaged to obtain the growth fraction, with MCTs divided into two groups: low (LGF; <23) and high (HGF; ≥23) growth fraction.

Prospective study

Histologic grading and histochemical evaluation. The retrospective study sample was consisted of 12 cases of cutaneous mast cell tumors (MCTs) diagnosed in dogs at the LPV-UFSM from August 2016 to May 2017. The neoplasms were separately fixed (in 10% neutral formalin) at the following fixation times: 24, 48, 72, and 96 hours. All samples were routinely processed for histopathology and embedded in paraffin. Only the samples that underwent 24h fixation were sectioned at 3µm and stained according to the hematoxylin and eosin (HE), toluidine blue (TB), and count of the argyrophilic nucleolar organizing regions (AgNOR) techniques. Subsequently, histologic grading was applied according to PS and KS, as previously described in the retrospective study.

For AgNOR staining, the 3µm-thick histologic sections were deparaffinized and hydrated in deionized water. They were then stained in a solution containing a mixture of 2% gelatin and 1% formic acid in deionized water and 50% silver nitrate solution at a ratio of 1:2, respectively. The slides were immersed in this solution at room temperature for 30 minutes. After that, they were washed with deionized water for 1 min, dehydrated, clarified, and assembled with synthetic mounting medium (Entellan new, Merck) (Bostock et al. 1989). The AgNORs were manually counted according to Bostock et al. (1989); to define the frequency of AgNOR, the black dots were counted within the nuclei in 100 randomly selected neoplastic mast cells throughout the tumor at 1000x magnification. Frequency was determined by dividing the AgNOR number by 100.

Immunohistochemistry (IHC). Two amplification systems were used: non-biotinylated (HRP-polymer; EasyLinkONE) and biotinylated (LSAB+System-HRP [Dako]). The 12 samples were tested with the same antibodies used in the retrospective study (KIT and Ki67) at 24, 48, 72, and 96h fixation times (Table 1). For the biotinylated system, after incubation with the primary antibody, the biotinylated secondary antibody (stage 1) was incubated at room temperature for 30 minutes. After washing with PBST (2x for 5 min), stage 2 consisted of incubation at room temperature for 30 min with the streptavidin-biotin-peroxidase complex. Interpretation of immunostaining for the primary antibodies followed the same criteria previously described in the retrospective study.

Table 1. Description of protocols performed to optimize immunohistochemistry with anti-KIT and anti-Ki67 antibodies

	Ab[c]	Antigen retrieval [h]	Dilution[d]	Incubation (time/ temperature)	Secondary antibody/ amplification	Substrate-Chromogen
R[a]	Anti-KIT	Tris-EDTA (pH 9.0)	1:200	1h/37C°	Non-biotinylated[e]	DAB[g]*
	Anti-Ki67	Citrate (pH 6.0)	1:50	1h/37C°	Non-biotinylated	DAB*
P[b]	Anti-KIT	Tris-EDTA (pH 9.0)	1:200	1h/37C°	Non-biotinylated	DAB*
	Anti-Ki67	Citrate (pH 6.0)	1:50	1h/37C°	Biotinylated[f] Non-biotinylated	DAB*

[a] R = retrospective, [b] P = prospective, [c] Ab = antibody, [d] antibody diluted in PBST (phosphate buffered saline with Tween® 20-Sigma), [e] HRP-polymer (EasyLink-One; EasyPath), [f] LSAB+System HRP = secondary antibody LSAB+System HRP and streptavidin-biotin-peroxidase complex (Dako), [g] DAB = liquid DAB (3,3' diaminobenzidine) + Substrate-Chromogen System (Dako), [h] antigen retrieval time of 10-15 min in maximal power microwave oven; * counterstained with Harris hematoxylin.

RESULTS

Retrospective study

Histologic grading and histochemical evaluation. Interrater agreement between the three evaluators was 88% (22/25) and 96% (24/25) for the Patnaik (PS) and Kiupel (KS) systems, respectively. Mitotic count in one case was a divergent aspect in the SK. Application of grading (consensus) according to the PS showed that 96% (24/25) of the neoplasms were grade II and 4% (1/25) of them were grade III.

Application of grading according to the KS resulted in 84% (21/25) of mast cell tumors (MCTs) with low grade (Fig.1A) and 16% (4/25) with high grade (Fig.1B). Of the 24/25 MCTs classified as grade II by the PS, 21/24 were low grade and 3/24 were high grade when the SK was applied. The only grade III (PS) case was classified as low grade (KS).

In the toluidine blue (TB) technique, the MCTs metachromatically stained (in purple) the cytoplasmic granules markedly in 52% (13/25), moderately in 24% (6/25), slightly in 20% (5/25), and no staining was observed in 4% (1/25), which presented grade III by the PS and high grade according to the KS.

Immunohistochemistry (IHC). Positive immunostaining was observed in all MCTs tested for KIT antigen and in 11/25 (44%) of the cases tested for Ki67 protein.

For KIT antigen, 64% (16/25) of the neoplasms presented immunostaining pattern II (cytoplasm; Fig.2A) and 36% (9/25) showed pattern I (membrane; Fig.2B). Regarding intensity, 56% (14/25) of the cases were markedly, 28% (7/25) were moderately, and 16% (4/25) were slightly stained. As for the number and distribution of immunostained cells, 52% (13/25) of the cases presented a large number of cells evenly distributed in the analyzed section and 48% (12/25) of them showed a few randomly distributed cells. Of the 16 MCTs that presented pattern II, 15 were grade II and one was grade III by the PS, whereas 14 were low grade and two were high grade according to the KS. Of the nine neoplasms that showed pattern I, all were grade II according to the PS, whereas seven were low grade and two were high grade by the KS.

All of the MCTs immunostained for Ki67 antigen (11/25; 44%) presented low growth fraction (LGF). No immunostaining was observed in 14/25 (56%) of the neoplasms.

Prospective study

Histologic grading and histochemical evaluation. According to the PS, 11/12 (92%) of the MCTs were grade II and 1/12 (8%) was grade III. Application of the KS showed that 8/12 (67%) of the neoplasms were low grade and 4/12 (33%) were high grade. In the TB technique, 6/12 of the MCTs stained the granules markedly, 4/12 moderately, and 2/12 slightly. In AgNOR, counting ranged from 1.22 to 1.97 (mean of 1.44) for grade II neoplasms and 2.89 for grade III neoplasms.

Immunohistochemistry (IHC). Positive immunostaining was observed in all MCTs at all formalin fixation times tested for KIT protein. Under microscopic examination, 59% (seven) of the cases presented pattern II (cytoplasm) and 41% (five) showed immunostaining pattern I (membrane). Of the seven neoplasms that presented pattern II, six were grade II and one was grade III by the PS, whereas four were high grade and three were low grade using the KS. Of the five MCTs that showed pattern I, all were grade II and low grade after application of the PS and KS, respectively. No difference between the two amplification systems (biotinylated and non-biotinylated) was observed regarding intensity of the immunostained cells. With respect to the number and distribution of immunostained cells, 75% (nine) of the neoplasms presented a large number cells evenly distributed in the analyzed section and 25% (three) of them showed few randomly distributed cells.

With respect to Ki67 antigen, at formalin fixation for 24 hours, high growth fraction (HGF) was observed in four and three MCTs in the non-biotinylated and biotinylated amplification systems, respectively (Figs.2C,D), whereas

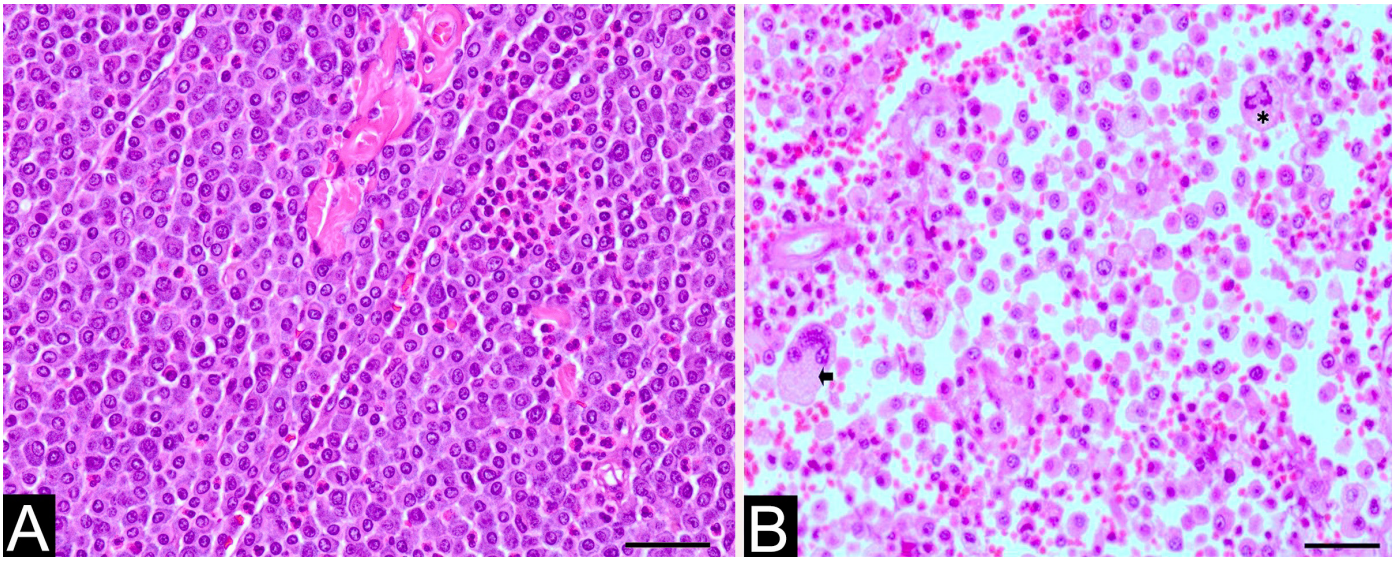

Fig.1. Histologic characteristics of cutaneous mast cell tumors in dogs. (**A**) Dog, skin. Low grade mast cell tumor according to the Kiupel system. HE, obj.40x. (**B**) Dog, skin. High grade mast cell tumor according to the Kiupel system. A pronounced pleomorphism is observed, with multinucleated cell (arrow) and mitotic figure (asterisk). HE, obj.40x.

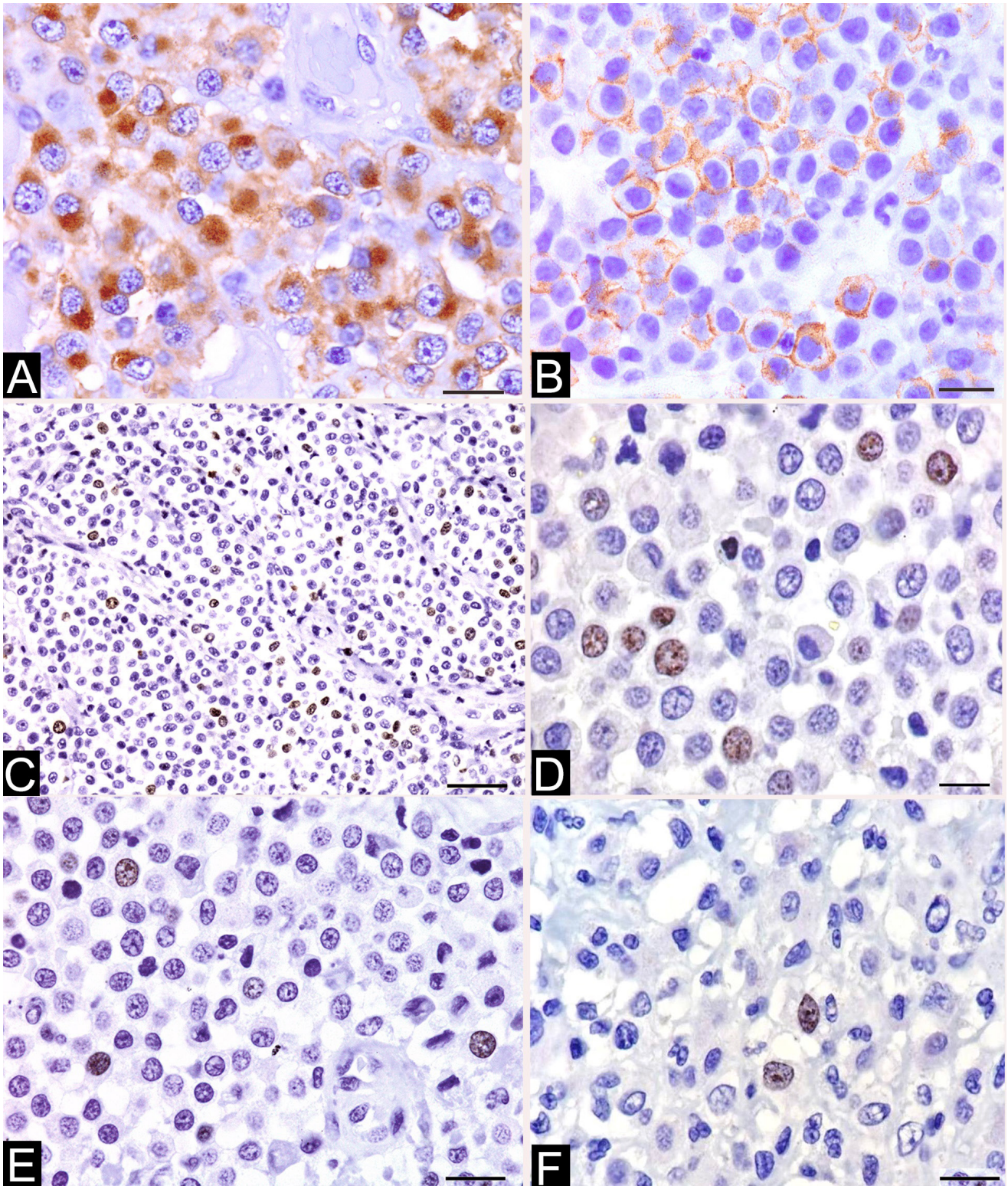

Fig.2. Immunohistochemical characteristics of cutaneous mast cell tumors in dogs. (**A**) Dog, skin. Mast cells showing intense cytoplasm immunostaining (pattern II) for KIT protein. Non-biotinylated method (HRP-polymer), obj.100x. (**B**) Dog, skin. Mast cells exhibiting cell membrane immunostaining (pattern I) for KIT protein. Non-biotinylated method (HRP-polymer), obj.100x. (**C**) Dog, skin. Mast cells presenting nuclear immunostaining for Ki67 protein. High cell growth fraction. 24h formalin fixation. Non-biotinylated method (HRP-polymer), obj.40x. (**D**) Neoplastic mast cells presenting nuclear immunostaining for Ki67 protein. High cell growth fraction. 24h formalin fixation. Biotinylated method (streptavidin-biotin-peroxidase complex), obj.100x. (**E**) Dog, skin. Neoplastic mast cells presenting nuclear immunostaining for Ki67 protein. Low cell growth fraction. 24h formalin fixation. Biotinylated method (HRP-polymer), obj.100x. (**F**) Dog, skin. Neoplastic mast cells presenting nuclear immunostaining for Ki67 protein. Low cell growth fraction. 24h formalin fixation. Biotinylated method (streptavidin-biotin-peroxidase complex), obj.100x.

LGF was found in eight and nine neoplasms in the first and latter systems, respectively (Figs.2E,F). Table 2 presents the growth fraction results based on Ki67 protein detection at the four formalin fixation times comparing the two amplification systems. Figure 3 depicts the Ki67 absolute values at the four fixation times comparing both systems.

In the non-biotinylated amplification system, 7/12 of the MCTs maintained the growth fraction at the four fixation times tested; in 3/12 of them, a change was observed in growth fraction detection, which shifted from high to low after 24 hours of fixation; no immunostaining was verified in 2/12 and 1/12 of the neoplasms after 48 and 72 hours of fixation, respectively.

In the non-biotinylated system, 5/12 of the MCTs maintained the growth fraction at the four fixation times; in 2/12 and 1/12 of them, a change was observed in growth fraction detection, which shifted from high to low after 24 and 72 hours of fixation, respectively; no immunostaining was verified in 1/12 and 3/12 of the neoplasms after 48 and 72 hours of fixation, respectively.

DISCUSSION

Concerning the histologic grading of the 25 mast cell tumors (MCTs) analyzed in the retrospective study, greater interrater agreement was observed with application of the Kiupel System (KS) (96%) compared with that of the Patnaik System (PS) (88%). These results are similar to those reported in the study where the KS was proposed, in which concordance between pathologists in the PS was 75% for grade III and approximately 60% for grades I and II; in the KS, regardless of the grade assigned, agreement was higher than 96% (Kiupel et al. 2011). Reduction in the variations between evaluators in the KS has been attributed to classification in only two grades, as it is based on more objective and simplified histologic criteria (Kiupel et al. 2011). The importance of establishing a high grade for MCTs lies in the fact that these neoplasms were more rapidly associated with animal metastasis or relapse, as well as with lower survival (Kiupel et al. 2011).

Table 2. Results of the cell growth fraction based on detection of Ki67 protein using the non-biotinylated and biotinylated amplification systems according to formalin fixation time in the prospective study

Case no.	Non-biotinylated				Biotinylated			
	24h	48h	72h	96h	24h	48h	72h	96h
1	↓	↓	↓	↓	↓	↓	•	•
2	↑	↓	•	•	↑	↑	↓	↓
3	↑	↓	↓	↓	↑	↓	↓	↓
4	↓	↓	•	•	↓	↓	↓	↓
5	↓	↓	↓	↓	↓	↓	↓	↓
6	↓	↓	↓	•	↓	↓	↓	•
7	↓	↓	↓	↓	↓	↓	↓	↓
8	↑	↑	↑	↑	↑	↓	↓	↓
9	↓	↓	↓	↓	↓	↓	↓	↓
10	↑	↓	↓	↓	↓	↓	↓	•
11	↓	↓	↓	↓	↓	↓	↓	•
12	↓	↓	↓	↓	↓	↓	↓	↓

↓ Low cell growth fraction, ↑ high cell growth fraction, • without immunostaining.

Counting of the argyrophilic nucleolar organizing regions (AgNOR) is a tool used to assist with assessing cell proliferation. This histochemical method quantitatively evaluates the rate at which tumor cells are proliferating (growth rate) (Derenzini 2000). The AgNOR values found in this study corroborate the specific scientific literature, which reports average AgNOR values for grade II MCTs from 1.28 to 3.96 (Bostock et al. 1989, Rech et al. 2004). The larger the number of AgNORs observed in the nucleus of the neoplastic cells, the greater the proliferative activity of the tumor (Derenzini 2000). Bostock et al. (1989) observed that the survival time for dogs with AgNOR <4 is almost three fold that of dogs with AgNOR >4. Ozaki et al. (2007), in addition to evaluating cell proliferation markers (Ki67, PCNA, and AgNOR), also assessed surgical resection, intratumoral vessel density, nuclear morphometry, and tumor depth and location.

Currently, two immunomarkers have been used to complement histologic grading in determination of biological behavior of MCTs, namely, KIT (tyrosine kinase membrane receptor) (Kiupel et al. 2004, Kiupel 2017) and Ki67 (marker of cell proliferation) (Webster et al. 2007). Sledge et al. (2016) created a flowchart on the prognostic evolution of MCTs based on the assessment of some criteria, surgical margin, histologic grading, clinical staging, and depending on the results, application of immunohistochemistry (IHC) (KIT and Ki67) and polymerase chain reaction (PCR) (mutations in exon 11), to determine the treatment to be used in each case. In this study, we sought to standardize and optimize the IHC technique for KIT and Ki67 proteins in samples received for routine diagnosis, without knowledge about formalin fixation time (retrospective study), because this is our current laboratory reality; subsequently, we worked with known fixation times (prospective study).

Immunostaining for KIT protein was not sensitive to prolonged fixation in formalin, with immunoreactivity observed in all cases, both in the retrospective and prospective studies. However, changes in the intensity, number, and distribution of immunostained cells were observed, which were heterogeneous but did not prevent identification of neoplastic cells as mast cells or determination of the immunostaining pattern. A factor considered important for the development of heterogeneous immunostaining for a given antigen is that formalin penetration is not homogeneous in relation to tissue fixation, occurring in centripetally (Ramos-Vara & Miller 2014).

Joint analysis of the retrospective and prospective studies showed immunostaining pattern II (cytoplasm) in 62% and pattern I (membrane) in 38% of the MCTs. Pattern III immunostaining was not observed in the present study. The vast majority of low grade neoplasms presented immunostaining pattern II, corroborating with the literature (Webster et al. 2007, Fonseca-Alves et al. 2015, Flores et al. 2016, Sledge et al. 2016). This information is important considering that it has been observed that MCTs that lose membrane expression and acquire cytoplasmic expression for KIT antigen, present a more aggressive biological behavior (Kiupel et al. 2004). Similarly, Webster et al. (2006) reported positive correlation between loss of membrane staining and presence of mutations in exon 11, suggesting that mutations may play a role in KIT protein localization. Mutations in the juxtamembrane domain of the

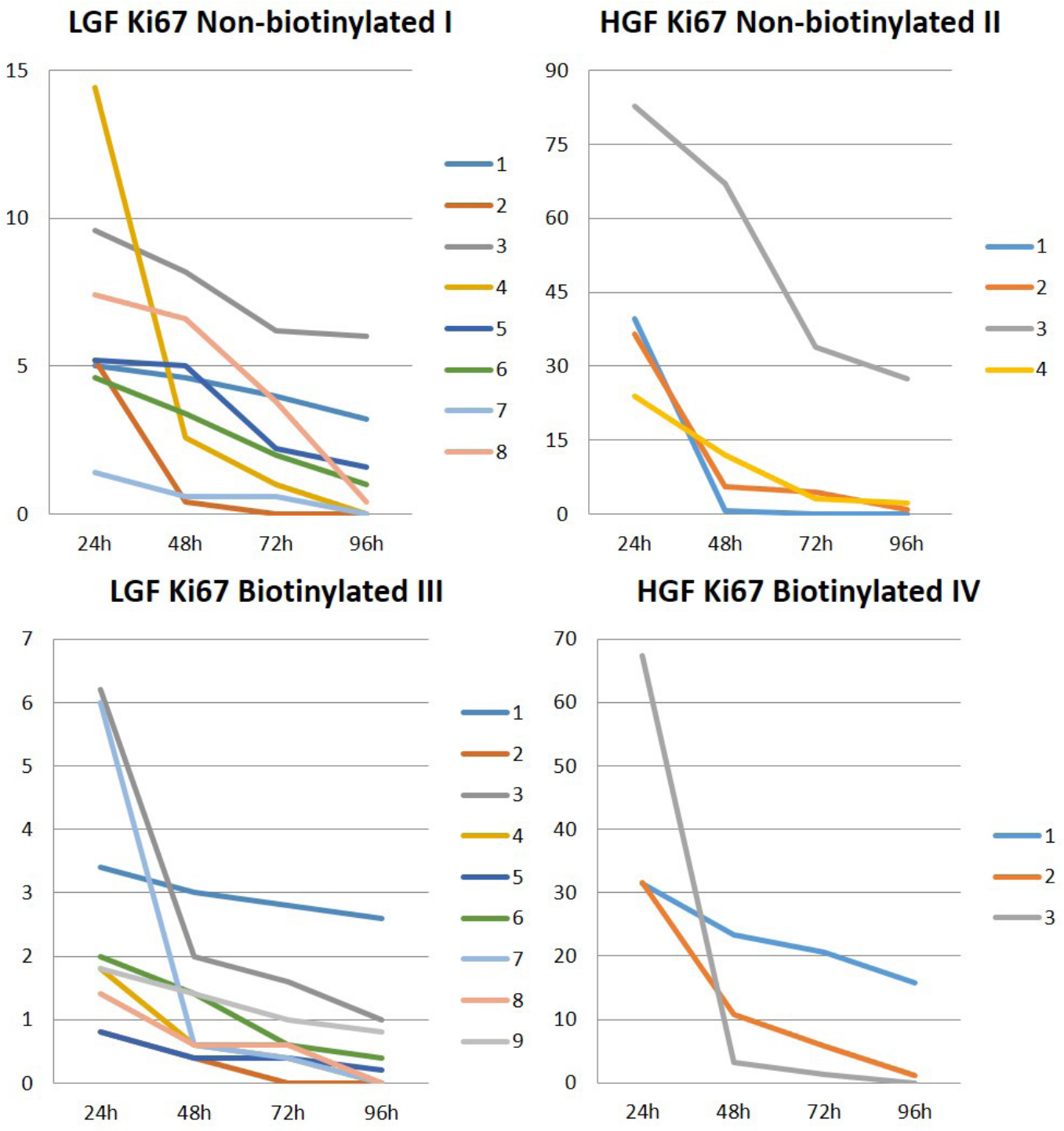

Fig.3. Absolute values for high (HGF) and low (LGF) cell growth fraction for Ki67 protein at the four formalin fixation times and in the two (non-biotinylated and biotinylated) amplification systems in the prospective study.

c-KIT gene are known to occur in biologically more aggressive neoplasms, but changes in the location of KIT immunostaining occur in all grades (Kiupel et al. 2004, Webster et al. 2006). As for pattern I immunostaining, it was observed mostly in cases classified as low grade by the KS, but also found in two high grade cases.

Failure in Ki67 antigen immunostaining, observed in 56% of the MCTs in the retrospective study, was possibly influenced by the prolonged formalin fixation time of some samples. A literature review conducted by Ramos-Vara & Miller (2014) associated cumulative effects of formalin fixation and factors relative to tissue processing (exposure

to alcohol and xylol) with failure to recognize Ki67 protein. That was the motivation for our prospective study, which sought to assess whether fixation time could be influencing the results of our retrospective study. Flores et al. (2016), using other samples from the same laboratory (LPV-UFSM), observed positive immunostaining for Ki67 protein in approximately 70% of the samples analyzed. The difference in the percentage of immunostaining between this study and that by Flores et al. (2016), even with different samples, could be associated with the systems of amplification (using a specific polymer for tissues of dogs and cats) and antigen retrieval (under controlled conditions of temperature and pressure) they used for Ki67 protein.

AgNOR is another method used to evaluate the cellular proliferation of a tumor. The AgNOR index could be used as an additional option for cases where IHC for Ki67 antigen could not be used, or in combination with the Ki67 result, as it will be discussed later. This is justified by the fact that the retrospective studies that used the AgNOR technique, possibly without knowledge of sample fixation time, did not mention the influence of fixation time on the results obtained (Rech et al. 2004, Lima et al. 2005).

Aiming to optimize the IHC technique for Ki67 protein, two amplification systems and four formalin fixation times were used in the prospective study. The advantage of the non-biotinylated system over the biotinylated system lies in the fact that the first is particularly simpler, has the same or even higher sensitivity, and presents lower background reaction compared with the latter (Ramos-Vara & Miller 2014). Galiza et al. (2014) found differences in specificity and sensitivity between different amplification systems in IHC for detection for aspergillosis and zygomycosis. For zygomycosis, the non-biotinylated method showed high sensitivity and specificity, whereas the biotinylated method presented high sensitivity and specificity for aspergillosis.

Although some advantages of the non-biotinylated system over the biotinylated system have been described (Ramos-Vara & Miller 2014), the present study observed changes (reduction in the number of immunostained cells) in determination of the growth fraction in the MCTs at fixation time of 48h in both systems. This information is important at the same time that it raises concern, because it represents the reality of most veterinary pathology laboratories, which receive material with unknown formalin fixation times. The impaired detection of cells that would be immunostained under ideal fixation conditions (up to 24 hours) may result in determination of a low (<23) instead of a high (>23) growth fraction, entailing a mistaken prognosis. This fact is important because dogs with MCTs with high growth fraction (HGF) presented lower survival (Webster et al. 2007).

It is worth highlighting that, in addition to the impaired detection in the growth fraction in the prospective study, there were no MCTs without immunostaining for Ki67 protein in the two amplification systems used, especially after 48 hours of fixation. Alves & Roman (2005) reported decreased positive immunostaining to proliferating cell nuclear antigen (PCNA) in tonsil samples, using the biotinylated system, in cases with formalin fixation longer than 24 hours. Other authors have found values of 43% (Fonseca-Alves et al. 2015) and 23% (Kandefer-Gola et al. 2015) failure in immunostaining for Ki67 protein in MCTs, and did not explain the reasons for such failures. According to Munakata & Hendricks (1993), the best results for Ki67 antigen were observed in tonsil samples after 4 hours of formalin fixation, with weak immunostaining in samples after 48 hours of fixation. Furthermore, it should be noted that the delay in exposing the sample to the tissue cross-linking fixative can significantly alter immunostaining with antibodies to cell proliferation rates (Ramos-Vara & Miller 2014), and this factor also depends on the veterinarian who sends the sample (clinician/surgeon), often without interference from the histopathological diagnostic laboratory.

Among the methods available to assess cell proliferation within a tumor, mitotic counting, although estimative, is the most commonly used by diagnostic laboratories (Romansik et al. 2007). This method, which is performed on slides stained using the hematoxylin and eosin (HE) technique, is simple and fast, and composes the criteria for histologic grading of MCTs (Kiupel et al. 2011, Souza et al. 2018). According to Sledge et al. (2016), cell proliferation is best and fully evaluated with the use of multiple methods. By multiplying the results of the AgNOR and Ki67 techniques (AgNOR x Ki67), it is possible to obtain the best cell proliferation rates within a neoplasm, because it associates growth rate with the cell growth fraction, respectively (Sledge et al. 2016, Kiupel 2017). Dogs diagnosed with AgNOR x Ki67 index >54 died within 12 months, whereas dogs with index <54 survived for two years (Webster et al. 2007). Although the literature describes that the best results of cell proliferation are obtained in combination, this study revealed that the combination of these techniques in the laboratory routine can be complicated, because the values for Ki67 antigen varied between fixation times. Likewise, it was observed that cases with formalin fixation time longer than 72 hours did not present immunostaining for this protein.

Using histologic grading, immunostaining pattern for KIT protein, and markers of cell proliferation, it is possible to choose the best therapy to be used in dogs with this neoplasm (Sledge et al. 2016). These techniques are offered by many laboratories to owners who wish, in addition to the histopathological diagnosis, other prognostic factors that enable the establishment of appropriate treatment for each situation and patient (Kiupel 2017).

CONCLUSIONS

This study demonstrates the importance to implement new tools in the search of the biological behavior of this neoplasm, but points to some barriers to be overcome in laboratory routine.

Immunostaining for KIT protein was efficient both at known (prospective study) and unknown, and possibly quite variable (retrospective study), fixation times.

Immunostaining for Ki67 antigen was highly sensitive to longer fixation time.

No differences were observed in immunohistochemical detection for KIT and Ki67 proteins in the comparison between the two amplification systems employed.

Assessment of Ki67 protein in previously formalin fixed samples, without knowledge of fixation time, presents variable results.

Thus, the AgNOR technique can be used as an alternative to evaluate cell proliferation rate in cases with fixation time longer than 24 hours.

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Original Article
Wildlife Medicine

Evaluation of tear production in juvenile opossum using three different methods[1]

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ABSTRACT.- Oria A.P., Raposo A.C., Araujo N.L.L.C., Romano J.V., Martins Filho E.F., Gomes Junior D. & Galera P.D. 2019. **Evaluation of tear production in juvenile opossum using three different methods.** *Pesquisa Veterinária Brasileira 39(1):61-65*. Departamento de Anatomia, Patologia e Clínicas Veterinárias, Universidade Federal da Bahia, Avenida Adhemar de Barros 500, Salvador, BA 41710-110, Brazil. E-mail: arianneoria@ufba.br

The establishment of parameters for tear production in different species is important for better understanding eye´s health and is one of the components of the ophthalmic semiological technique. Particularities derived from the anatomophysiology of non-domestic species induce the search for more reliable methodologies. The aim was to evaluate and compare tear production of white-eared opossum (*Didelphis albiventris*) and Brazilian common opossum (*Didelphis aurita*) by three different methods. Fifteen individuals of each species, juveniles, healthy, of both sexes, with 60 to 90 days of life, were physically restrained. Phenol red thread test (PRTT), endodontic absorbent paper point tear test (EAPPTT) and modified -Schirmer tear test (mSTT) were performed. PRTT was the most difficult to perform because of the wire malleability, while EAPPTT was more feasible for both species. The median ± semi-quartile range for PRTT were 19.79±2.61mm/15 "and 5.22±2.92mm/15", for EAPPTT were 16.25±1.82mm/min and 10.9±3.04mm/min, and for STTm were 0±1.63mm/min and 0±1.63mm/min for white-eared opossum and Brazilian common opossum respectively. There was no difference between the right and left eye neither sex. A significant difference was obtained for the same test to different species. No significant correlation was found between the tests for both species. The description of tear production parameters for juvenile white-eared opossum and Brazilian common opossum may be used as a tool, which will allow the early diagnosis of ocular diseases.

INDEX TERMS: Tear production, juvenile opossum, marsupials, phenol red thread test, endodontic absorbent paper point tear test, modified-Schirmer tear test, wild animals.

RESUMO.– [**Avaliação da produção lacrimal em gambás filhotes por três diferentes métodos.**] O estabelecimento do parâmetro de produção lacrimal nas diferentes espécies é importante para o entendimento da saúde do olho e é um dos componentes da semiotécnica oftálmica. Particularidades derivadas da anatomofisiologia das espécies não domésticas induzem a busca de metodologias que sejam mais fidedignas aos parâmetros. Objetivou-se com este estudo avaliar e comparar a produção lacrimal de gambás-de-orelha-branca (*Didelphis albiventris*) e gambás-de-orelha-preta (*Didelphis aurita*) por três diferentes métodos. Quinze indivíduos de cada espécie, juvenis, hígidos, de ambos os sexos, com 60 a 90 dias de vida, foram contidos fisicamente para realização do teste lacrimal do vermelho de fenol (TLVF), da ponta de papel absorvente estéril e do teste lacrimal de Schirmer modificado (TLSm). O TLVF foi o mais difícil de ser executado devido à maleabilidade do fio, enquanto a TEPA se mostrou mais exequível para ambas as espécies. A mediana ± intervalo semi-interquartil para o TLVF foi de 19,79±2,61mm/15" e 5,22±2,92mm/15", para a TEPA foram de 16,25±1,82mm/min e 10,93±3,04mm/min, e para o TLSm foram de 0±1,63mm/min e 0±1,63mm/min, para gambás-de-orelha-branca e gambás-de-orelha-preta,

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respectivamente. Não houve diferença entre o olho direito e esquerdo e nem quanto ao sexo. Obteve-se diferença significativa para um mesmo teste entre as espécies. Não foi encontrada correlação significativa entre os testes para ambas as espécies. A quantificação da porção aquosa da lágrima poderá auxiliar no diagnóstico precoce de doenças oculares nas espécies estudadas.

TERMOS DE INDEXAÇÃO: Produção lacrimal, gambás filhotes, teste lacrimal do vermelho de fenol, tira endodôntica de papel absorvente, teste lacrimal de Schirmer modificado, sariguê, marsupiais, animais silvestres.

INTRODUCTION

The opossums, genus *Didelphis,* are marsupials with oval ears and prehensile tail, widely distributed in South America, which have nocturnal, omnivorous and opportunistic habits, and record of occurrence in a peri-urban environment (Cáceres & Monteiro-Filho 1998, Cáceres 2000, Astua de Moraes et al. 2015, Costa et al. 2015).

These animals were described as an experimental model for eye studies due to their immature condition at birth (Mcmenamin & Krause 1993) and it often is referred to rehabilitation centers due to the death of the progenitor (Cáceres 2000). Ulcerative lesions and bacterial conjunctivitis are common in opossum; however, there are few descriptions on ophthalmic parameters of marsupials (Mcmenamin & Krause 1993, Cáceres 2000).

Quantitative measurement of tear film is one of the stages of the ophthalmic semiotechnique, and the composition and adequate volume of tears are responsible for the ocular surface homeostasis (Ghaffari et al. 2012, Trbolova & Ghaffari 2012). Schirmer tear test (STT) is considered the gold standard for the quantitative evaluation of this fluid, with reports for humans, domestic and wild animals (Beech et al. 2003, Trost et al. 2007, Ghaffari et al. 2012, Trbolova & Ghaffari 2012). However, modifications were suggested to species with palpebral fissure length smaller than the width of the strip (5.0mm), adapting it to 2.5mm (mSTT) (Conceição et al. 2011, Lange et al. 2012, Silva et al. 2013, Somma et al. 2015).

As alternative to animals with small eyes or decreased basal and reflex tear production, the phenol red tear test (PRTT) was used (Araujo et al. 2017). It is a cotton thread impregnated with phenol red dye, sensitized in contact with the normal pH of the skin. Its use was described in birds, which had small eyelid and required rapid physical restraint (Hida et al. 2005, Holt et al. 2006, Trost et al. 2007, Blackwood et al. 2010, Lange et al. 2012). The endodontic absorbent paper point tear test (EAPPTT) was used in wild species, among them mammals, reptiles and birds (Lima et al. 2010, Lange et al. 2012, 2014, Huaringa et al. 2015, Oriá et al. 2015b, 2015c, Rajaei et al. 2015, 2016a, 2016b, Monção-Silva et al. 2016a, 2016b, Araujo et al. 2017). This test is composed of a strip of absorbent material, with a small width, and the reading is performed by measuring the moist portion after 60 seconds (Lange et al. 2012).

The objective of this study was to evaluate and compare tear production in two species of juvenile opossums (*D. albiventris* e *D. aurita*) using PRTT, EAPPTT and mSTT.

MATERIALS AND METHODS

The study was approved by the Biodiversity Authorization and Information System (27489), the Ethics and Animal Welfare Committee of the Federal University of Bahia (73/2016) and conducted according to the precepts described by the Association for Research in Vision and Ophthalmology (ARVO).

Fifteen healthy juveniles white-eared opossums (7 males, 8 females) and fifteen Brazilian common opossums (8 males, 7 females), aged 60-90 days, from the Wild Animals Triage Center and the Getulio Vargas Zoobotanical Park (Salvador, Bahia, Brazil) were used in this research. Age was estimated based on phenotypic characteristics (D'Andrea et al. 1994, Shaw & Renfree 2006). These animals were rescued after being found in peri-urban regions near the dead progenitor or in conditions requiring health care. Before this study, the animals were submitted to physical examination and palpebral reflexes, pupillary light reflexes, and menace responses of both eyes, by the local veterinary staff to exclude individuals with indications of systemic changes or gross abnormalities in the eye or periocular region. After collection, the selected animals were submitted to the fluorescein test (Ophthalmos®, São Paulo, Brazil) to exclude specimens with corneal lesions. The data were collected with opossum physically restrained, and tests were performed with a 48-hour interval between 09:00 and 10:00 am. The ambient humidity and temperature were 60 to 63% and 28 to 29°C, respectively.

The phenol red tear test (Zone-Quick®, Oasis Medical, California, USA) was performed by inserting the cotton thread into the lower conjunctival sac of both eyes and maintained for 15 seconds (Fig.1A). Immediately after the removal, the reading was performed using a digital caliper (Mitutoyo®, São Paulo, Brazil).

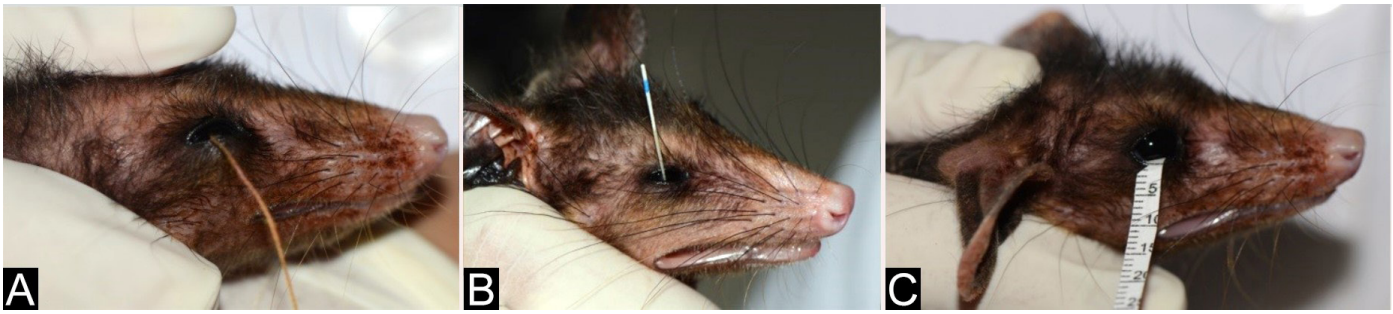

Fig.1. Quantitative measurement of tear production of *Didelphis aurita*. (**A**) Phenol red tear test, (**B**) endodontic absorbent paper point tear test, (**C**) modified Schirmer tear test.

Table 1. Evaluation of tear production in juvenile white-eared opossum (*Didelphis albiventris*) and juvenile brazilian common opossum (*Didelphis aurita*) by three different methods

Specie	Parameter	Median (±S-IQR)	CI	P value
White-eared opossum	PRTT	19.79 (±2.61)[a]	18.53 - 22.16	0.95
	EAPPTT	16.25 (±1.82)[b]	14.96 - 17.57	0.54
	mSTT	0.00 (±1.63)[c]	0.00 - 3.00	<0.01
Brazilian common opossum	PRTT	5.22 (±2.92)[A]	3.91 - 8.06	0.02
	EAPPTT	10.93 (±3.04)[B]	7.95 - 11.87	0.30
	mSTT	0.00 (±1.63)[c]	0.00 - 3.00	<0.01

S-IQR = Semi-interquartil range, CI = confidence interval, PRTT = phenol red tear test, EAPPTT = endodontic absorbent paper point tear test, mSTT = modified Schirmer tear test; [a, b, c, A, B] Difference between uppercase and lowercase letters show statistical difference between the same test for the species studied (P<0.05); P value for the distribution of values for a given test in the same species.

The endodontic absorbent paper point tear test (Roeko color® - Color size 30, Langenau, Germany) was inserted into the lower conjunctival sac and maintained for 60 seconds (Fig.1B). Immediately after the removal, the reading was made using a digital caliper (Mitutoyo, São Paulo, Brazil).

The modified Schirmer tear test (Ophthalmos®, São Paulo, Brazil) was performed with the strip cut in half using a No. 15 scalpel blade, to reduce its width from 5.0mm to 2.5mm (Conceição et al. 2011). The modified strip was inserted into the lower conjunctival sac and maintained for 60 seconds. After this period, the strip was removed and read immediately (Fig.1C).

Statistical analysis was performed with the IBM® SPSS® software, version 20.0, for the Windows® operating system (IBM Corporation, Somers, New York, USA). Quantitative data were assessed using the Shapiro-Wilk test. The Mann-Whitney and Wilcoxon tests were used for comparison between the PRTT, EAPPTT and mSTT, sex, and right and left eye. Spearman correlation was used to verify association between the variables. The level of significance was 5%.

RESULTS

Difficulties were found to execute the tests due to the size and proximity of the vibrissae to the eye: when the strips leaned against the vibrissae, the animals reacted with palpebral incursion, which removed the test from the conjunctival sac. When compared, the execution of PRTT, despite having a shorter execution time, proved to be more difficult due to the excessive malleability of the wire. EAPPTT was easier to manipulate due to its relative stiffness, which made it easier to access the lower conjunctival sac. mSTT was considered intermediate compared to previous tests related to its use. Data for STT, PRTT and EAPPTT of both species, were not normally distributed by Shapiro-Wilk test (P≥0,034). No significant differences were found in the comparison between the right and left eye and between sex (P>0.05). The medians of three evaluation methods are shown in Table 1. According to Mann-Whitney, there was a statistical difference when compared the same test between two species (P<0.05), except for mSTT. No significant correlations were found between tests for both species (P>0.05).

DISCUSSION

Reports of tear production evaluation in opossum species were not found until now by the authors, although diseases in these animal's eyes were reported (Cáceres 2000). Once the establishment of tear production parameter is relevant for an adequate ophthalmic semiotechnique, such as the detection of differences between healthy and diseased eyes, this study presents 3 different methods, which provides information on values and adequacy of the methodology for the species, mainly in case of animals with a small eyelid cleft.

The choice of the most suitable test for a given species should be guided by the possibility of acquiring the results associated with minimum stress (Montiani-Ferreira et al. 2006). To obtain data on tear production, caution should be taken in the manipulation prior to the tests, since contact with the vibrissae during manipulation induces the flashing protective reflex. As reported in horses (Hendrix 2005, Grant et al. 2013), this can cause changes in the dynamics of the tear in the conjunctival sac (Tsubota & Nakamori 1995).

PRTT-related performance difficulties were not reported in equines and cats, although these animals have abundance in vibrissae. Possibly, due to the size of the eyelid cleft (Sindak et al. 2010, Oriá et al. 2015a). When comparing different methods of tear production evaluation in an experimental model, EAPPTT is reported as a method that causes less discomfort to the ocular surface (Lima et al. 2010). In the present study, no differences were observed in the behavior of the animals in relation to the tests. However, features regarding execution have been reported, and it was pointed out that the use of EAPPTT allowed less contact with the vibrissae. For cats, Schirmer tear test caused greater discomfort (Oriá et al. 2015a), when compared the three tests. For opossums, mSTT has been considered easier than PRTT for being more rigid strip, but even the modified method was wider than the EAPPTT, causing more difficulties for execution.

There have already been reported different methods of tear production quantification, in rabbits (Lima et al. 2015), parrots (Monção-Silva et al. 2016a), macaws (Monção-Silva et al. 2016b) and green-iguanas (Araújo et al. 2017). In addition, the present study reports the use of different methods of measuring tear production in animals with small palpebral fissure length, such as marmoset and tortoise (Lange et al. 2012, Oriá et al. 2015b). Modifications on Schirmer tear test were suggested for neonatal dogs due to palpebral cleft size (Silva et al. 2013). However, studies of tear production in marsupials are scarce, with reports for koalas and kangaroos, using Schirmer tear test (Herring et al. 2000, Takle et al. 2010, Grundon et al. 2011).

Similar results obtained for PRTT in white-eared opossums were described for swine, 16±4.7mm/15s (Trost et al. 2007); owls (*Megascops asio*), 15±4.3mm/15s (Harris et al. 2008); bats of the genus *Pteropus*, 20.23±1.28 mm/15s (Blackwood et al. 2010); chinchillas (*Chinchilla lanigera*), 14.6±3.5mm/15s (Lima et al. 2010); marmoset (*Callithrix penicillata*), 13.27±5.41 (Lange et al. 2012); and parrots (*Amazona amazonica*), 21.9±2.3mm/15s (Monção-Silva et al. 2016a).

Despite the phylogenetic proximity, the Brazilian common opossum presented reduced values in comparison to the white-eared opossum. A value close to that obtained in this species was also observed in Syrian hamster (*Mesocricetus auratus*), 5.57±1.51mm/15" (Rajaei et al. 2016a).

Prior study with EAPPTT for marmosets obtained values of 9.32±3.09mm/min, similar to values for Brazilian common opossum (Lange et al. 2012). Assessments in animals with small eyelid cleft, as saffron finch (*Sicalis flaveola*) obtained 5.10±0.26mm/min; chestnut-bellied seed-finch (*Sporophila angolensis*), 4.11±0.34mm/min; mouse (*Rattus norvegicus*), 6.18±2.06mm/min; mice (*Mus musculus*), 4.39±1.45mm/min (Lange et al. 2014), demonstrated values different to this study. Similar values to white-eared Opossum were obtained to parrots (*Amazona amazonica*), 14.9±1.65mm/min (Monção-Silva et al. 2016a); New Zealand rabbits, 13.8±1.5mm/min (Lima et al. 2015); english angora, 18.8±2.1mm/min; and dutch rabbits, 16.9±1.7mm/min (Rajaei et al. 2016b).

Results of mSTT were similar to those obtained in marmosets (Lange et al. 2012), red-eared tortoise (Somma et al. 2015) and prairie dog (Meekins et al. 2015), ranging from 0 to 7mm, 0 to 5mm, and 0 to 4mm respectively. Null or negative value in this type of quantitative test may suggest non-validation for these species, which highlights the need for adaptations of the methodology. Studies on marsupials with the unmodified Schirmer tear test, such as koalas (*Phascolarctus cinereus*), found values of 10.3±3.6mm/min and in red kangaroo (*Macropus rufus*), which is higher than those found in this study (Herring et al. 2000, Takle et al. 2010, Grundon et al. 2011).

Due to different characteristics of the three tests, extrapolate their values are not recommended, based in the non-correlation found between them. Studies of tear production in neonates and juveniles are reported for dogs (Silva et al. 2013), with a difference for adult individuals. From this, it is suggested to carry out further studies with evaluation of tear production in individuals of larger age groups. There was a notable difference in tear production between species, even with similar age and the same genus, which highlights the importance of knowledge regarding species-specific parameters.

The three tests show feasible options for the evaluation of tear production in white-eared opossum and Brazilian common opossum, and the implementation of EAPPTT was less difficult due to the stiffness of the strip that allowed less contact with the vibrissae. White-eared opossums have greater tear production than Brazilian common opossum for the evaluated tests, except for the modified Schirmer tear test.

CONCLUSION

The determination of these parameters for different tests of the tear film on white-eared opossum (*Didelphis albiventris*) and juvenile Brazilian common opossum (*Didelphis aurita*) represent important aid in the diagnosis of ocular diseases.

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

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Original Article
Wildlife Medicine

The urban and rural capybaras (*Hydrochoerus hydrochaeris*) as reservoir of *Salmonella* in the western Amazon, Brazil[1]

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ABSTRACT.- Farikoski I.O., Medeiros L.S., Carvalho Y.K., Ashford D.A., Figueiredo E.E.S., Fernandes D.V.G.S., Silva P.J.B. & Ribeiro V.M.F. 2019. **The urban and rural capybaras (*Hydrochoerus hydrochaeris*) as reservoir of *Salmonella* in the western Amazon, Brazil.** *Pesquisa Veterinária Brasileira 39(1):66-69*. Programa de Pós-Graduação em Sanidade e Produção Animal Sustentável da Amazônia Ocidental, Universidade Federal do Acre, Rodovia BR-364 Km 4, Distrito Industrial, Rio Branco, AC 69915-900, Brazil. E-mail: itacir.farikoski@gmail.com

The capybara (*Hydrochoerus hydrochaeris*) is the largest rodent in the world. In the state of Acre, Brazil, populations of capybaras have been increasing significantly. The role of capybaras in the transmission of certain bacterial zoonotic infections is not well understood, including bacteria of the genus *Salmonella. Salmonella* spp. generally cause enteritis or septicemia in mammals, however many mammalian species can carry the bacteria asymptomatically and shed it in their feces. To better understand the possible role of capybaras as reservoirs of *Salmonella* spp., we conducted a study of *Salmonella* within fecal samples from capybara in Acre. In a convenience sample, 54 capybaras from two urban and two rural areas of Acre were captured and kept for three to four days for sampling. None of the animals were symptomatic of any intestinal illness. Three separate fecal samples were collected from each animal, during their stays in captivity. Each sample was cultured for the presence of *Salmonella* spp. at the bacteriology laboratory of the Veterinary College of the Federal University of Acre. Samples were seeded in tetrationate pre-enrichment broth and in pre-enrichment broth peptone. After a 24 hour of incubation all samples were streaked on MacConkey Agar (MC) and *Salmonella-Shigella* Agar (SS). Suggestive colonies were submitted to biochemical analysis. Salmonella compatible colonies according to biochemical profile were submitted to serotyping (Sorokit for *Salmonella* - Probac do Brasil). In addition, the first sample from each of the 54 capybara was tested for *Salmonella* spp. using PCR targeting gene hilA. Eight (5%) of the 162 samples examined by bacterial culture were positive for *Salmonella* spp., while four (7%) of the 54 examined by PCR were positive. From the eight positive animals on culture, five were from urban area and three from rural area. On PCR, only one positive animal was from urban area and four were from rural area. Overall, by either test, one of the 54 animals was positive. All samples were collected in free - living animals with no apparent clinical signs of salmonellosis, indicating the potential of capybara as reservoir on this ecosystem.

INDEX TERMS: Capybaras, *Hydrochoerus hydrochaeris*, *Salmonella*, Amazon, Brazil, zoonosis, wild animals.

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RESUMO.- [**As capivaras urbanas e rurais (*Hydrochoerus hydrochaeris*) como reservatório de *Salmonella* no oeste da Amazônia, Brasil.**] A capivara (*Hydrochoerus hydrochaeris*) é o maior roedor do mundo. No estado do Acre, Brasil, as populações de capivaras têm aumentado significativamente. O papel das capivaras na transmissão de certas infecções zoonóticas bacterianas não é bem compreendido, incluindo as bactérias do gênero *Salmonella*. *Salmonella* spp. geralmente causam enterite ou septicemia em mamíferos, porém muitas

espécies de mamíferos podem carregar a bactéria de forma assintomática e eliminá-la em suas fezes. Para entender melhor o possível papel das capivaras como reservatórios de *Salmonella* spp., realizamos um estudo para identificação de *Salmonella* spp. em amostras fecais de capivaras no Acre. Em uma amostra de conveniência, 54 capivaras de duas áreas urbanas e duas áreas rurais do Acre foram capturadas e mantidas por três a quatro dias para amostragem. Nenhum dos animais era sintomático de qualquer doença intestinal. Três amostras fecais foram coletadas de cada animal, durante sua permanência em cativeiro. Cada amostra foi cultivada para a presença de *Salmonella* spp. no Laboratório de Bacteriologia Veterinária da Universidade Federal do Acre. As amostras foram semeadas em caldo de pré-enriquecimento tetrationato e em peptona de caldo de pré-enriquecimento. Após 24 horas de incubação, todas as amostras foram semeadas em ágar MacConkey (MC) e ágar *Salmonella-Shigella* (SS). Colônias sugestivas foram submetidas a análises bioquímicas. Colônias compatíveis com *Salmonella* de acordo com o perfil bioquímico foram submetidas à sorotipagem (Sorokit para *Salmonella* - Probac do Brasil). Além disso, a primeira amostra de cada uma das 54 capivaras foi testada para *Salmonella* spp. usando PCR, visando gene hilA. Oito (5%) das 162 amostras examinadas por cultura bacteriana foram positivas para *Salmonella* spp. Enquanto quatro (7%) das 54 examinadas pela PCR foram positivas. Dos oito animais positivos em cultura, cinco eram de área urbana e três de área rural. Na PCR, apenas um animal positivo era de área urbana e quatro de área rural. Considerando o diagnóstico conjunto por ambos os testes, PCR e cultura, um animal foi considerado positivo. Todas as amostras foram coletadas em animais livres, sem sinais clínicos aparentes de salmonelose, indicando o potencial da capivara como reservatório nesse ecossistema.

TERMOS DE INDEXAÇÃO: Capivaras urbanas e rurais, *Hydrochoerus hydrochaeris*, *Salmonella*, Amazônia, Brasil, zoonoses, animais silvestres.

INTRODUCTION

Capybaras (*Hydrochoerus hydrochaeris*), family Caviidae and subfamily Hidrochoerinae, are the largest rodents in the world and can be found in most of the South American continent, excluding only the more arid basins (Hosken & Silveira 2002, Oliveira & Bonvicino 2011). Capybara lives in close proximity to humans, within their range, and often come in contact with domestic animals and people. Capybaras may have an important role in the transmission of zoonotic etiological agents (Chiacchio et al. 2014), and reports on bacterial zoonotic diseases among wild animals are scarce and usually based only on serologic surveys (Nogueira & Cruz 2007, Siembieda et al. 2011).

The global burden of nontyphoidal *Salmonella* gastroenteritis has been estimated to be 93,8 million cases of gastroenteritis each year, with 155,000 deaths (Majowicz et al. 2010). Lower mammals play a critical role in the maintenance and transmission of *Salmonella* spp. to humans, primarily in food-borne transmission. Wild animals can serve as a direct source of *Salmonella* infection for humans through contact with fecal contamination or through the secondary infection of domestic animals from wild animal sources. While capybara may be a source of zoonoses, there are only two reports of *Salmonella* were recorded in capybara (Bandarra et al. 1995, Nogueira 1998). In both cases the capybaras had been in captivity for several years. Only one study has previously been conducted on the *Salmonella* carriage rates of free-ranging capybara (Chiacchio et al. 2014).

PCR is an efficient technique for the diagnosis of *Salmonella*. It can be used to replace blood culture, but for a precise diagnosis a standard technique must be ensured in order to avoid false negatives (Sánchez-Jiménez & Cardona-Castro 2004). In order to correct this problem, it is necessary to use enrichment broths that reduce these inhibitors (Pathmanathan et al. 2003). PCR using hILA gene is an important tool for the identification of *Salmonella* spp., additionally the use of multiplex PCR allows to differentiate some serovars (Kim et al. 2006, Crăciunaș et al. 2012).

To better understand the role of capybaras on spread of *Salmonella* spp., we conducted a study with a convenience sample of free ranging capybara from both urban and rural settings in the state of Acre, Brazil.

MATERIALS AND METHODS

Capybara sample. The study was conducted from June 2014 to November 2015. During this period, capybaras were captured in a convenience sample from free roaming herds in two urban areas and two rural areas. The two urban areas included the Federal University of Acre campus, UFAC (9°57'33.0" S 67°52'23.3" W) and at Farmhouse Ipê (9°57'51.4" S 67°52'14,9" W), a closed urban housing area made up of condominiums and about 100 families. The two rural areas were the Farm São Raimundo (09°56'49.7" S 67°44'9.4" W) and the Farm Piracema (10°00'39.7" S 67°56'14.9"W).

After the capture, all the animals were identified with microchip and transported to the Catuaba Experimental Farm, located in the municipality of Senador Guiomard, Acre (10°3'42.6" S 67°36'7.3" W) for further study. After completion of the study, the animals were released unharmed at the initial sites of their capture.

All animals were captured and anesthetized according to the protocol approved by CEUA/UFAC No. 23107.016723/2014-41. The capture and collection of samples of Brazilian wildlife was authorized by the Chico Mendes Institute for Biodiversity Conservation (ICMBIO) through the System of Authorization and Information on Biodiversity (SISBIO) No. 44791-1.

Collection of fecal samples. Each animal was sampled at the time of capture and then at day six and day 12 of captivity. Each fecal sample was collected using two sterile swabs per animal. The swabs were introduced rectally and rotated so as to cover the whole surface of the swab with animal feces. After the collection, the materials were labeled, kept chilled (4°C), and transported to the Veterinary Bacteriology laboratory of the Federal University of Acre. All samples were processed for culture in less than 12 hours following collection. As a result of duplicating samples at each time of sampling, there were a total of 324 fecal samples taken for analysis from a total of 54 animals.

Sample processing. Of the 162 fecal samples collected, one of each sample was seeded in replicate on tetrationate pre-enrichment broth and on pre-enrichment broth peptone. After a 24 hour of incubation all samples were streaked on MacConkey Agar (MC) and *Salmonella-Shigella* Agar (SS) as shown below and incubated in at 37°C, with readings at 24 and 48 hours.

After incubation for 24-48 hours, the morphologically suggestive colonies of *Salmonella* spp., production of hydrogen sulphide (H_2S) and no fermentation of lactose, were plated onto a blood agar plate and incubated at 37°C for a further 24 hours. After growth on blood

agar, colonies were submitted to biochemical analysis. The following parameters were evaluated: bacterial motility, lysine decarboxylase production, glucose fermentation in depth and sucrose on the surface of the medium, production of hydrogen sulphide (H_2S), gas production, and use of the amino acid L-tryptophan (deamination), hydrolysis of Urea and the formation of indole (Quinn et al. 1994, Koneman et al. 2005). TSI-Triple Sugar Iron Agar was used to verify the fermentation of glucose, lactose and sucrose. *Salmonella* compatible colonies according to biochemical profile were submitted to serotyping, according the Kauffmann-White classification, (Sorokit for *Salmonella* - Probac do Brasil) using somatic (O) and flagellar (H) sera to identify the most frequent serogroups and the most clinically significant serotypes according to the manufacturer 's specifications (Wattiau et al. 2011).

Polymerase chain reaction (PCR). A 2ml aliquot of tetrathionate (TT) broth from each of the 162 samples was frozen at -4°C for detection of *Salmonella* spp. by PCR. Colonies with a biochemical profile compatible with *Salmonella* spp. were stored in glycerol and skimmed milk powder and frozen according to Malik (1988) and Thompson (1987) and also submitted to PCR for confirmation.

The aliquots of 1ml of the TT broth and the sugestive isolated colonies were subjected to DNA extraction and purification with DNeasy® merican Food Kit (Qiagen®), to the quantification of DNA by fluorometry with Qubit 2.0 (Invitrogen®). PCR assays were performed in duplicate for amplification of the hILA gene with primers hILA 2-F (5'-CTGCCGCAGTGTTAAGGATA-3') and hILA 2-R (5'-CTGTCGCCTTAATCGCATGT-3'), with initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C at 1min, 58°C at 1min, 72°C at 1min and final extension at 72°C for 10min. Using a reaction mixture 5µl of buffer (Invitrogen®), 0.2mM dNTP Fermentas®), 1.5 U of recombinant Taq polymerase (Platinum® Taq-Invitrogen®), 5mM MgCl2 (Invitrogen®) and 25pMol of each primer (Invitrogen®) with a final volume of 25µL. Were analyzed by 1.5% ultrapure agarose gel electrophoresis and stained with GelRed™ for visualization of the 497 base pair (bp) confirmatory fragment of Salmonella spp.

Statistical analysis. The chi-square test was used to compare diagnostic methods. Differences were considered significant when $p<0.05$.

RESULTS

Among the 162 fecal samples collected over three separate samplings of 54 capybaras, eight samples were positive for *Salmonella* spp. by culture. All eight of these samples grew *Salmonella* spp. that were positive by agglutination testing to A and E, but all were negative to the flagellar agglutination test. Four of these eight came from the first sampling of the animals, two from the second sampling, and two from the third sampling. From the eight positive animals on culture, five were from urban area and three from rural area. One culture isolate, from urban area, was confirmed as *Salmonella* spp. by PCR.

In addition to one sample being positive by both PCR and culture, five additional samples were positive by PCR alone when examining the TT aliquots for DNA. One of the PCR positive animals (2%) was from urban area animal and four (7%) of positive animals was from a rural area. Thirteen of the 54 animals analyzed (24.07%) presented positive results when considering the results of the culture and PCR together.

DISCUSSION

Capybara represents an important link between wild, urban and production animals in rural and urban areas of South America. In addition, the fact that Capybaras are well adapted to urban and suburban environments means that they represent an additional direct threat to humans for zoonotic pathogens. Considering this potential for infection and transmission, we conducted this study to confirm the possible role of capybara as a reservoir of *Salmonella* spp. Our study confirms that capybaras are potential reservoirs or sources of infection for salmonellosis, when considering the results of the culture and PCR together 24.07% presented positive results. This result is higher than that observed by Nogueira (1998), who investigated enterobacteria in capybaras and found 4.92% of animals bearing *Salmonella* spp. when only the conventional culture is used.

Nogueira (1998) identified a strain of *Salmonella belem* and two of *Salmonella paratyphi B*. The author used the conventional technique of isolation by bacterial culture and serology in peri-urban breeding animals. In this work the suspicion was raised that these animals may have been contaminated by handlers or visitors, due to the specificity of Paratyphi serovar. The non-agglutination of the samples obtained from free-living capybara when exposed to flagellar sera indicates that these strains were probably of lower pathogenic potential for man. However, according to Acha & Szyfres (2001), excluding serotypes *S. typhi*, *S. paratyphi A* and *S. parathyphi C*, which are unique to humans, all serotypes of *Salmonella* are considered to be zoonotic. We did not have achieved serovar identification nor pathogenicity assessments in our study to compare to these earlier publications.

The similar results obtained in the pre-enrichment culture with buffered peptone water and TT broth demonstrated efficacy and complementarity for the diagnosis of *Salmonella* spp. allowing the cultivation of eight suspected isolates. Among the isolates only one was confirmed by PCR. This result is not unexpected because several factors limit the efficiency of the PCR technique for detecting bacteria. Biological samples may be accompanied by artifacts that have inhibitors, reducing the efficacy of the technique. Among the artifacts we can mention blood, bile salts found in feces and some milk proteins (Al-Soud & Rådström 1998). Thus, as the isolates from this study were cryopreserved using milk and glycerol, the presence of milk inhibitors may have resulted in a lower PCR efficiency.

PCR from enrichment broths showed a better result in the detection of *Salmonella* spp. when compared to bacteriological culture ($P<0.05$). The two techniques associated with repeat sampling make the diagnosis of *Salmonella* spp. more effective. Especially in asymptomatic animals as in the case of capybaras under study. Miller et al. (2008) observed intermittent elimination of *Salmonella* spp. in asymptomatic rhinoceroses. This was even more evident in this study because both the isolation and PCR were able to identify the presence of *Salmonella* spp. on different samples, increasing the joint diagnostic sensitivity in the group of animals studied.

Cohen et al. (1996) observed that positive samples in PCR were negative in culture; however the majority of the positive cultures were also positive in PCR. Although traditional techniques of culture and bacterial identification are used as official for analysis, it is fundamental to complement molecular techniques, since they present a higher sensitivity in a shorter

time (Gandra et al. 2008). Samples from five animals that were PCR positive (samples from the TT broth) were not confirmed by bacteriological culture which may indicate different differentiated bacterial profiles. The absence of bacterial growth in samples with positive PCR can also be explained by the predilection that some bacterial strains present by specific cuture media, then it is expected that molecular methods detect as positive samples considered negative in bacterial isolation (Sugimoto et al. 2009).

Differences were also found on the isolation pattern between the urban and rural groups. More suggestive colonies of *Salmonella* spp. were recovered from animals from urban areas, while PCR was able to identify as positive more samples collected in the Rural Zone. This fact may indicate that these animals may be exposed to different strains, suggesting a different sensitivity in the identification of distinct strains in both techniques (Murray et al. 2014).

CONCLUSION

These findings confirm the importance of free living capybaras as potential carriers and disseminators of *Salmonella* spp. in urban and rural areas of Brasil.

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

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Original Article
Morphophysiology

Effects of atracurium besylate on corneal endothelium of chickens: *in vitro* study[1]

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ABSTRACT.- Guimarães C.B., Albuquerque L.,Torikachvili M., Vargas E.V., Dall'Agnol C.C., Silva T.C. & Pigatto J.A.T. 2019. **Effects of atracurium besylate on corneal endothelium of chickens: *in vitro* study.** *Pesquisa Veterinária Brasileira 39(1):70-74.* Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves 9090, Agronomia, Porto Alegre, RS 91540-000, Brazil. E-mail: pigatto@ufrgs.br

The aim of this study was to investigate the acute effects of atracurium besylate on cellular damage in corneal endothelium of chickens. Twenty healthy chicken eyes were assigned to the following groups: Group 1 (G1), experimental group (n=10); and Group 2 (G2), control (n=10). Excised corneoscleral buttons were immediately placed on glass microscopy slides with endothelial region faced up. Corneal endothelium of eyes in G1 were covered with AB (0.2mL, 10mg/mL) for 3 min and then rinsed with balanced salt solution (BSS), while the corneal endothelium of eyes in G2 were covered with BBS for 3 min. Corneas from both groups were stained with alizarin red/trypan blue and visualized by light microscopy. Ten random photographs were taken from each cornea. The area of cellular damage was measured by software in all samples and cell loss of each group was averaged and compared. Endothelial area of denudation and Descemet's membrane exposure were higher in G1 than G2. In conclusion, atracurium besylate induced an acute damage on corneal endothelium of chickens.

INDEX TERMS: Atracurium besylate, corneal endothelium, chickens, *in vitro*, avian, mydriasis, damage, alizarin red.

RESUMO.- [**Avaliação dos efeitos do besilato de atracúrio no endotélio corneano de galinhas: estudo *in vitro*.**] Objetivou-se avaliar os efeitos agudos do besilato de atracúrio sobre o endotélio corneano de galinhas. Vinte olhos saudáveis de galinhas foram aleatoriamente separados em dois grupos com 10 olhos cada, sendo G1 o grupo controle e G2 o grupo tratamento. Imediatamente após a excisão dos botões corneoesclerais estes foram colocados em lâminas de microscopia de vidro com o lado endotelial voltado para cima. No Grupo 1, o endotélio corneano foi recoberto com 0,2ml de besilato de atracúrio (10mg/ml) durante 3 minutos e depois lavado com solução salina balanceada. No Grupo 2, o endotélio corneano foi recoberto apenas com solução salina balanceada durante 3 min. As córneas de ambos os grupos foram coradas com vermelho de alizarina e azul de tripano e visualizadas com microscópio óptico. Foram obtidas dez fotografias aleatórias de cada amostra. As imagens foram analisadas e com auxílio de um software as áreas com ausência de células endoteliais calculadas. A perda celular endotelial foi significativamente maior no grupo tratamento comparativamente ao grupo controle. Com base nos resultados apresentados foi possível concluir que o besilato de atracúrio induziu dano agudo nas células do endotélio da córnea de galinhas.

TERMOS DE INDEXAÇÃO: Besilato de atracúrio, endotélio corneano, galinhas, *in vitro*, aves, midríase, córnea, dano endotelial, vermelho de alizarina.

INTRODUCTION

The corneal endothelium is an interlocking polygonal cell monolayer which comprises the posterior surface of the cornea (Yee et al. 1987). The endothelial integrity and metabolic activities are essential for the maintenance of corneal transparency (Joyce 2012, Albuquerque et al. 2015). Changes in endothelial cells can occur depending on

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age, applied drugs, ocular diseases or intraocular surgical procedures (Gwin et al. 1982, Bercht et al. 2015). Ocular diseases, such as cataracts, are common causes of blindness in birds and surgical treatment are possible and have been implemented with success (Gonçalves et al. 2006, Carter et al. 2007). Neuromuscular blocking agents are used to produce mydriasis in birds, because their iris has striated muscle which is unresponsive to the usual mydriatic and cycloplegic drugs (Oliphant et al. 1983, Barsotti et al. 2012). The use of atracurium as a mydriatic in cataract surgery in birds has been described by some authors as an effective method (Gonçalves et al. 2006, Carter et al. 2007). In these atracurium use reports in intraocular surgery, only intracameral applications have been described. The topical ocular use of neuromuscular blocking agents usually does not cause a sufficient mydriasis. Rocuronium and vecuronium have been successfully used to dilate the pupil of birds. However complications such as corneal ulcer and paralysis of the eyelids were found. (Barsotti et al. 2010a, 2010b, 2012, Petritz et al. 2016).

Among the main methods used for endothelial analysis are specular microscopy, confocal microscopy and scanning electron microscopy (Pigatto et al. 2006, 2008, Nagatsuyu et al. 2014, Kobashigawa et al. 2015, Coyo et al. 2016, Terzariol et al. 2016). In addition, it is possible to analyze and photograph the endothelium of the cornea under an optical microscope after staining with alizarin red dye (Saad et al. 2008, Ruggeri et al. 2009, Park et al. 2012). Endothelial cell loss is inevitable during intraocular surgeries (Terzariol et al. 2016). In this sense, it is important to select substances inert to the endothelium of the cornea in an attempt to avoid endothelial damage that may induce decompensation of irreversible cornea. The intracameral atracurium has been routinely employed to cause mydriasis in birds during intraocular surgery (Gonçalves et al. 2006, Carter et al. 2007). However, the possible effects of this treatment on the corneal endothelium have never been evaluated in birds. The aim of this study was to investigate the acute effects of atracurium besylate on the corneal endothelium of chickens.

MATERIALS AND METHODS

Twenty corneas from 10 healthy chickens (*Gallus gallus domesticus*) of the Cobb500 lineage, male and female, with 21 days old, obtained from a local slaughterhouse were studied. All animals were sacrificed for reasons unrelated to this study. The research was conducted according to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Visual Research. Immediately after humane slaughter enucleation was performed. Ophthalmic examination was realized before the start of the experiment. The examination consisted of evaluation with slit-lamp biomicroscopy (Portable Slit Lamp SL 15, Kowa, Japan) and fluorescein stain (Fluorescein, Allergan, SP, Brazil). Eyes that showed evidence of ocular disease were excluded.

Corneas were randomly divided into two groups of 10 corneas each. Group 1, G1, was the experimental group composed of corneas from the left eyes, and group 2, G2, was the control group composed of corneas from the right eyes. Excised corneoscleral buttons were immediately placed on glass microscopy slides with endothelial region faced up. In G1, the corneal endothelium was covered with 0.2 ml of commercially available atracurium besylate (Cristália, São Paulo, Brazil) for 3 min and then rinsed with balanced salt solution (BSS, Ophthalmos, São Paulo, Brazil). In G2, the corneal endothelium was covered only with BSS for 3 min (Fig.1A). Corneal buttons were placed endothelial side up on a glass slide and stained with 0.1% trypan blue (Ophthalmos, São Paulo, Brazil) followed by rinsing in BSS and staining with alizarin red (Fig.1B) (Sigma-Aldrich, St Louis, USA) for 90 seconds.

After a final rinse with BSS, the corneal disc was mounted endothelial side up on a microscope slide and examined and photographed using an optical microscope (Nikon Eclipse E200, Japan) at 10x magnification. Ten random images of the corneal endothelium of each cornea were obtained. In each imagine areas with loss endothelial cells were manually measured and quantified using a software for morphometric analysis (UTHSCSA Image tool 3.0, Texas, USA), and then the percentage of endothelial damage was described as mean ± SD. Statistical data analysis was conducted using Student's t-test using a confidence level of 95%, in order to check the statistical difference between the level of damage in the treatment eyes (G1) and control eyes (G2).

RESULTS

With optical microscopy after the use of the dyes in all analyzed images it was possible to observe sharp cellular borders (Fig.1C). In all the analyzed images the areas with absence of cells were idenditified and demarcated (Fig.1D). With this, the percentage of cellular damage was calculated. The results given for endothelial cell loss in the control group were 0.75%±1.13% and in the treatment group were 22.23%±13.67%.

The results showed that the average of the level of damage in experimental group was significantly higher than the media of the control group (p=0.005).

DISCUSSION

In order to avoid the systemic effects of atracurium, and benefit only from its local action on iridian muscles to generate mydriasis in intraocular surgery, atracurium has been administered intracamerally with satisfactory effects (Gonçalves et al. 2006, Carter et al. 2007). The topical application of atracurium on the cornea has not been described, probably because atracurium has low lipid solubility and, therefore, does not easily penetrate biological membranes. The ocular use of neuromuscular blocking, however, has been reported with the use of other drugs such as rocuronium and vecuronium, producing effective mydriasis in different species of birds, but with some reports of corneal ulcers and transitional eyelid paralysis (Barsotti et al. 2010a, 2010b, 2012, Baine et al. 2016, Petritz et al. 2016). Gonçalves et al. (2006) applied 0.1ml of atracurium at a concentration of 10 mg/ml intracameral in the right eyeballs in 15 chickens of the same age. In the left bulbs, 0.1ml of normal saline was applied (control group). These authors conclude that the intracameral use of atracurium in chickens produces satisfactory mydriasis for intraocular procedures without clinical signs of systemic or contralateral effects. However, the ophthalmologic examination was performed only with focused light, which showed conjunctiva with no detectable evidence of inflammation, transparent chambers and a normal iridian movement, but no tools were used for more accurate evaluation, such as corneal pachymetry or microscopy techniques. Therefore, it was not possible to state the absence of endothelial damage.

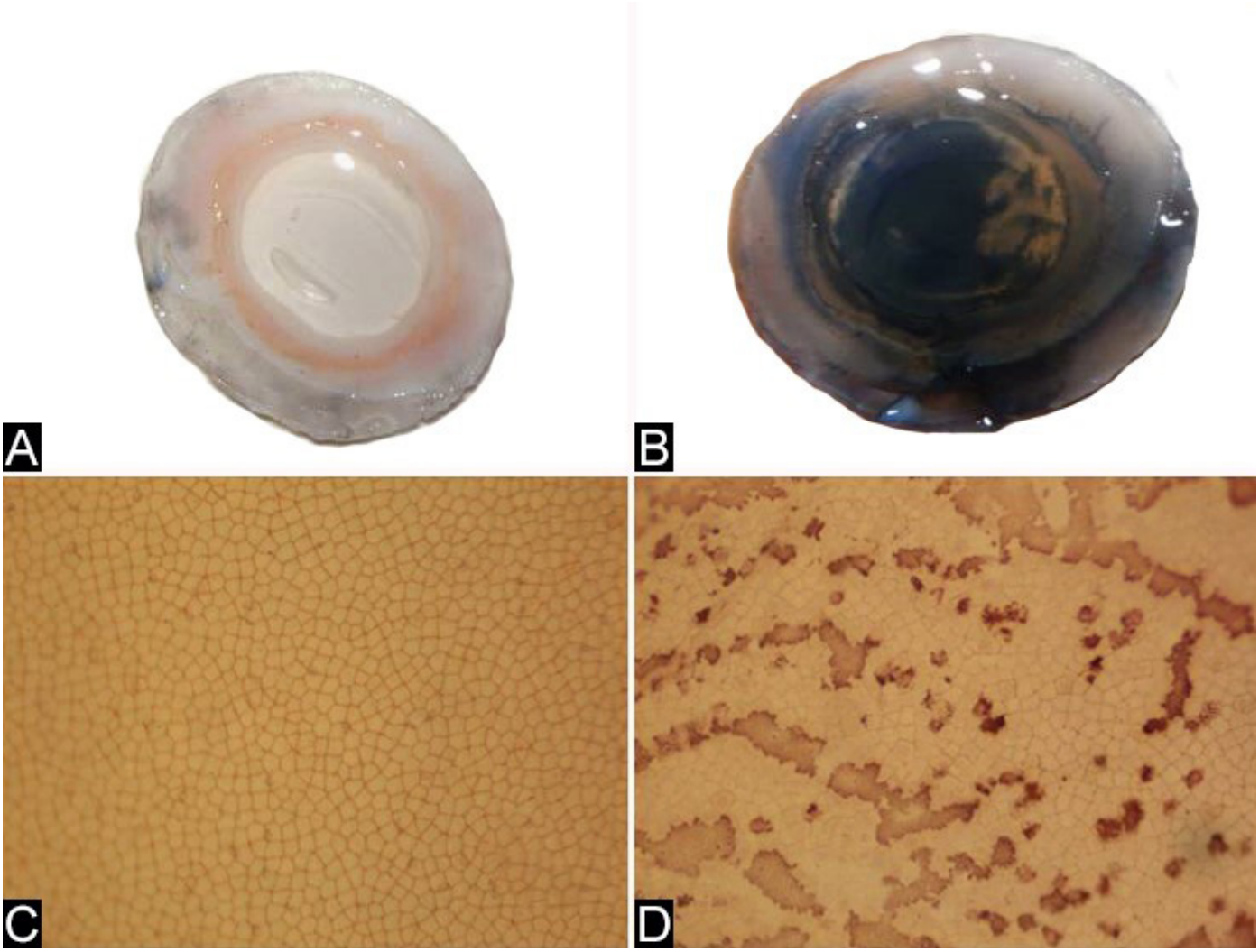

Fig.1. (**A**) Excised corneoscleral button of chicken eye with the endothelial side face up covered with BSS. (**B**) Corneoscleral button with the endothelial side face up after staining with trypan blue and alizarin red. (**C**) Optical photomicrograph of corneal endothelium from G2 stained with alizarin red. The endothelium has a regular polygonal appearance. Obj.10x. (**D**) Optical photomicrograph of experimental corneal endothelium stained with alizarin red showing areas of cell loss. Obj.10x.

Among used techniques to analyze the corneal endothelium are including mainly SEM, specular microscopy, and optical microscopy (Pigatto et al. 2006, 2008, Nagatsuyu et al. 2014, Kobashigawa et al. 2015, Coyo et al. 2016, Faganello et al. 2016).

SEM has been applied mainly for the analysis of endothelial cell ultrastructure of different species of animals and to evaluate the effects of medications, chemicals or surgical procedures on the endothelium (Pigatto et al. 2009, Tamayo-Arango et al. 2009, Terzariol et al. 2016). Specular microscopy is the technique most used for clinical evaluations in humans and animals (Pigatto et al. 2006, 2008, Franzen et al. 2010, Nagatsuyu et al. 2014, Albuquerque et al. 2015, Bercht et al. 2015, Kobashigawa et al. 2015, Coyo et al. 2016). Among the limitations of specular microscopy technique is the difficulty of obtaining images in corneas that are not transparent (Saad et al. 2008). The evaluation method of the corneal endothelium through the use of vital staining enables a simple, fast and convenient way to detect cell damage (Taylor & Hunt 1981, Faganello et al. 2016). This technique was applied in this study using alizarin red and trypan blue dyes and it has demonstrated to be a protocol that can be used to stain the corneal endothelium of chickens, since clear images were obtained of the endothelial monolayer. The alizarin red dyes the intercellular spaces and exposure of Descemet's membrane in areas where cell loss occurs, and the trypan blue stains the nuclei of dead cells, where the cell membrane is not intact (Saad et al. 2008). Some species of birds have had their corneal endothelium studied in relation to endothelial cell density, area, shape and cell morphology (Yee et al. 1987, Pigatto et al. 2009). Albuquerque et al. (2015) studied the corneal endothelium of chickens from different age groups by specular microscopy, which showed a regular pattern in the distribution of cells. The corneal endothelium of chickens was similar to that of other vertebrate species. Thus, the importance of the subject, coupled with the lack of data about the impact of atracurium besylate in the corneal endothelium, motivated this study. Despite there being no reports about cataract removal surgery in chickens, this becomes a viable model to investigate because of its corneal endothelium similarities with other species of birds.

The option of an *ex vivo* study using chicken's eyes from the slaughter line was a viable alternative, particularly from an ethical point of view, since avoided the death of animals for reasons relating solely to this research. Previous studies with enucleated eyes proved it is possible to analyze the cornea within six hours postmortem without structural changes occurring in the endothelium (Pigatto et al. 2008, 2009, Terzariol et al. 2016). The samples were divided into two groups, the experimental group consisting of the left eyes and the control group consisting of the right eyes. Previous studies reported a lack of differences regarding endothelial parameters obtained from the right and left eyes (Pigatto et al. 2008, 2009, Terzariol et al. 2016). Therefore, the evaluation of one eye allowed us to infer the same results for the contralateral eye. The existence of the control group is critical for the study since it evaluates the endothelium after handling performed in the processes of enucleation of the eye and excising the corneoscleral button. This analysis later helped in the statistical calculation to differentiate the damage caused by atracurium and manipulation, which would not be possible with a previous endothelial analysis with specular microscopy before the excision of buttons, for example. In this experiment it was possible to evaluate immediate effects in the chicken's corneal endothelium caused by exposure to atracurium in all corneas of G1. The areas of cell loss and endothelial denudation with Descemet's membrane exposure were stained by alizarin red. The corneas of G2 also had areas stained with alizarin red. The technique used for corneal excision and handling during the staining procedure can be responsible for those areas of cell loss. Corneal endothelial toxicity is related to substances that come in contact with the endothelium, based on their chemical compositions, pH and osmolarities (Edelhauser et al. 1981, Carter et al. 2007). The pH of atracurium besylate is 3.25 to 3.65, and its osmolarity is 10-30 mOsm/L which is below the range tolerated by the corneal endothelium in tested species. Therefore, the fact that it is acidic and hyposmotic relative to the aqueous humor can explain its toxic effect. The results of the present investigation as it was performed showed that atracurium induced significant damage in the cells of the endothelium of the cornea of chickens. It is important to emphasize that in the current study atracurium was placed in direct contact with the corneal endothelium of chickens for three minutes. When used intracameral the atracurium will be diluted by the aqueous humor and probably the endothelial damage will be smaller than that evidenced in the present study. In addition, the results obtained in the current study are related to the acute effect of atracurium on the corneal endothelium of chickens. The observed endothelial changes were not correlated with functional impairment of the endothelium. In this sense, future research evaluating the corneal endothelium using live animals after intracameral atracurium injection is necessary to elucidate these facts.

CONCLUSION

The results obtained with this research indicated that atracurium bezilate induced an acute damage on corneal endothelium of chickens.

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

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Original Article
Morphophysiology

B-mode and Doppler ultrasonography in the assessment of the common carotid arteries of equines and mules and the relation with body mass, age and neck circumference[1]

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ABSTRACT.- Fogaça J.L., Castiglioni M.C.R., Vettorato M.C., Andrade D.G.A., Puoli-Filho J.N.P., Fernandes M.A.R. & Machado V.M.V. 2019. **B-mode and Doppler ultrasonography in the assessment of the common carotid arteries of equines and mules and the relation with body mass, age and neck circumference.** *Pesquisa Veterinária Brasileira 39(1):75-84.* Departamento de Reprodução Animal e Radiologia Veterinária, Faculdade de Medicina Veterinária e Zootecnia, Universidade Estadual Paulista, Campus Botucatu, Rua Prof. Dr. Walter Mauricio Correra s/n, Rubião Junior, Botucatu, SP 18618-970, Brazil. E-mail: vania.mv.machado@unesp.br

As age increases, changes in cardiovascular anatomy and physiology occur, even in the absence of disease. Thus, studies of vessel hemodynamics are considered primordial to detect any cardiovascular changes. The objective of this study has been to describe the parameters of B-mode and spectral Doppler ultrasonography in the evaluation of the common carotid arteries of 11 equine and 11 mules, and correlate with age, body mass and neck circumferences. The diameters, intima - media thickness (IMT), resistivity index (RI), pulsatility index (PI), systolic velocity (SV), diastolic velocity (DV), maximum velocity (MV), vascular flow index (VFI), body mass, age, circumference and neck length. Ultrasonographic variables were evaluated in three different region called cranial, middle and caudal. Equine females presented higher values regarding the body mass, age and neck length, as compared to the neck circumferences of the animals, those of the mules were superior. The age of the mules had a positive correlation with the body mass, diameter and neck circumferences, it has a negative correlation between age and vessel diameters. The body mass of the mules had a positive correlation with age and vessel diameters, and with vessel diameters and neck circumferences in equine females. The RI and PI variables had a positive correlation with body mass for mules, and with age for equine females. The DV had a negative correlation with body mass for both equine and mule females. Regarding the variables MV and VFI, age correlated negatively for mules, while it was not significant for equine females. It found a difference between equine females and mules in the correlations performed, with body mass, age, neck circumferences and between B-mode and Doppler ultrasonography variables.

INDEX TERMS: B-mode, Doppler, ultrasonography, carotid arteries, equines, mules, body mass, age, neck circumference, horses.

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RESUMO.- [**Estudo de ultrassonografia modo-B e Doppler na avaliação das artérias carótidas comuns de equinos e muares e a relação com o peso, idade e a circunferência do pescoço.**] Com o aumento da idade ocorrem alterações na anatomia e fisiologia cardiovascular, mesmo na ausência de doenças. Assim, os estudos da hemodinâmica dos vasos são considerados primordiais para detectar quaisquer alterações cardiovasculares. Esse trabalho tem como objetivo descrever

as variáveis de ultrassonografia modo-B e Doppler espectral na avaliação das artérias carótidas comuns de 11 fêmeas equinas e 11 muares, e correlacionar com idade, massa corpórea e circunferências dos pescoços. Para tais procedimentos foram avaliados os diâmetros, espessura da camada íntima média (EIM), índice de resistividade (IR), índice de pulsatilidade (IP), velocidade sistólica (VS), velocidade diastólica (VD), velocidade máxima (VM), índice de vascularização de fluxo (IVF), massa corpórea, idade, circunferências e comprimentos dos pescoços. As variáveis ultrassonográficas foram avaliadas em três regiões diferentes denominados de crania médio e caudal. As fêmeas equinas apresentaram valores maiores referente ao massa corpórea, idade e comprimento dos pescoços, já em relação às circunferências dos pescoços dos animais, as dos muares foram superiores. A idade dos muares possuiu correlação positiva com a massa corpórea, diâmetro e com as circunferências dos pescoços, com as fêmeas equinas, possui correlação negativa entre idade e os diâmetros dos vasos. A massa corpórea dos muares teve correlação positiva com idade e diâmetros dos vasos, já às fêmeas equinas com diâmetros dos vasos e as circunferências dos pescoços. As variáveis IR e IP tiveram correlação positiva com a massa corpórea para os muares, e com idade para fêmeas equinas. O VD teve correlação negativa com a massa corpórea tanto para as fêmeas equinas quanto nos muares. Já as variáveis VM e IVF, a idade correlacionou negativamente para os muares, enquanto não foi significativo para as fêmeas equinas. Averiguou diferença entre fêmeas equinas e muares nas correlações realizadas, com a massa corpórea, idade, circunferências dos pescoços e entre as variáveis da ultrassonografia modo-B e Doppler.

TERMOS DE INDEXAÇÃO: Ultrassonografia, modo-B, Doppler, artéria carótida, equinos, muares, peso, idade, circunferência do pescoço.

INTRODUCTION

In equines, the common carotid artery is considered an important vessel. It irrigates the brain, neck and head and originates the internal carotid, external carotid and occipital arteries, besides several collateral branches, among them the muscular, tracheal and esophageal branches and also the cranial and caudal thyroid, ascending pharyngeal and parotid arteries (Getty 1981, Khamas et al. 2002, Ozgel et al. 2004, Krupinski et al. 2006). The diseases of the central nervous system (CNS) in equines and mules are a relatively important part of those treated by the veterinarian (Pessoa et al. 2014, Aguiar et al. 2014, Aguiar 2015).

In human medicine, the evaluation of the carotid is a fundamental routine examination due to the frequent occurrence of atheromas. However, atherosclerotic plaques are not so frequent in veterinary medicine, although there are already reports of its incidence in dogs and horses (Hess et al. 2003, Rosa et al. 2003, Ribeiro & Shintaku 2004, Aguiar et al. 2014). Another disturbance that affects the common carotid arteries is stenosis secondary to traumatic, neoplastic or inflammatory processes (Bonamigo & Lucas 2007, Freitas et al. 2008, Kandiah et al. 2014). Other diseases that can also affect these great arteries are atherosclerosis, stiffening secondary to senility or chronic lesions and dissection (Mendes & Themudo Barata 2008).

The guttural pouch is a structure which exists in horses and mules and is positioned between the base of the skull and the atlas. This structure consists of air-filled diverticula of the ear tubes (Eustachian), which communicate between the middle ear and the pharynx (Borges et al. 2005, Hayah 2011). There are diseases that affect the guttural sac and some of them are related to the carotid arteries, such as the aneurysm and fungal erosion of the internal carotid artery, which can seriously compromise this structure and even lead to the death of the animal in a few minutes. This is why diagnostic methods such as conventional or two-dimensional mode (B-mode) and spectral Doppler ultrasound can aid in the diagnosis of these diseases (Hayah 2011, Aguiar 2015).

B-mode ultrasonography is a two-dimensional, non-invasive and safe imaging modality that can be used to evaluate organs and tissues in real time. This technique allows the study of the diameter and thickness of the intima-media layer (IMT) of the common carotid arteries (Carvalho 2009). Spectral Doppler ultrasonography, on the other hand, is a tool that, coupled with conventional ultrasonography, allows the evaluation of the blood flow through the analysis of some variables such as resistivity index (RI), pulsatility index (PI), systolic velocity (SV), diastolic velocity (DV), maximal velocity (MV) and vascular flow index (VFI) (Cipone et al. 1997, Khamas et al. 2002, Torres et al. 2007, Aguiar 2015). Besides supplying information on the blood flow, the resistivity index (RI) and pulsatility index (PI) also inform on vessel impedance and compliance (Bailey et al. 2012, Aguiar 2015).

As a consequence of aging alterations take place in cardiovascular anatomy and physiology which are not considered pathological (Mendes & Themudo Barata 2008). In these cases, studies of vessel hemodynamics are primordial to detect any cardiovascular alterations, whether they are due to senility or to pathological processes (Paiva-de-Souza et al. 2010, Aguiar 2015). This study aimed at describing the B-mode and Doppler ultrasonography variables in the common carotid arteries of equine females and mules and at correlating them with age, body mass and neck circumferences in these animals.

MATERIALS AND METHODS

This study has been approved by the Animal Ethics Committe (Comissão de Ética no Uso de Animais, CEUA) of Faculdade de Medicina Veterinária e Zootecnia de Botucatu (FMVZ/Unesp) (Protocol no. 0100/2017) and carried out at Fazenda Edgárdia, Unesp Campus Botucatu, Faculdade de Medicina Veterinária e Zootecnia (FMVZ). Twenty-two animals belonging to the division of education, research and extension in equidae of FMVZ, Unesp Botucatu were used. The animals were classified in two groups: 11 healthy equine females of undefined breed (UB), with body mass between 348 and 486 kilograms (kg) and aged from 5 to 25 years, and 11 healthy mules (six males and five females) with body mass between 350 and 462 kg, aged from 4 to 12 years.

For the ultrasound examinations, the animals were taken to the individual containments by professional handlers. During the exams the animals were not subjected to fluid or solid food fasting nor given sedatives, to avoid any hemodynamic changes. For the ultrasound examinations, isopropyl alcohol was applied in the region to be examined at the concentration of 30% water and 70% alcohol, followed by silicone gel to protect the transducers. Alcohol dispenses with the need for trichotomy and, along with the gel, improves the conduction of ultrasound waves. The animals were positioned according to their convenience; however, all were kept with their heads above their withers, which became the standard, comfortable positioning.

Ultrasound examinations were performed using equipment of the MyLab®30 model from the Italian manufacturer Esaote. A cardiac *pre-set* was used for the analysis of the flow of the common carotid arteries. The *pre-set* allows the evaluation of high-flow structures without alteration of the angle of insonation. A linear transducer (3.0 to 11.0 megahertz (MHz), made by Esaote, Italy) was used to obtain the B-mode images, and a convex transducer (1.0 to 8.0 MHz, also made by Esaote, Italy) to obtain spectral Doppler images.

The B-mode and spectral Doppler ultrasounds were done in three regions of the neck: caudal, middle and cranial (Fig.1). They were then analyzed and processed to select the PI for the spectral Doppler ultrasonography images, to obtain the blood flow variables (PI, RI, SV, DV, MV and FVI). The B-mode images, on the longitudinal and transversal planes, were used for measuring the intima-media thickness (IMT) and the diameters of the common carotid arteries of the animals.

The anatomic spots in these regions were classified as caudal, middle and cranial measurement points. The caudal point was determined by a dorsoventral line tangent caudally to the seventh cervical vertebra. The middle point was considered as a dorsoventral line tangent caudally to the articular fovea of the fourth cervical vertebra. The cranial point was identified as a line tangent dorsally to the condyles of the occipital bone and ventrally to the angle of the jaw. All values of PI, RI, SV, DV, MV, VFI, IMT and the diameters of the common arteries, right and left, were analyzed and, after that, entered into a table drawn up using Microsoft Excel 2013 for statistical analysis, using The SAS System 9.0 software for the calculation of the mean and standard deviation in each group. Spearman's correlation test was applied between the variables of interest. The base of the neck, middle and cranial (near the head) circumferences of the necks were measured using a millimetric measuring tape, along with the length of the neck, to evaluate their correlation with body mass, age, diameter and IMT of the walls of the common carotid arteries, right and left, of the animals (Fig.2) and immediately analyzed statistically. This study considered a significance of 5%, that is, the null hypothesis was rejected when p-value was smaller or equal to 0.05.

RESULTS

Table 1 illustrates the mean and standard deviation of the variables body mass, age, neck and base of the neck, middle and cranial circumferences in the equine and mule females.

It should be noted that this sample had an average body mass of 368 kg in mules and 413 kg in equine females, while the average age was 8 years for mules and 14 for equine females.

As for the neck circumferences, the base of the neck averaged 104 centimeters (cm) in the mules as well as in the equine females. The medium region averaged 87cm in the mules and 83cm in the equine females; the cranial variable averaged 71cm both in the mules and in the equine females. Neck length measured an average of 39cm in the mules and 44cm in the equine females. Table 2 illustrates two statistics: the correlation value (r) and the p-value. The correlation values (r) indicate whether the correlation is positive, that is, if the increase in one variable is associated with the increase of the associated variable, or negative, when the variables are indirectly proportional.

In Table 2 it can be observed that the age of the mules had positive correlations with the body mass ($p=0.0140$), base of the neck ($p<0.001$), medium ($p=0.0003$), cranial ($p<0.001$) and longitudinal ($p<0.001$) and transversal diameter of the vessel ($p<0.001$).

The body mass variable showed positive correlations with age ($p=0.0140$), longitudinal diameter ($p=0.0407$), transversal diameter ($p=0.0403$) and base of the neck ($p<0.001$). There

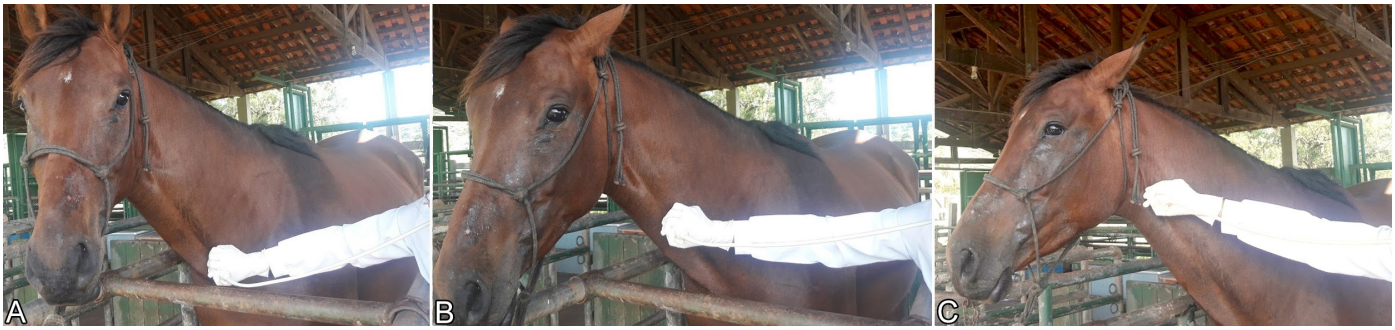

Fig.1. The images were obtained in the longitudinal and transversal plane of the animals, being (**A**) caudal, (**B**) medium and (**C**) cranial.

Fig.2. Measurements of the necks of the animals, being (**A**) cranial circumference, (**B**) medium and (**C**) base of the neck.

Table 1. Mean and standard deviation of descriptive variables in equine females and mules

Variables	Mules (N=11)				Equine females (N=11)			
	Mean	Standard deviation	Minimum	Maximum	Mean	Standard deviation	Minimum	Maximum
Body mass (kg)	368	72	175	462	413	42	348	486
Age (years)	8	3	3	12	14	7	5	25
Base of the neck (cm)	104	5	96	113	104	5	96	110
Medium (cm)	87	5	75	92	83	4	77	90
Cranial (cm)	71	7	52	77	71	6	63	83
Neck length (cm)	39	3	36	48	44	3	38	50

p-value<0.05.

Table 2. Spearman's correlation test between the variables of interest: age, body mass, base of the neck, medium, cranial, neck length, longitudinal and transversal diameter and longitudinal and transversal wall of mules

Variables	Statistic	Age	Body mass	Base of the neck	Medium	Cranial	Neck length
Age	r	1.00	0.32	0.53	0.46	0.61	0.31
	p-value		0.0140*	<0.001*	0.0003*	<0.001*	0.0165*
Body mass	r	0.32	1.00	0.60	-0.04	-0.04	-0.02
	p-value	0.0140*		<0.001*	0.7608	0.7623	0.8505
Base of the neck	r	0.53	0.60	1.00	0.60	0.65	0.69
	p-value	<0.001*	<0.001*		<0.001*	<0.001*	<0.001*
Medium	r	0.46	-0.04	0.60	1.00	0.88	0.85
	p-value	0.0003*	0.7608	<0.001*		<0.001*	<0.001*
Cranial	r	0.61	-0.04	0.65	0.88	1.00	0.83
	p-value	<0.001*	0.7623	<0.001*	<0.001*		<0.001*
Neck length	r	0.31	-0.02	0.69	0.85	0.83	1.00
	p-value	0.0165	0.8505	<0.001*	<0.001*	<0.001*	

Variables	Statistic	Longitudinal diameter	Transversal diameter	Longitudinal wall	Transversal wall
Age	r	0.70	0.63	0.09	0.08
	p-value	<.0001*	<.0001*	0.5265	0.5824
Body mass	r	0.29	0.29	-0.28	-0.06
	p-value	0.0407*	0.0403*	0.0497*	0.6710
Base of the neck	r	0.64	0.71	-0.02	0.05
	p-value	<.0001*	<.0001*	0.8925	0.7176
Medium	r	0.55	0.58	0.28	-0.02
	p-value	<0.001*	<0.001*	0.0397*	0.8589
Cranial	r	0.58	0.62	0.19	0.03
	p-value	<0.001*	<0.001*	0.1607	0.8322
Neck length	r	0.49	0.57	0.21	0.04
	p-value	0.0001*	<0.001*	0.1187	0.7719

* p-value<0.05.

was also a negative correlation with the longitudinal wall (p=0.0497).

For the base of the neck variable there were positive correlations with age (p<0,001), body mass (p<0.001), medium (p<0.001), cranial (p<0.001), neck length (p<0.001), longitudinal diameter (p<0.001), and transversal diameter (p<0.001).

Regarding the middle of the neck variable, there were positive correlations with age (p=0.0003), base of the neck (p<0.001), cranial (p<0.001) and neck length (p<0.001), longitudinal diameter (p<0.001), transversal diameter (p<0.001) and longitudinal wall (p=0.0397).

The cranial variable had positive correlations with age (p<0.001), base of the neck (p<0.001), middle of the neck (p<0.001), neck length (p<0.001), longitudinal diameter (p<0.001) and transversal diameter (p<0.001).

Table 3 shows the correlation of the variables of interest: body mass, age, diameters and IMT of the vessels and neck measurements in the equine females.

In Table 3 it can be observed that the age of equine females showed a positive correlation with the longitudinal wall (p=0.0431) and negative correlations with body mass (p=0.0040) and base of the neck (p=0.0217).

As for the body mass variable, there were positive correlations with the base of the neck (p<0.001), middle of the neck (p<0.001), cranial (p<0.001), longitudinal diameter (p=0.0022) and transversal diameter (p=0.0122), and negative correlation with age (p=0.0040).

For the base of the neck variable there were positive correlations with body mass (p<0.001), middle of the neck (p<0.001), cranial (p<0.001) and longitudinal diameter (p=0.0364), and negative correlation with age (p=0.0217).

For the middle of the neck variable there were positive correlations with body mass (p<0.001), base of the neck (p<0.001) and cranial (p<0.001).

The cranial variable showed positive correlations with body mass (p<0.001), base of the neck (p<0.001) and middle of the neck (p<0.001).

As for the neck length variable in the equine females, no statistically significant correlation was observed with the variables analyzed. Table 4 illustrates the correlation of the variables of interest: Doppler, age, body mass, longitudinal and transversal diameter, longitudinal and transversal wall of the blood vessels of mules.

Table 4 shows that the RI variable has positive correlations with PI (p<0.001), SV (p= 0.0367) and body mass (p=0.0044), and negative correlations with the transversal diameter (p=0.0254) and DV (p=0.0010).

For the PI variable there were positive correlations with RI (p<0.001) and body mass (p=0.0002), and negative correlations with DV (p=<0.001) and MV (p=0.0045).

The SV variable had positive correlations with MV (p<0.001), VFI (p<0.001), DV (p<0.001) and RI (p=0.0367), and negative correlations with age (p=0.0367), longitudinal diameter (p=0.0010) and transversal diameter (p=0.0011).

The DV variable had positive correlations with MV (p<0.001), VFI (p<0.001) and SV (p<0.001), and negative correlations with RI (p=0.0010), PI (p<0.001), body mass (p=0.0264) and age (p=0.0336).

As for the MV variable, positive correlations were found with VFI (p<0.001), SV (p<0.001) and DV (p<0.001), and negative correlations with age (p<0.001), body mass (p=0.0111), longitudinal diameter (p=0.0014), transversal diameter (p=0.0036) and PI (p=0.0045).

With respect to the VFI variable, there were positive correlations with MV (p<0.001), SV (p<0.001) and DV (p<0.001), and negative correlations with age (p=0.0006), longitudinal diameter (p=0.0055) and transversal diameter (p=0.0055). Table 5 illustrates the correlation of the variables: Doppler, age, body mass, longitudinal and transversal diameter, longitudinal and transversal blood vessel wall of equine females; and shows that the RI variable had positive correlations with PI (p<0.001), SV (p<0.001), VFI (p=0.0355), longitudinal (p<0,001) and transversal diameter (p=0.0002),

Table 3. Spearman's correlation test between the variables of interest: age, body mass, base of the neck, medium, cranial, longitudinal and transversal diameter and longitudinal and transversal wall of equine females

Variables	Statistic	Age	Body mass	Base of the neck	Medium	Cranial	Neck length
Age	r	1.00	-0.34	-0.28	-0.22	-0.05	-0.21
	p-value	_	0.0040*	0.0217*	0.0682	0.7097	0.0797
Body mass	r	-0.34	1.00	0.76	0.53	0.59	-0.20
	p-value	0.0040*	_	<0.001*	<0.001*	<0.001*	0.1010
Base of the neck	r	-0.28	0.76	1.00	0.77	0.72	0.01
	p-value	0.0217*	<0.001*	_	<0.001*	<0.001*	0.9535
Medium	r	-0.22	0.53	0.77	1.00	0.84	-0.16
	p-value	0.0682	<0.001*	<0.001*	_	<0.001*	0.1808
Cranial	r	-0.05	0.59	0.72	0.84	1.00	0.04
	p-value	0.7097	<0.001*	<0.001*	<0.001*	_	0.7563
Neck length	r	-0.21	-0.20	0.01	-0.16	0.04	1.00
	p-value	0.0797	0.1010	0.9535	0.1808	0.7563	_

Variables	Statistic	Longitudinal diameter	Transversal diameter	Longitudinal wall	Transversal wall
Age	r	0.07	0.08	0.28	0.09
	p-value	0.6314	0.5602	0.0431*	0.5375
Body mass	r	0.41	0.35	0.11	-0.01
	p-value	0.0022*	0.0122*	0.4435	0.9342
Base of the neck	r	0.29	0.17	-0.06	-0.14
	p-value	0.0364*	0.2148	0.6529	0.3171
Medium	r	0.01	0.03	-0.09	-0.14
	p-value	0.9173	0.8397	0.5211	0.3221
Cranial	r	0.22	0.17	0.10	-0.12
	p-value	0.1212	0.2277	0.4788	0.4085
Neck length	r	0.11	-0.09	-0.05	-0.07
	p-value	0.4401	0.5266	0.7367	0.6358

* p-value<0.05.

Table 4. Spearman's correlation test between the variables of interest: RI, PI, SV, DV, MV, VFI, age, body mass, longitudinal and transversal diameter and longitudinal and transversal wall of mules

Variables	RI	PI	SV	DV	MV	VFI
RI	1.00	0.93	0.26	-0.40	-0.15	-0.05
		<0.001*	0.0367*	0.0010*	0.2352	0.7107
PI	0.93	1.00	0.11	-0.48	-0.35	-0.23
	<0.001*		0.3703	<0.001*	0.0045*	0.0579
SV	0.26	0.11	1.00	0.61	0.85	0.84
	0.0367*	0.3703		<0.001*	<0.001*	<0.001*
DV	-0.40	-0.48	0.61	1.00	0.81	0.72
	0.0010*	<0.001*	<0.001*		<0.001*	<0.001*
MV	-0.15	-0.35	0.85	0.81	1.00	0.94
	0.2352	0.0045*	<0.001*	<0.001*		<0.001*
VFI	-0.05	-0.23	0.84	0.72	0.94	1.00
	0.7107	0.0579	<0.001*	<0.001*	<0.001*	

Variables	Age	Body mass	Longitudinal diameter	Transversal diameter	Longitudinal wall	Transversal wall
RI	-0.03	0.36	-0.23	-0.30	-0.11	-0.22
	0.8309	0.0044*	0.0945	0.0254*	0.4280	0.1077
PI	0.14	0.47	-0.07	-0.17	-0.17	-0.18
	0.2784	0.0002*	0.6001	0.2211	0.2031	0.1760
SV	-0.42	-0.12	-0.43	-0.42	-0.06	-0.18
	0.0008*	0.3653	0.0010*	0.0011*	0.6734	0.1836
DV	-0.27	-0.29	-0.22	-0.19	0.04	0.05
	0.0336*	*0.0264	0.0992	0.1504	0.7742	0.6948
MV	-0.52	-0.33	-0.42	-0.38	0.02	-0.03
	<0.001*	*0.0111	0.0014*	0.0036*	0.8829	0.8053
VFI	-0.43	-0.18	-0.37	-0.36	0.01	-0.10
	0.0006*	0.1580	0.0055*	0.0063*	0.9437	0.4603

RI = resistivity index, PI = pulsatility index, SV = systolic velocity, DV = diastolic velocity, MV = maximum velocity, VFI = vascular flow index; * p-value<0.05.

longitudinal blood vessel wall (p=0.0328) and age (p=0.0045), and negative correlation with DV (p=0.0029).

In the case of the PI variable, there were positive correlations with RI (p<0.001), SV (p<0.001), age (p=0.0020), longitudinal (p<0.001) and transversal diameter (p=0.0003), and negative correlation with DV (0.0043).

For the SV variable, there were positive correlations with MV (p<0.001), VFI (p<0.001), RI (p<0.001), PI (p<0.001) and DV (p=0.0002).

The DV variable showed positive correlations with MV (p<0.001), SV (p=0.0002) and VFI (p<0.001), and negative correlations with RI (p=0.0029), PI (0.0043), body mass (0.0392), longitudinal diameter (p=0.0009) and transversal diameter (p=0.0123).

The MV variable had positive correlations with VFI (p<0.001), SV (p<0.001) and DV (p<0.001).

The VFI variable had positive correlations with MV (p<0.001), SV (p<0.001), DV (p<0.001) and RI (p=0.0355).

DISCUSSION

Several studies about the carotid arteries have been done in a great number of animals, such as camels (Darweesh et al. 1989), buffaloes (Prakash & Rao 1976), bovines (Khamas & Mahdi 1984, Braun & Fohn 2005) and dogs (Hess et al. 2003), among others. However, studies involving B-mode and spectral Doppler ultrasonography have been few, especially involving these animals and equines. Some studies with B-mode and spectral Doppler ultrasonography of the common carotid arteries of equines have been reported (Cipone et al 1997, Schmucker et al. 2000, Aguiar 2015). However, reports involving studies on mules or any large species except equines have not been found in the literature consulted.

Mules are hybrid equidae originating from the crossing between mares and donkeys and, although equines and donkeys share a common ancestry, they have significant morphological differences. Thus, it is to be expected that mules will present some anatomo-physiological differences from equines. However, there are few studies on equines which do not involve domestic horses, and so there is a dearth of information on mules (Burnhan 2002, Alsafy et al. 2008, Smith 2009). It is important that studies about these animals be undertaken, and especially about their common carotid arteries, which are responsible for carrying the oxygen-rich blood flow directly to the brain. Any change in the functioning of these vessels can cause severe neurological issues in the individual (Rosa et al. 2003, Chequer et al. 2006, Kobayashi & Karino 2016).

The intima - media layer thickness (IMT) and the diameter of common carotid arteries provide structural and anatomic information, just as the cardiac cycle variation provides

Table 5. Spearman's correlation test between the variables of interest: RI, PI, SV, DV, MV, VFI, age, body mass, longitudinal and transversal diameter and longitudinal and transversal wall of equine females

Variables	RI	PI	SV	DV	MV	VFI
RI	1.00	0.94	0.59	-0.36	0.21	0.26
	_	<0.001*	<0.001*	0.0029*	0.0845	0.0355*
PI	0.94	1.00	0.57	-0.34	0.14	0.17
	<0.001*	_	<0.001*	0.0043*	0.2545	0.1646
SV	0.59	0.57	1.00	0.44	0.84	0.81
	<0.001*	<0.001*	_	0.0002*	<0.001*	<.0001*
DV	-0.36	-0.34	0.44	1.00	0.72	0.65
	0.0029*	0.0043*	0.0002*	_	<0.001*	<0.001*
MV	0.21	0.14	0.84	0.72	1.00	0.91
	0.0845	0.2545	<0.001*	<0.001*	_	<0.001*
VFI	0.26	0.17	0.81	0.65	0.91	1.00
	0.0355*	0.1646	<0.001*	<0.001*	<0.001*	_
Variables	Age	Body mass	Longitudinal diameter	Transversal diameter	Longitudinal wall	Transversal wall
RI	0.34	0.06	0.53	0.50	0.30	0.19
	0.0045*	0.6376	<0.001*	0.0002*	0.0328*	0.1889
PI	0.37	0.01	0.55	0.48	0.21	0.16
	0.0020*	0.9339	<0.001*	0.0003*	0.1273	0.2641
SV	0.16	-0.15	0.04	0.12	0.16	0.26
	0.1960	0.2171	0.7947	0.4000	0.2504	0.0612
DV	-0.04	-0.25	-0.45	-0.34	-0.08	0.09
	0.7424	0.0392*	0.0009*	0.0123*	0.5860	0.5465
MV	-0.01	-0.17	-0.26	-0.13	0.13	0.26
	0.9491	0.1727	0.0623	0.3508	0.3482	0.0611
VFI	-0.05	-0.18	-0.21	-0.10	0.15	0.23
	0.6900	0.1524	0.1408	0.4639	0.2912	0.0958

RI = resistivity index, PI = pulsatility index, SV = systolic velocity, DV = diastolic velocity, MV = maximum velocity, VFI = vascular flow index; * p-value<0.05.

functional information (Caviezel et al. 2013, Aguiar 2015). IMT and the diameter of common carotid arteries are essential variables to diagnose stenosis and the presence of atherosclerotic plaques, including in the equine and canine species (Rosa et al. 2003, Hess et al. 2003, Coll et al. 2013, Aguiar et al. 2014, Kiyota 2014).

Krejza et al. (2006) have carried out work involving B-mode and spectral Doppler ultrasonography for the evaluation of common carotid arteries in humans (306 women and 194 men). Measurements of the circumferences and lengths of necks were used to obtain the correlation with the diameter and IMT of the common carotid arteries. It was found that women had lower values for the diameter, IMT, body mass, circumference and neck length. The present research evaluated the common carotid arteries in three regions: caudal, middle and cranial. This was done because in equines and mules the common carotid arteries vary along their length (Furuhata 1964).

Mules averaged higher values for neck circumference in the middle region, while at the base of the neck and in terms of cranial circumference both mules and equine females showed equivalent values. Mules probably have higher values for neck circumference due to being small and robust animals (Chirgwin et al. 2000). As for body mass, age and neck length, it was found that equine females had higher values.

In the evaluation according to age, mules had positive correlations with body mass, longitudinal and transversal diameter and neck circumference (base of the neck, middle region and cranial). In equine females, age had a positive correlation with the vessels' longitudinal wall; similar to what was found by Aguiar (2015) in the Quarter horse breed, and negative correlations with body mass and the base of the neck. It was found that the age of equine females did not have significant correlations with the diameters of the common carotid arteries, unlike the results obtained by Aguiar (2015) with equines and Krejza et al. (2006), Torres et al. (2007), Freitas et al. (2008) and Coll et al. (2013) in humans.

Regarding the body mass variable, mules showed positive correlations with age, longitudinal and transversal diameter of the neck and base of the neck. On the other hand, equine females were found to have positive correlations with the longitudinal and transversal diameter and with neck circumference, including neck length. It was possible to ascertain that the body mass of all animals influenced in the longitudinal and transversal diameter of the common carotid arteries, similarly to what had been found by Higa (2009) and Denarie et al. (2000) and Zanini (2012) in humans and by Cipone et al. (1997) and Aguiar (2015) in equines.

Body mass also influenced the neck circumference of equine females (base of the neck region and head), as did age in mules.

This correlation of body mass with neck circumference had already been mentioned by Krejza et al. (2006) in humans, and males showed a wider variability.

Regarding spectral Doppler parameters, it was observed that the RI variable of mules showed a positive correlation with PI, SV and body mass, and negative correlations with DV and transversal diameter. In equine females there were positive correlations with PI, SV, VFI, age and longitudinal and transversal diameter, and negative correlation with DV, that is, there was a positive correlation between the variable RI with body mass in mules, while in equine females that correlation is with age, as reported by Aguiar (2015) in equines and by Barbosa et al. (2006) in humans. A positive correlation was also found between RI and the longitudinal and transversal diameter of the neck in equine females, while in mules this correlation was negative.

Very high values of RI reduce DV, and, depending on the issue that is causing the increase in resistance of the vessel, DV absence may be observed. SV is the most reliable measuring variable to determine the degree of obstruction of the vessel studied, since the reduction in blood flow occurs after stenosis, but can also be observed when there is a reduction of DV (Bragato 2013). Thus, hemodynamic indices such as RI, PI and SV help in the comparison between SV and DV, collaborating in the diagnosis of stenosis or thrombosis of peripheral vessels with increased resistance (Hedrick et al. 1995).

In humans it is observed that, depending on the degree of stenosis of the common or internal carotid artery, an alteration of the distal blood flow may occur. Light degrees of stenosis result in reduced blood flow (VFI), increase of SV near the point of stenosis and increase of RI and PI. These alterations point to an increase in resistance and a reduction of the vascular compliance of the carotid artery, that is, a reduction of the arterial blood flow through the artery in question. Severe degrees may result in significant reduction of the blood flow speed (SV, MV, DV), or the flow may become absent or even negative (reverse) (Johnston et al. 1981, Kaproth-Joslin et al. 2014).

These findings are normally more prominent the closer the measurement point is to the change, although they may also be observed in cases of vascular spasm, carotid dissection and in inflammatory processes. Another common occurrence is increased arterial blood flow through the contralateral carotid to compensate for the reduced blood flow in the brain and thus avoid ischemic brain lesions. On the other hand, the alteration of the blood flow in the two common carotids is more indicative of a diffuse arterial inflammatory process or of intracranial changes such as increased intracranial pressure or diffuse intracerebral vascular spasm (Johnston et al. 1981, Kaproth-Joslin et al. 2014).

The PI has been suggested for the standardization of blood flow, as it is more sensitive to differentiate abnormal waves (Moreira et al. 2008), and both PI and RI are measured independently of the angle of insonation (Pozor & McDonnell 2004, Bailey et al. 2012).

Regarding the PI variable of mules, positive correlations were observed with RI and body mass, and negative correlations with DV and MV. On the other hand, in equine females the PI variable showed positive correlations with RI, SV, age and longitudinal and transversal diameter, and negative correlation with DV, that is, a positive correlation was observed between the PI and body mass in mules. As for female equines, that correlation was observed with age, as found by Aguiar (2015) in equines and Barbosa et al. (2006) in humans.

The RI normally has a positive correlation with the PI and negative correlation with DV (Carvalho 2009, Kiyota 2014), and this correlation has been observed in both equine and mule females, as this procedure allows the evaluation of vessel resistance and blood flow speed. High values of RI indicate lower arterial elasticity, that is, greater vascular resistance in the irrigated region. There is, however, a correlation between the increase of carotid RI and atherosclerosis risk factors (Carvalho 2009, Kiyota 2014).

For the SV variable, positive correlations were observed with MV, VFI, DV and RI, and negative correlations with age, longitudinal and transversal diameter in mules. In the case of equine females, positive correlations were found with MV, VFI, DV, PI and RI, and no negative correlation, contrasting with what has been reported by Cipone et al. (1997), who observed a negative correlation with vessel diameters in equines. In the present study this was observed statistically only in mules, unlike what Hansen et al. (1995) reported in humans, in whom there is a positive correlation between these variables.

According to Appleton & Hatle (1992), in humans SV is inversely proportional to age, and in this work this has been statistically identified only in mules, and was not found to be relevant in equine females.

As for the DV variable in mules, it showed positive correlations with MV, VFI and SV, and negative correlations with RI, PI and body mass. In equine females positive correlations were found with MV, VFI and SV, and negative correlations with RI, PI, body mass and transversal diameter. It was observed that both in mules and equine females body mass is inversely proportional to the DV variable, just as pointed out by Appleton & Hatle (1992) in humans.

In mules, the MV variable had positive correlations with the VFI, SV and DV variables, and negative correlations with PI, age, body mass, longitudinal and transversal diameter. Regarding equine females, positive correlations were observed with VFI, SV and DV and no negative correlation, unlike what was reported by Cipone et al. (1997), who found a negative correlation with vessel diameters in equines. However, this was statistically verified only in mules.

Schmidt-Trucksass et al. (1999) observed an indirectly proportional correlation between MV and age in humans, a response identical to that found in mules. However, the association between these variables has been studied and correlated with age (Barbosa et al. 2006).

As for the VFI variable, positive correlations were noticed with MV, SV and DV, and negative correlations with age, longitudinal and transversal diameter in mules. In equine females only positive correlations with MV, SV, DV and RI were observed. In spite of the results noted about VFI, there are still no reports of any correlation involving this variable in equines in the consulted literature.

CONCLUSIONS

The correlations analyzed showed variations between equine and mule females.

Neck circumference is greater in mules than in equine females, but length is greater in equines.

In mules age influences neck circumference, while in equine females it influences body mass.

All neck regions are positively correlated in all animals.

Vessel diameters are related with all regions in mules, but in equine females only with the base of the neck.

The resistivity index (RI) and pulsatility index (PI) are positively correlated with body mass in mules and with age in equine females.

Systolic velocity (SV) influences age negatively only in mules, and diastolic velocity (DV) influences body mass in all animals.

Maximum speed (MV) and vascular flow index (VFI) are negatively connected with age and with diameters only in mules.

Conflict of interest statement. - The authors report no conflict of interest.

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Original Article
Animal Morphophysiology

DNA damage and primordial follicle activation after *in vitro* culture of sheep ovarian cortex in *Morus nigra* leaf extract[1]

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ABSTRACT.- Gouveia B.B., Barberino R.S., Menezes V.G., Macedo T.J.S., Cavalcante A.Y.P., Gonçalves R.J.S., Almeida J.R.G.S. & Matos M.H.T. 2019. **DNA damage and primordial follicle activation after *in vitro* culture of sheep ovarian cortex in *Morus nigra* leaf extract.** *Pesquisa Veterinária Brasileira 39(1):85-92*. Colegiado de Medicina Veterinária, Laboratório de Biologia Celular, Citologia e Histologia, Universidade Federal do Vale do São Francisco, Rodovia BR-407 Km 12, Lote 543, Projeto de Irrigação Nilo Coelho s/n C1, Petrolina, PE 56300-990, Brazil. E- mail: bruna.bortoloni@gmail.com

This study evaluated the effect of *Morus nigra* leaf extract, with or without supplementation, on morphology, activation and DNA damage of preantral follicles cultured within sheep ovarian tissue. Ovaries were collected and divided into fragments, being one fixed for histological and Terminal deoxynucleotidyl transferase (TdT) mediated dUTP nick-end labeling (TUNEL) analysis (fresh control). The remaining fragments were cultured for 7 days in alpha minimum essential media (α-MEM) supplemented with bovine serum albumin (BSA), insulin, transferrin, selenium, glutamine, hypoxanthine and ascorbic acid (α-MEM$^+$; control medium) or into medium composed of *M. nigra* extract without supplements (0.1; 0.2 or 0.4mg/mL) or supplemented with the same substances described above for α-MEM$^+$ (MN 0.1$^+$; 0.2$^+$ or 0.4$^+$mg/mL). Then, tissues were destined to histological and TUNEL analysis. The α-MEM$^+$ treatment had more morphologically normal follicles than all *M. nigra* extract treatments. However, α-MEM$^+$ treatment also showed signs of atresia because the percentage of TUNEL positive cells was similar in α-MEM$^+$ and in 0.1mg/mL *M. nigra* without and with supplements. Moreover, a reduction in the primordial follicles and an increase in the growing ones were observed in all treatments, except 0.2mg/mL *M. nigra*. In conclusion, the follicles cultured at 0.1mg/mL *M. nigra* extract were in good condition and able to continue their development, as demonstrated by the same rates of DNA damage and follicular activation as the control medium.

INDEX TERMS: DNA damage, *Morus nigra,* follicle growth, in vitro culture, ovarian cortex, leaf extraction, Oocyte, medicinal plant, apoptosis, ovarian tissue culture, sheep.

RESUMO.- [**Dano ao DNA e ativação do folículo primordial após a cultivo *in vitro* do córtex ovariano de ovelha em extrato de folha de *Morus nigra*.**] Este estudo avaliou o efeito do extrato das folhas de *Morus nigra*, com ou sem suplementos, sobre a morfologia, a ativação e o dano ao DNA de folículos pré-antrais cultivados inclusos em tecido ovariano. Os ovários foram coletados e divididos em fragmentos, sendo um fixado para análise histológica e ensaio de marcação de terminações dUTP mediada por desoxinucleotidil transferase terminal (TUNEL) (controle fresco). Os fragmentos restantes foram cultivados durante 7 dias em meio essencial mínimo alfa (α-MEM) suplementado com albumina sérica bovina (BSA), insulina, transferrina, selênio, glutamina, hipoxantina e ácido ascorbico (α-MEM$^+$; meio controle) ou em meio composto de extrato de *M. nigra* sem suplementos (0,1; 0,2 or 0,4mg/mL) ou suplementado com as mesmas substâncias descritas para α-MEM$^+$ (MN 0,1$^+$; 0,2$^+$ or 0,4$^+$mg/mL). Então, os tecidos foram destinados à análise histológica e TUNEL. O tratamento do

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α-MEM+ apresentou mais folículos morfologicamente normais que todos os tratamentos do extrato de *M. nigra*. No entanto, o tratamento com α-MEM+ também mostrou sinais de atresia, pois a porcentagem de células TUNEL positivas foi semelhante em α-MEM+ e em 0,1mg/mL *M. nigra* sem e com suplementos. Além disso, observou-se uma redução nos folículos primordiais e um aumento nos folículos em crescimento em todos os tratamentos, exceto 0,2mg/mL *M. nigra*. Em conclusão, os folículos cultivados com 0,1mg/mL de extrato de M. nigra estavam em boas condições e aptos a continuar seu desenvolvimento, como demonstrado pelas taxas de dano ao DNA e de ativação folicular semelhantes ao meio controle.

TERMOS DE INDEXAÇÃO: DNA, folículo primordial, cultivo *in vitro*, córtex ovariano, ovelha, extrato de folha, *Morus nigra*, crescimento folicular, Oócito, planta medicinal, apoptose, cultivo de tecido ovariano, ovinos.

INTRODUCTION

To maximize the reproductive potential of fresh and cryopreserved ovarian tissue it is necessary to develop culture systems that support the activation of primordial follicles as this is the most abundant stage of follicle development present in mammalian ovaries. Therefore, culture of ovarian tissue would support the initiation and maintenance of primordial and primary follicle growth to the secondary stage of development, which could be isolated and cultured to the antral stage, supporting oocyte growth to full size. The development of these technologies in association with ovarian cryopreservation holds many possibilities for clinical practice, animal production technology, and can also be used as a research tool to investigate the biology and toxicology of oogenesis in both animals and humans (Picton et al. 2008).

To achieve this goal, further optimization of the culture medium is required to sustain high levels of primordial follicle activation and growth *in vitro*. Studies have shown that α-Minimum Essential Medium (α-MEM) to which different supplements (hormones, antioxidants and/or growth factors) have been added promoted goat (Berrocal et al. 2016) and sheep (Santos et al. 2014) follicular development *in vitro*. However, in order to reduce the costs of the media and supplements used for follicle culture, extracts of medicinal plants have attracted increasing attention as natural compounds with antioxidant properties (Barberino et al. 2016, Gouveia et al. 2016).

Morus nigra L. is an arboreal plant found in temperate to subtropical regions of the Northern hemisphere to the tropics of the Southern hemisphere (Ercisli & Orhan 2007), being widely used in folk medicine as anti-inflammatory, diuretic, antioxidant and antidiabetic (Nickavar & Mosazadeh 2010, Radojkovic et al. 2016). Phenolic compounds with antioxidant activities (flavonoids, stilbenes, coumarins, rutin, isoquercetin and kaempferitrin) have been isolated from the bark and leaves of this plant (Mazimba et al. 2011, Cavalcante et al. 2017), which may reduce the levels of reactive oxygen species (ROS) and modulate the expression of antioxidant enzymes in different cell types (Mata-Campuzano et al. 2012, Nafees et al. 2015).

A recent study by our team has shown that 0.05mg/mL of *M. nigra* leaf extract can be used as a preservation medium for ovine ovarian tissue at 4°C, maintaining follicular survival and decreasing DNA fragmentation in comparison to control medium (MEM) (Cavalcante et al. 2017). Other study showed that *M. nigra* extract has a protective action against peroxidative damage to biomembranes and biomolecules of rats and human (Naderi et al. 2004). Knowing that oxidative stress initiates apoptosis through the increase of ROS after the culture of follicular cells (Devine et al. 2012), it can be hypothesized that *M. nigra* extract and its antioxidant properties may be a useful and cheap substitute for follicle culture medium.

As there are no reports in which *M. nigra* extract has been used as a culture medium for ovine ovarian tissue, this study was conducted to evaluate the effect of this extract on *in vitro* morphology, primordial follicle activation and DNA fragmentation of follicles cultured within slices of the ovine ovarian cortex. Furthermore, the effectiveness of the addition of supplements to *M. nigra* leaf extract was studied.

MATERIALS AND METHODS

Unless indicated, supplements and chemicals used in the present study were purchased from Sigma Chemical Co. (St Louis/MO, USA). This project was approved by the Ethics Committee on Human and Animal Studies of Federal University of São Francisco Valley (protocol No. 0008/040713).

Plant material and extract preparation. Fresh leaves of M*orus nigra* were collected in Petrolina (09°23'55" S and 38-40°30'03" W, Pernambuco, Brazil). A voucher specimen (1764) is deposited at the Herbário Vale do São Francisco (HVASF) of the Universidade Federal do Vale do São Francisco. The leaves were dried in an oven at 40ºC and pulverized and extracted at room temperature with 95% ethanol (Vetec, Duque de Caxias-RJ, Brazil) for 72h. The extract was dried at 45ºC using a rotavapor and this method yields approximately 10%, thus obtaining the crude ethanolic extract of the leaves of *M. nigra*. Thereafter, the extract was dissolved in 0.9% saline solution, corresponding to concentrations of 0.1; 0.2 or 0.4mg/mL.

Ovaries collection. Ovarian cortical tissues (n=8 ovaries) were collected at a local abattoir from four adult (1-3 years old) mixed-breed sheep. Immediately postmortem, pairs of ovaries were washed once in 70% alcohol (Dinâmica, São Paulo, Brazil) and then twice in Minimum Essential Medium buffered with HEPES (MEM-HEPES) and supplemented with antibiotics (100µg/mL penicillin and 100µg/mL streptomycin). Next, the ovaries were transported within 1h to the laboratory in tubes containing MEM-HEPES with antibiotics at 4°C (Chaves et al. 2008).

Experimental design for the in vitro culture. The *in vitro* culture was performed according to a previous study (Santos et al. 2014). In the laboratory, the surrounding fatty tissues and ligaments were stripped from the ovaries; large antral follicles and corpora lutea were removed. The pair of ovaries from each animal were divided into 8 fragments approximately 3×3mm (1mm thick), under sterile conditions. For each animal, one slice of tissue was randomly selected and immediately fixed for histological and TUNEL analysis (fresh control). The remaining slices of ovarian cortex were cultured individually in 1mL of culture medium in 24-well culture dishes for 7 days; the culture conditions were 39°C in an atmosphere of 5% CO_2 in air. The base culture medium (control) consisted of α-MEM (Gibco, Invitrogen, Karlsrushe, Germany, pH 7.2-7.4) supplemented with 10ng/mL insulin, 5.0µg/mL transferrin, 5.0ng/mL sodium selenite, 2mM glutamine, 2mM hipoxanthine, 1.25mg/mL BSA and 50µg/mL ascorbic acid and then referred as α-MEM+. To test the effect of the plant extract, ovarian fragments were cultured in α-MEM+ (control medium) or in medium composed of different concentrations of *Morus nigra* extract (*M. nigra* diluted in saline solution) without supplements (MN 0.1, 0.2 or 0.4mg/mL, pH 7.2-7.4) or *M. nigra*

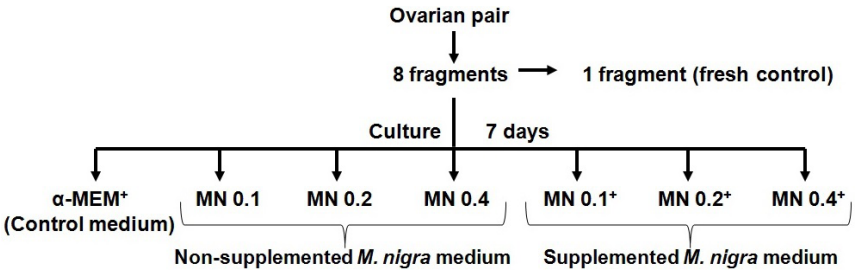

Fig.1. General experimental protocol for *in situ* culture of ovine preantral follicles in *Morus nigra* extract.

extract supplemented with the same substances described above for α-MEM$^+$ supplementation. The *M. nigra* supplemented medium was named MN 0.1$^+$; 0.2$^+$ or 0.4$^+$mg/mL (Fig.1). The culture medium was replenished every other day. Each treatment was repeated 4 times, thus using the ovaries of 4 different animals.

Morphological analysis of preantral follicles. Ovarian fragments from the fresh control and each cultured treatment were fixed individually in 4% buffered paraformaldehyde (Dinâmica, São Paulo, Brazil) for 18 hours. Subsequently, fragments were dehydrated in a graded series of ethanol (Dinâmica, São Paulo, Brazil), clarified with xylene (Dinâmica, São Paulo, Brazil) and embedded in paraffin wax (Dinâmica, São Paulo, Brazil). Tissues were serially sectioned at a thickness of 5µm and sections were stained using standard protocols with hematoxylin-eosin (Vetec, Duque de Caxias/RJ, Brazil). Sections were examined by light microscopy (Nikon, Tokyo, Japan) at 400× magnification (Cavalcante et al. 2017).

Preantral follicles were counted and evaluated in the section where the oocyte nucleus was visible. These follicles were classified individually, according to the quality aspect, as histologically normal when an intact oocyte is present and surrounded by granulosa cells that are well organized in one or more layers and have no pyknotic nuclei. Atretic follicles were defined as those with a retracted oocyte, pyknotic nucleus, and/or disorganized granulosa cells detached from the basement membrane. Overall, 120 follicles were evaluated in each treatment (30 follicles per treatment x 4 replicates = 120 follicles), totaling 960 preantral follicles.

The evaluation of follicular activation (transition from primordial to growing follicles) was performed by quantifying normal follicles at different classes of follicular development (Silva et al. 2004), as primordial (one layer of flattened granulosa cells around the oocyte) or growing follicles (intermediate: one layer of flattened to cuboidal granulosa cells; primary: one layer of cuboidal granulosa cells, and secondary: two or more layers of cuboidal granulosa cells around the oocyte). The proportion of primordial and growing follicles was calculated at day 0 (fresh control) and after 7 days of culture.

Assessment of DNA fragmentation by TUNEL assay. Terminal deoxynucleotidyl transferase (TdT) mediated dUTP nick-end labeling (TUNEL) assay was used for a more in-depth evaluation of ovine preantral follicle quality before (fresh control) and after culture in the different media. TUNEL was performed using a commercial kit (In Situ Cell Death Detection Kit, Roche Diagnostics Ltd., Indianapolis, USA) following the manufacturer's protocol and as previously described (Santos et al. 2014), with some modifications. Briefly, sections (5µm) mounted on glass slides were deparaffinized and rehydrated through graded alcohols, then rinsed in PBS (pH 7.2). The slides were incubated in citrate buffer (Dinâmica) at 95°C in a deckloaking chamber (Biocare, Concord/CA, USA) for 40min to retrieve antigenicity, and endogenous peroxidase activity was prevented by incubation with 3% H_2O_2 (Dinâmica) and methyl ethanol (QEEL, São Paulo, Brazil) for 10 min. After rinsing in Tris buffer (Dinâmica), the sections were incubated with TUNEL reaction mixture at 37°C for 1h. Then, the specimens were incubated with Converter-POD in a humidified chamber at 37°C for 30 min. The DNA fragmentation was revealed by incubation of the tissues with diaminobenzidine (DAB; 0.05% DAB in Tris/HCl pH 7.6, 0.03% H_2O_2) during 1 min. Finally, sections were counterstained with Harry's haematoxylin in a dark chamber at room temperature for 1 min, dehydrated in ethanol, cleared in xylene, and mounted with balsam (Dinâmica). For negative controls (reaction controls), slides were incubated with label solution (without terminal deoxynucleotidyl transferase enzyme) instead of TUNEL reaction mixture.

Only follicles that contained an oocyte nucleus were analyzed by TUNEL assay (Santos et al. 2014). The number of brown TUNEL positive cells (oocyte and granulosa cells) was counted in ten randomly fields per treatment using Image-Pro Plus® software. The percentage of DNA fragmentation was calculated as the number of TUNEL-positive cells out of the total number of cells (x 100).

Statistical analysis. Percentages of morphologically normal follicles and follicular activation were submitted to ANOVA and the Tukey´s test was applied for comparison among treatments. Data from TUNEL-positive cells were submitted Chi-squared test and expressed as percentages. Differences were considered to be statistically significant when $P<0.05$.

RESULTS

Follicular morphology and development after *in vitro* culture

The preantral follicles from the control tissue showed centrally located oocytes and granulosa cells surrounded by normal intact basement membranes (Fig.2A). Normal follicles could also be observed after culture in control medium (Fig.2B). However, after 7 days of culture in 0.2mg/mL of *Morus nigra* extract without supplements, atretic follicles with a retracted oocyte and disorganized granulosa cells could be observed (Fig.2C).

The percentage of morphologically normal follicles (follicular survival) decreased ($P<0.05$) after 7 days of culture in all treatments, compared to the fresh control (76%; Fig.3). In addition, there were more ($P<0.05$) morphologically normal follicles in α-MEM$^+$ (53.5%) than in *M. nigra* extract (average of 40.3%). However, there was no significant difference in the rate of follicular survival among the *M. nigra* concentrations, with or without supplements ($P>0.05$).

In all culture conditions, a significant reduction in the percentage of primordial follicles, reflecting an increase in the percentage of growing ones, was observed in all treatments

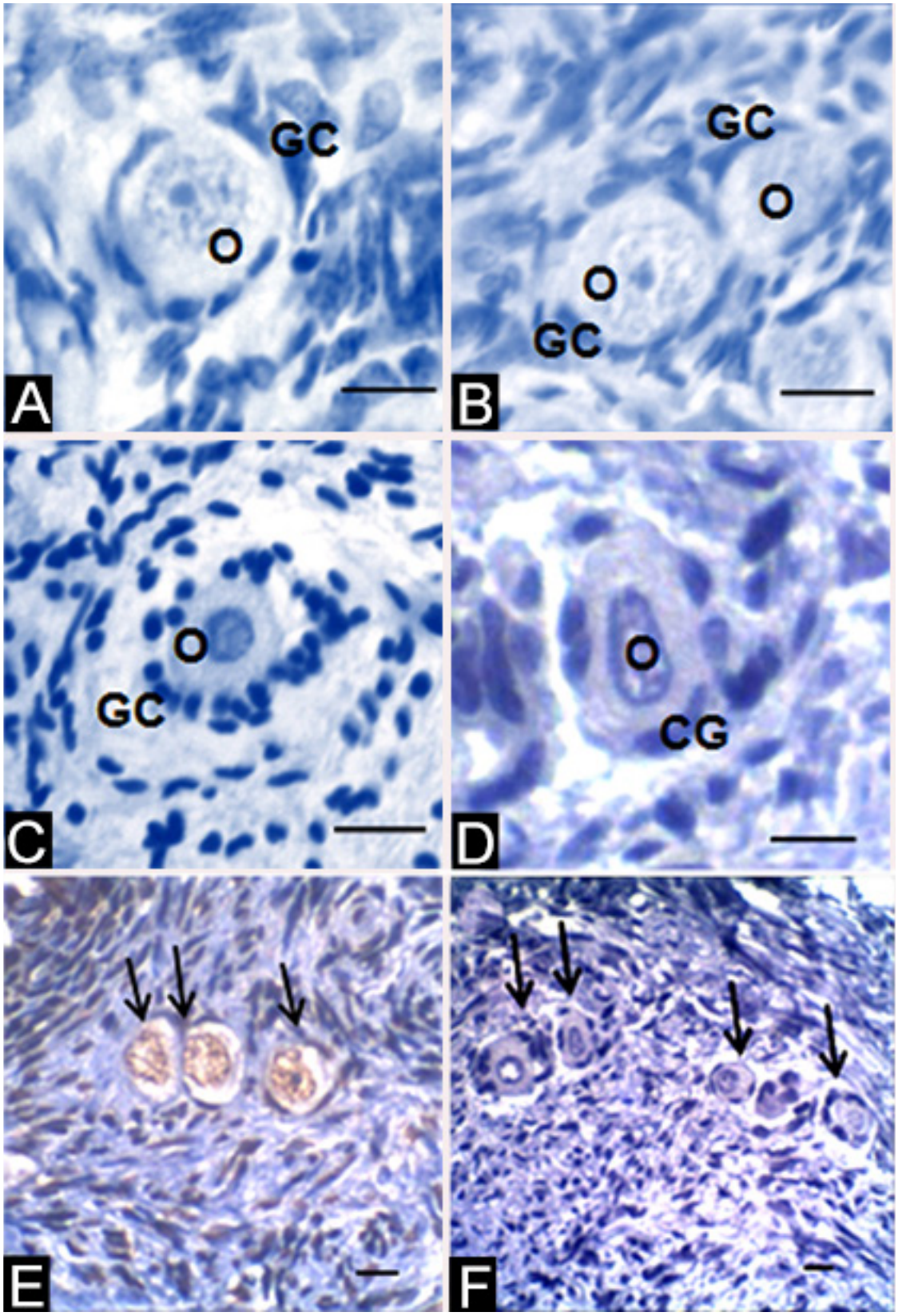

Fig.2. Histological sections of ovine ovarian fragments showing (**A**) morphologically normal follicles in the fresh control and (**B**) normal follicle after 7 days of culture in α-MEM⁺ or (**C**) atretic follicle in 0.2mg/mL *Morus nigra* extract without supplements. (**D**) Normal preantral follicle in 0.1mg/mL *M. nigra* extract, DNA damage follicle in 0.4mg/mL *M. nigra* extract (E) and negative control (F). (**E**,**F**) TUNEL-positive cells detection in ovine ovarian tissue after 7 days of culture. O = oocyte, GC = granulosa cell. Arrows indicate the follicles. TUNEL counterstained by hematoxylin, bar = 30μm.

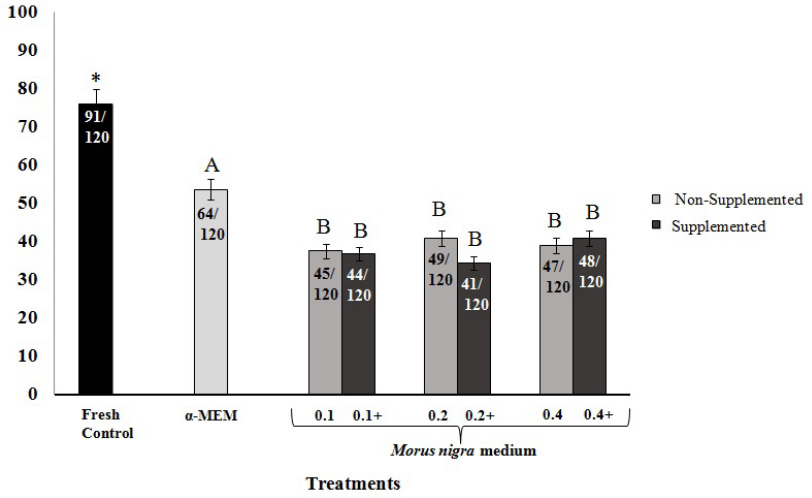

Fig.3. Percentages (mean ± SEM) of morphologically normal follicles in the fresh control and after culture in α-MEM or *Morus nigra* extract. MN 0.1 = 0.1mg/mL non-supplemented *M. nigra* extract, MN 0.1⁺ = 0.1mg/mL supplemented *M. nigra* extract, MN 0.2 = 0.2mg/mL non-supplemented *M. nigra* extract, MN 0.2⁺ = 0.2mg/mL supplemented *M. nigra* extract, MN 0.4 = 0.4mg/mL non-supplemented *M. nigra* extract, MN 0.4⁺ = 0.4mg/mL supplemented *M. nigra* extract. * Differs significantly from fresh control (P<0.05); [A, B] different letters denote significant differences among treatments (different media, P<0.05).

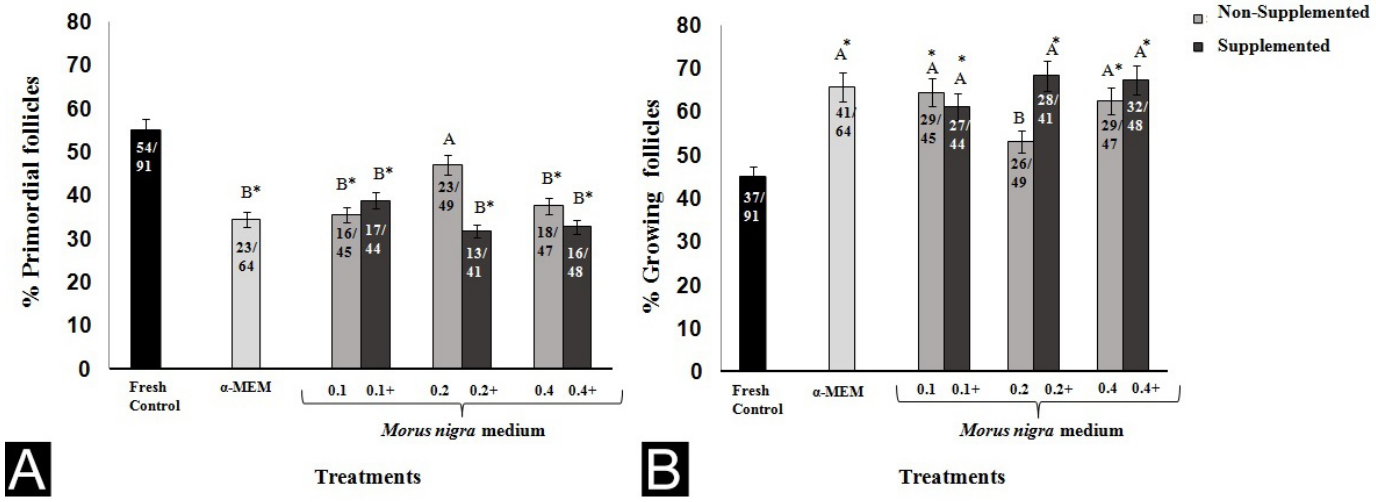

Fig.4. Percentages (mean ± SEM) of (**A**) normal primordial and (**B**) growing follicles in the fresh control and after 7 days of *in vitro* culture in α-MEM or *Morus nigra* extract. MN 0.1 = 0.1mg/mL non-supplemented *M. nigra* extract, MN 0.1⁺ = 0.1mg/mL supplemented *M. nigra* extract, MN 0.2 = 0.2mg/mL non-supplemented *M. nigra* extract, MN 0.2⁺ = 0.2mg/mL supplemented *M. nigra* extract, MN 0.4 = 0.4mg/mL non-supplemented *M. nigra* extract, MN 0.4⁺ = 0.4mg/mL supplemented *M. nigra* extract. * Differs significantly from fresh control (P<0.05); [A, B] different letters denote significant differences among treatments (P<0.05).

compared to fresh control group, except in ovarian tissue cultured in 0.2 mg/mL of *M. nigra* extract without supplements (Fig.4).

DNA fragmentation after culture

Ovarian follicles cultured in 0.1mg/mL of *M. nigra* without supplements did not show or showed less TUNEL-positive cells (Fig.2D). Nevertheless, oocyte DNA damage was commonly found after culture in 0.4mg/mL of *M. nigra* without supplements (Fig.2E). Negative controls did not show staining for TUNEL analysis (Fig.2F).

The percentage of TUNEL-positive cells in ovine preantral follicles before and after *in vitro* culture is shown in Figure 5. Culture of ovarian tissue for 7 days increased (P<0.05) the percentage of TUNEL-positive cells in all treatments compared to the fresh control. The percentage of DNA fragmentation in follicles cultured in α-MEM⁺ was similar (P>0.05) to that observed in 0.1mg/mL of *M. nigra* extract without or with supplements. Nevertheless, culture of follicles in 0.2 or 0.4mg/mL of *M. nigra* (in the absence or presence of supplements) increased (P<0.05) the percentage of TUNEL-positive cells when compared to other treatments.

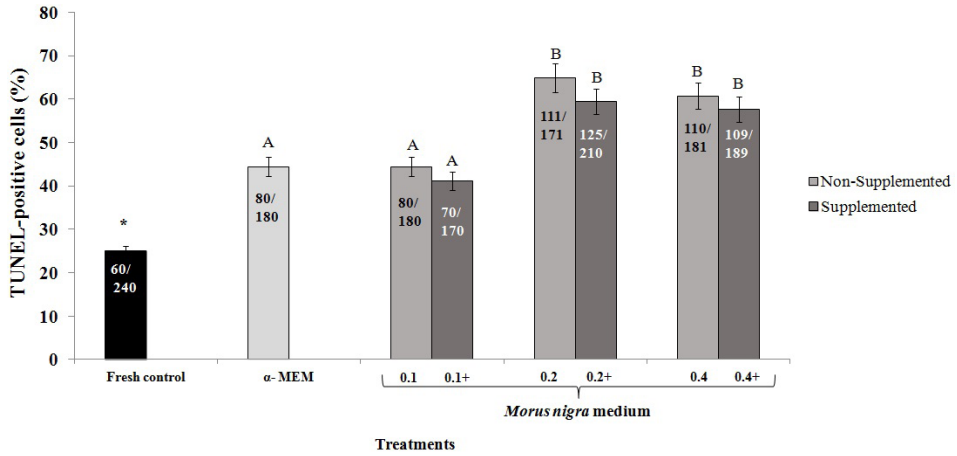

Fig.5. Percentage of total of TUNEL-positive cells in the fresh control, in α-MEM or in *Morus nigra* extract. MN 0.1 = 0.1mg/mL non-supplemented *M. nigra* extract, MN 0.1⁺ = 0.1mg/mL supplemented *M. nigra* extract, MN 0.2 = 0.2mg/mL non-supplemented *M. nigra* extract, MN 0.2⁺ = 0.2mg/mL supplemented *M. nigra* extract, MN 0.4 = 0.4mg/mL non-supplemented *M. nigra* extract, MN 0.4⁺ = 0.4mg/mL supplemented *M. nigra* extract. * Differs significantly from fresh control (P<0.05); $^{A, B}$ different letters denote significant differences among treatments (P<0.05).

DISCUSSION

The present study evaluated for the first time the effect of *Morus nigra* leaf extract as a culture medium for ovine preantral follicles. The control medium (α-MEM⁺) showed more histologically normal follicles than all *M. nigra* concentrations, in the absence or presence of supplements. The α-MEM⁺ is a complex medium containing electrolytes, antioxidants, amino acids, energy substrates, vitamins, and it has been routinely used as a base medium in the *in vitro* culture systems for preantral follicles (bubaline: Gupta et al. 2008, canine: Serafim et al. 2010, caprine: Magalhães et al. 2011, equine: Haag et al. 2013, ovine: Santos et al. 2014). Moreover, the addition of supplements to α-MEM seems to be important for the maintenance of follicular survival after culture (Silva et al. 2004, Peng et al. 2010, Gouveia et al. 2016).

Although the follicles cultured in α-MEM were classified as being of good morphology, this treatment showed the same rate of TUNEL-positive cells as 0.1mg/mL of *M. nigra* extract. It is noteworthy that classification by traditional microscope observation is more subjective for quality assessment than ideally desired. Moreover, nuclear apoptosis at an early stage is difficult to identify with routine Haematoxylin-Eosin staining (Wang & Roy 2007). Nevertheless, the TUNEL assay of labeling fragmented DNA has been used as a valid method for identifying cell death (Luyckx et al. 2014, Furlong et al. 2015). As DNA fragmentation in follicular cells could be detectable before other morphological and biochemical signs of degeneration (Sreejalekshmi et al. 2011), the DNA damage in follicles cultured in the control medium (α-MEM) was only noticeable after TUNEL analysis.

On the other hand, reduced DNA fragmentation in the medium composed of 0.1mg/mL of *M. nigra* (without or with supplements), compared to other plant concentrations, and could be explained by compounds present in the extract used, which contains rutin, isoquercetin and kaempferitrin (Cavalcante et al. 2017). These substances are flavonoids, a large group of polyphenolic compounds found in many plant-based foods, with antioxidant properties (Moretti et al. 2012). More specifically, rutin decreased lipid peroxidation, ROS and DNA fragmentation in thawed deer spermatozoa after incubation at 37°C (Mata-campuzano et al. 2012) and can be used as the single antioxidant present in the base medium during *in vitro* culture of ovine secondary follicles, maintaining follicular viability and increasing GSH levels (Lins et al. 2017). Isoquercetin protects neuroblastome from the oxidative damage *in vitro* (Soundararajan et al. 2008), being described as anti-inflammatory and antiapoptotic factor (Wang et al. 2013). Moreover, kaempferitrin inhibits mieloperoxidase, a cytosolic enzyme that participates in ROS production in neutrophils (Regasini et al. 2008). It can be suggested that these three natural antioxidants may act isolated or in association with each other or with substances added to the medium to reduce the DNA damage in preantral follicles cultured in 0.1mg/mL of *M. nigra*.

Nevertheless, in high concentrations antioxidants can become prooxidants (Carocho & Ferreira 2013). Therefore, it is possible that higher concentrations (0.2 or 0.4mg/mL) of *M. nigra* have potentiated the cytotoxic effect of some compounds, increasing the rates of DNA damage. A previous study has shown that *in vitro* culture with 1000µg/mL or 666µg/mL of *M. nigra* extract after 18 or 72h, respectively, increased caspases-3/7 activity in human prostate cancer cells (Turan et al. 2017).

In the current study, all treatments promoted primordial follicle activation, except 0.2mg/mL of *M. nigra* without supplements. Others authors have also demonstrated follicular activation using the supplemented α-MEM (Santos et al. 2014, Lima et al. 2016). Moreover, it is thought that the supplements could interact positively with the extract compounds to promote activation. A possible explanation for the activation observed in *M. nigra* without supplements may be the presence of rutin in the extract. Rutin increases the survival and prevents the decrease of proliferation of neural crest stem cells after damage caused by aflatoxin during *in vitro* culture (Nones et al. 2015).

CONCLUSIONS

Despite follicle loss after culturing, overall, our findings appear to suggest that the remaining follicles cultured at 0.1mg/mL of *Morus nigra* extract were in good condition and able to resume their development, as demonstrated by the same rates of DNA damage as the control medium and by primordial follicle activation.

Due to the higher cost of α-MEM (about 40 times more expensive), we recommend the use of *M. nigra* as a culture medium under the condition tested.

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

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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, Lousiana. (Abstract D-50)

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

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

(Note: Consult entire papers instead of only Abstracts)

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CONTENTS Volume 39, Number 1, January 2019

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