Morphoquantitative description of bovine digital cushion[1]

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ABSTRACT.- Borges L.C., Leonardo A.S., Barreto-Vianna A.R.C., Borges J.R.J., Castro M.B. & Lima E.M.M. 2015. **Morphoquantitative description of bovine digital cushion.** *Pesquisa Veterinária Brasileira 35(7):671-676*. Departamento de Anatomia Veterinária, Faculdade de Agronomia e Medicina Veterinária, Universidade de Brasília, ICC Ala Sul, Campus Darcy Ribeiro, Cx. Postal 4508, Brasília, DF 70760-701, Brazil. E-mail: limaemm@unb.br

The digital cushion is characterized as a modified subcutaneous tissue that absorbs the shock during gait, assists venous return of the hoof and supports a considerable part of body weight. Digital cushions have particular importance in the pathogenesis of the hoof, since they need to properly work in order to prevent compression and traumas in soft tissues. This study aimed to measure and determine how is the arrangement of these structures, and for this it was established the proportions of connective, adipose, vascular tissues and collagen fibers and collagen types found in palmar and plantar digital cushion of bovine using fore and hindlimbs of twelve adult zebu cattle of both sexes, 11 male and one female, with 269kg average carcass weight and without limb disorders. Fragments of cushions were subjected to conventional histology, cut to a thickness of 4µm and stained with Red Picrosirius. With digital optical microscope, the quantification of the connective tissue and differentiation of types of collagen used the Image Pro Plus® software, and of adipose and vascular tissue, the test point system. The mean and standard error were estimated with the GraphPad Prism 5.0 software, and then data were subjected to Kolmogorov-Smirnov normality test and Student's t-test with significance level set at 5% for determining the amount of different tissues between fore and hindlimbs of studied animals. In forelimbs the mean and standard error of the connective tissue proportion was $50.10\% \pm 1.54$, of the adipose tissue was $21.34\% \pm 1.44$, and of vascular tissue was $3.43\% \pm 0.28$. Hindlimbs presented a proportion of connective tissue of $61.61\% \pm 1.47$, $20.66\% \pm 1.53$ of adipose tissue, and $3.06\% \pm 0.20$ of vascular tissue. A significant difference ($p<0.001$) was detected in the connective tissue proportion between fore and hindlimbs. Types I and II collagen fibers have presented, respectively, a proportion of 31.89% and 3.9% in forelimbs and 34.05% and 1.78% in hindlimbs. According to the used methodology, digital cushions had a clear differentiation relative to adipose tissue between fore and hindlimbs.

INDEX TERMS**:** Digital cushion, cattle, claudication, tissue measurement, collagen differentiation, internal structures of the hoof.

RESUMO.- [**Descrição morfoquantitativa do tórus digital de bovinos.**] O tórus digital é caracterizado como um tecido subcutâneo modificado que atua na absorção do impacto durante a locomoção, auxilia o retorno venoso do casco e mantêm o suporte de uma considerável parte do peso corporal. Os tórus possuem particular importância nas patogêneses de casco, já que eles precisam trabalhar corretamente para prevenir compressões e traumas nos tecidos moles. Assim, o objetivo deste estudo foi quantificar e com isso determinar, como se arranjam estas estruturas, para tanto, foram estabelecidas as proporções dos tecidos conjuntivo, adiposo, vascular e ainda das fibras colágenas e dos tipos de colágenos encontrados nos tórus digitais palmares e plantares de bovinos. Foram utilizados membros torácicos e pélvicos de doze bovinos zebuínos adultos, de ambos os sexos, sendo onze machos e uma fêmea, com peso

médio de carcaça com 269kg e sem afecções nos membros. Os fragmentos dos tórus foram submetidos à técnica histológica convencional, cortados em espessura de 4µm e corados com Picrosirius Red. Com o uso de microscópio óptico digital, o tecido conjuntivo e a diferenciação dos tipos de colágeno foram quantificados empregando-se o programa de análise de imagem Image Pro Plus® e para a quantificação dos tecidos adiposo e vascular foi utilizada o sistema teste de pontos. Através do programa GraphPad Prism 5.0 foram obtidas por meio de uma analise descritiva a media e o erro padrão da media, em seguida os dados foram submetidos à aplicação do teste de normalidade de Kolmogorov-Smirnov e ao teste "T" Student com nível de significância de 5% para a determinação da quantidade encontrada dos diferentes tecidos entre os membros torácicos e pélvicos dos animais estudados. Nos membros torácicos a média e o erro padrão da proporção de tecido conjuntivo foi de 50,10%±1,54, a de tecido adiposo foi de 21,34%±1,44 e a de tecido vascular foi de 3,43%±0,28. Os membros pélvicos apresentaram uma proporção de tecido conjuntivo de 61,61%±1,47, de tecido adiposo de 20,66%±1,53 e de tecido vascular de 3,06%±0,20. Verificou-se diferença estatística na proporção de tecido conjuntivo entre membros torácicos e pélvicos (p<0,001). As fibras colágenas tipo I e III apresentaram, respectivamente, uma proporção de 31,89% e 3,9% nos membros torácicos e 34,05% e 1,78% nos membros pélvicos. Os tórus digitais, de acordo com a metodologia utilizada, apresentaram diferenciação evidente em relação ao tecido adiposo entre membros torácicos e pélvicos.

TERMOS DE INDEXAÇÃO: Tórus digital, bovinos, claudicação, quantificação de tecidos, diferenciação de colágeno, estruturas internas do casco.

INTRODUCTION

The bovine hoof is made up by skin, hoof capsule and several associated structures, such as bones, ligaments, tendons, fat, digital cushions, connective tissue, blood vessels and nerves. The hoof quality is determined by the arrangement, anatomy and physiology of its internal structures (Greenough 2007). Inside the capsule of the hoof, the supporting mechanism is given by the solear corium, associated with the loose connective tissue and digital cushions with variable amounts of adipose tissue (Bergsten 2003). These digital cushions are complex in thickness and size, operating in shock absorption to the distal phalanx and heel, dissipating the forces inside the hoof, these structures still works to maintain the support of a considerable part of the animal weight (Räber et al. 2004).

The lameness in cattle is an important economic and sanitary problem, which causes economic losses in herds in which its incidence is high, these losses are result of diseases, primarily emerging as result of disease and not by the cost of treatment itself (Kester et al. 2014).

Digital cushions are of particular importance in the pathogenesis of hoof lesions, once must operate properly to prevent trauma and compression of adjacent structures. In this context, this study aimed to describe and assess morphological and quantitative aspects of tissues constituents of digital cushions of zebu cattle, by means of determining the amount of connective, adipose and vascular tissues, and characterizing the types of collagen fiber using histochemical and morphometric techniques.

MATERIALS AND METHODS

The studied material consisted of twelve bovine digital cushions, six palmar and six plantar, collected at random from adult zebu animals of both sexes, eleven males and one female, with 269 kg average carcass weight and without disorders in structures related to the locomotor system. The study was approved by the Ethics Committee on Animal Use of the Institute of Biological Sciences of the University of Brasilia, according to the protocol #86551/2010.

With the aid of a bandsaw, hooves were sectioned at the level of the functional axis of the member, separating the fingers III and IV which in turn were longitudinally sectioned, allowing thus the visualization and dissection of cushions. They were fixed in 10% formaldehyde solution for 48 hours. After this, two fragments, one axial and one abaxial, of each digital cushion were randomly collected and subjected to conventional histological technique and subsequently sliced with a manual microtome (Leica RM 2125RT) to a thickness of 4µm placed on slides and stained with Red Picrossirius to disclose the connective tissue.

Photomicrographs were obtained from ten random fields of each slice by the BX51 Olympus® optical microscope coupled to a software for capturing and analyzing images, the ProgRes® Capture Pro 2.5. For the connective tissue quantification, it was used the software Image Pro Plus.6.0® and for differentiation of collagen fibers, polarized light microscopy. The area occupied by blood vessels and adipocytes was estimated by a test point system (Gundersen et al. 1988) (Fig.1). The total area calculation was based on the Delesse principle (Fig.2).

Data were shown as mean±standard error of the mean. The Kolmogorov-Smirnov test was used to test normality, followed by

Fig.1. Schematic drawing of a photomicrograph superimposed by the test point system.

$$A\,[est] = \frac{P\,[est]}{Pt}$$

Fig.2. Calculation of the total area by the Delesse Principle, in which $A_{[est]}$ - area occupied by the structure, $P_{[est]}$ - number of points counted on the structure and P_t - total number of points of the test point system.

Student's t-test and Pearson Correlation, considering P≤0.05 as statistically significant. All analyses were run using the software GraphPad Prisma® 5.

RESULTS

Bovine digital cushion of both limbs were predominantly formed by dense connective tissue (fig.3b), which presented a complex arrangement, made up by elastic fiber bundles organized in the periphery of the adipose tissue and irregular bundles of collagen fibers that in turn formed projections delimiting this tissue, and forming large lobes filled with adipose tissue, constituting thus a support network in mesh (fig.3). Areas of fibrocartilage and hyaline cartilage, as well as of myxoid tissue, were not observed in the stroma of cushions.

Digital cushions were predominantly formed by connective tissue, contributing with 50.10%±1.54 in forelimbs and 61.61%±1.47 in hindlimbs (p<0.001) (Fig.3a, 3b and 4). There was a large presence of adipose tissue in their composition, constituting 21.34%+1.44 of forelimbs and 20.66%+1.53 of hindlimbs (Fig.3a,3b and 4). A negative correlation (r= -0.11) was found between animal weight and proportion of connective tissue, for digital cushions of both fore- and hindlimbs (Fig.6).

The differentiation of collagen fibers revealed the predominance of type I collagen fibers in digital cushions, representing 31.89% in forelimbs and 34.05% in hindlimbs (Fig.5). For the type III collagen fibers it was observed the proportion of 3.09% in forelimbs and 1.78% in hindlimbs (fig.3c, 3d and 5).
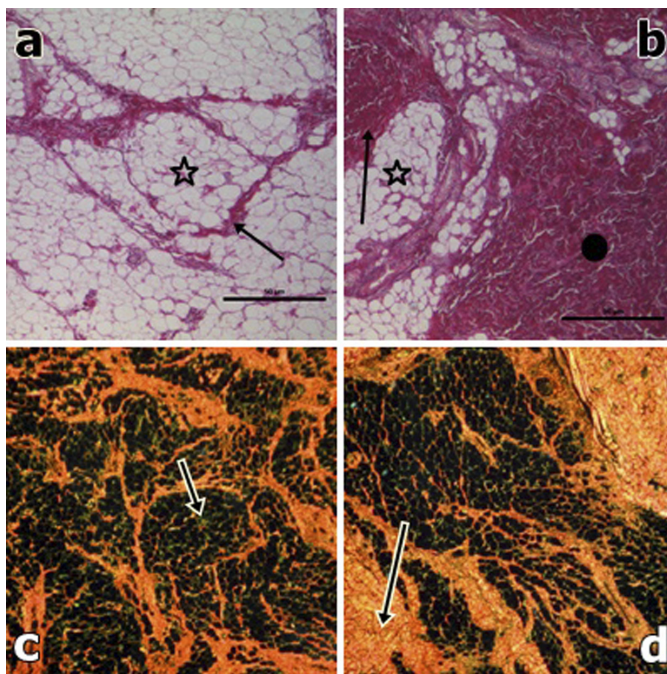

Fig.3. Tissue components of bovine digital cushion. (**a**) Adipose tissue lobes delimited by connective tissue. Star: adipocytes. Arrow: connective tissue. (**b**) Sphere: connective tissue. Star: adipocytes. Arrow: reticular fibers. (**c**) Arrow: type III collagen (green refringence). (**d**) Arrow: type I collagen (red refringence). Red Picrosirius staining, Bar = 50μm
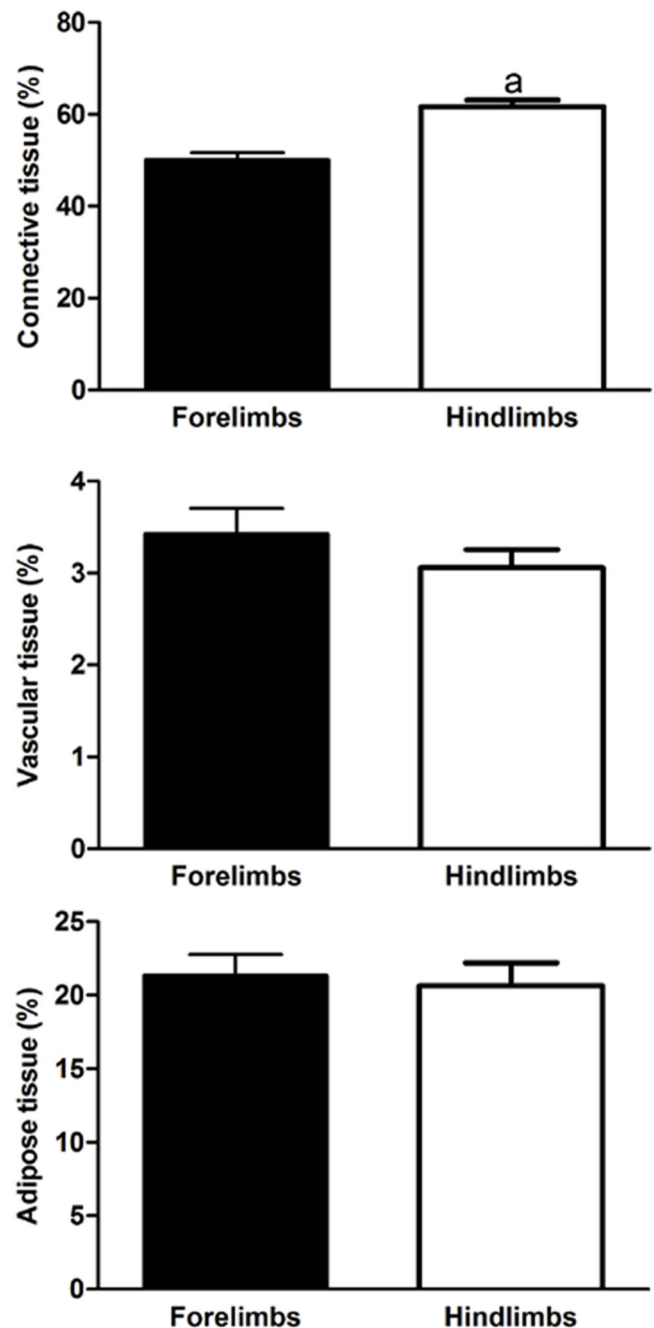

Fig.4. Mean and standard errors (*n*=12) of connective, adipose and vascular tissue in bovine digital cushions. Letter 'a' on the column indicates significant difference (p≤0.05).

The adipose tissue was observed forming lobes delimited by connective tissue (Fig.3a and 3b). The vascular tissue was distributed diffusely and randomly throughout the cushion, where 3.43%+0.28 was in forelimbs and 3.06%+0.20 in hindlimbs (Fig.3b and 4). The amount of blood vessels had no significant difference between limbs (Fig.4), and likewise the adipose tissue was proportional between different limbs (Fig.4). The amount of adipose tissue was positively correlated to the animal weight, for both forelimbs (r=0.49) and hindlimbs (r=0.31) (Fig.6).

DISCUSSION

The distal portion of limbs is related to the support and movement of cattle, in this way the digital cushion structures quantified and described herein are of paramount importance because if injured can generate severe problems in the locomotor system, leading to great economic losses. This is particularly observed in chronic laminitis with adipose tissue fibrosis and predisposition to sole ulcers (Lischer & Ossent 2002) compromising the microcirculation of the digit and worsening the symptoms (Baldwin & Pollitt 2010). The subclinical laminitis, cracking and erosion of the hoof can also damage the digital cushion structures, so the correction of weight bearing aplomb and hoof trimming ensure the integrity of the digital cushion, preventing the appearance of lesions on the hoof (Higuch 2005, Schöpke 2013).

The presence of connective tissue aims providing support and structure to the body, and participating in processes of defense and repair of lesions (Zhan 2015). According to our findings, digital cushions of hindlimbs presented a greater amount of connective tissue than forelimbs (Fig.4). Probably due to the mode of locomotion of cattle, since forelimbs are responsible for the greater support of animal body weight, and hindlimbs are associated to movement traction. This still suggests that the particular arrangement
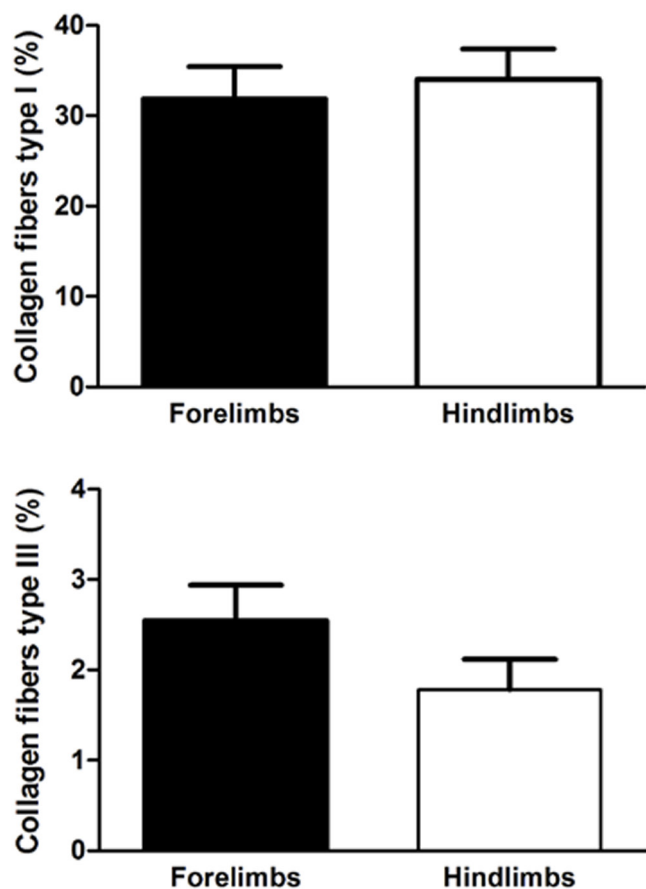

Fig.5. Quantification of different types of collagen fibers (I and III) found in bovine digital cushions of forelimbs (FL) and hindlimbs (HL).
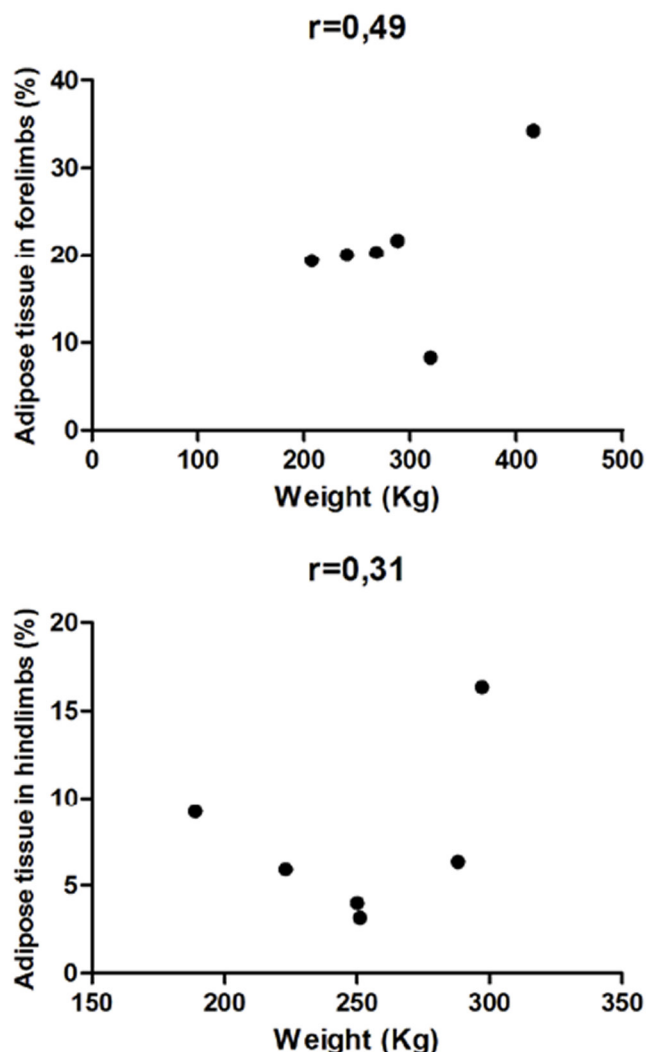

Fig.6. Correlations found between animal weight and proportion of adipose tissue of bovine digital cushions of forelimbs and hindlimbs.

had been related to the extensive production system imposed to animals, which expose them to environments quite diverse, leading to the belief that there was a functional adaptation.

The bovine digital cushion structure is similar to elephant digital cushion, both presenting a tough connective tissue that includes variable amount of adipocytes, forming lobules (Fig.3) (Weissengruber et al. 2006). The structural similarity is because both bear a greater proportion of body weight in relation to other animals, such as horses that have a more developed support apparatus due to a flexible hoof, which gives in the pressure impact with the ground and dissipates the resulting concussion, reinforced accessory ligaments especially superficial and deep flexor tendons. Also differently from horses, the bovine digital cushion has no complex architecture made up by elastic fiber bundles, fibrocartilage and myxoid tissue, but a complex architecture that consists of elastic fiber bundles arranged in the periphery of the adipose tissue and irregular bundles of collagen fibers that, in turn form projections that delimi-

ted this tissue, and forming large lobes filled with adipose tissue, constituting thus a support network in mesh (Fig.3) (Räber et al. 2004, Weissengruber et al. 2006).

The main characteristic of the adipose tissue in other regions of the body is to work as a thermal insulator in heat dissipation, but in the digital cushion it works as mechanical insulator, an efficient absorber of forces, providing a soft and tough consistency at the same time (Räber et al. 2006). In the present study, it was considered that this tissue assists in preventing against mechanical shocks and in venous return, with 21% on average of adipose tissue for both members, similar to found for brown-Swiss heifers (Räber et al 2006), which presented 26.4% on average. Given the small difference, our finding can be within a range observed for cattle, indicating small variations among different breeds.

The amount of adipose tissue found in digital cushions tends to increase to 36.7%, as higher parity, lactation and aging of animals (Räber et al. 2006). In the same way, the composition of this tissue is altered according to the diet (Baird et al. 2010). Probably the increase in lipid content occurs by the change of saturated fatty acids by monounsaturated fatty acids, in the composition of digital cushions during the first calving and lactation, making less effective the absorption system, which explains the higher incidence of sole lesions in primiparous (Baird et al 2010). Once the age, number of calvings, and diet nature were not taken into account in the present study, it was not possible to establish the relationship between them for the animals studied.

Digital cushions of forelimbs have a greater amount of adipose tissue relative to hindlimbs (Räber et al. 2004), which was also observed in the present study, but with no statistical difference. This proportion can be because forelimbs are responsible for supporting the major part of animal body weight (Räber et al. 2004). In other words, the need for tissues that actually promote the maintenance of structure and integrity of the digit, such as the connective tissue, revealed to be more important than the presence of a great amount of adipose tissue.

Moreover, several blood vessels of different sizes were described as dispersed throughout the connective tissue and intertwined with the connective tissue in elephants (Weissengruber et al. 2006). During the walking and standing, the hoof is subjected to forces in which the digital cushion expands and compresses, responding by the efficient venous return of the hoof (Csuti et al. 2001, Benz et al. 2005). In the animals evaluated, although without significant difference relative to the quantification of this tissue in digital cushions between fore- and hindlimbs (fig.4), it was understood that their presence complies with the functional active role performed by cushions, especially when combined together with the function of the digits in ruminants.

Types I and III collagen fibers were present in digital cushions of bovine fore- and hindlimbs. Type I collagen fibers, the most abundant in mammals, provide resistance to forces, stresses and stretching of fibers, i.e., a structural function. Type III collagen fibers, reticular fibers, have been almost always associated with type I and contribute when there is loss of connective tissue, i.e., replacement

fibers (Junqueira & Carneiro 2013). Similarly to evidence in elephants (Weissengruber et al. 2006), there was a network of collagen fibers surrounding the digital cushions and the adipose tissue present, this covering has determined the maintenance of the entire structure, allowing the reestablishment of the original shape after compression from the gait or even from the body support. The digital cushion of hindlimbs presented a greater proportion of type I collagen compared with forelimbs, however with no statistical difference. Conversely, the type III collagen had a greater amount in forelimb digital cushions (Fig.5), but without significant difference. This result suggested that digital cushions of forelimbs have been under constant remodeling, justifying thus the highest proportion found for the type III collagen.

CONCLUSIONS

Considering our results it was possible to describe, by means of quantification, the structures constituent of bovine digital cushions. There was a greater amount of connective tissue in forelimbs, probably owing the mode of locomotion of these animals that employ more strength on those members to move.

The arrangement of collagen fibers in different limbs suggests that digital cushions of forelimbs have been under constant remodeling, since it was registered a greater proportion of type III collagen than observed in hindlimbs.

In this study, a greater presence of connective tissue than of adipose tissue indicated its highest importance for the perfect functioning of this structure.

As for the blood vessels, they are spread throughout the structure, being related to the active functional role performed by the digital cushion when associated with the function of the digits in ruminants.

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