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Ultrastructure of foot coronary corium in Nelore cattle.

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ABSTRACT

Franco da Silva LA, Belett ME, Eurides D, Rabelo RE, Ribeiro de Freitas SL, Bastos Queiroz PJ, Alves da Silva J., Ultrastructure of foot coronary corium in Nelore cattle, Onl J Vet Res., Nelore cattle are known to be rustic and have greater resistance to foot diseases. Nevertheless, their occurrence, even at extensive farming system, is still observed. The ultrastructural examination shows up as an alternative to identify the pathophysiology of most foot problems. This study aimed to identify the coronary band structures of Nelore cattle hooves by means of transmission electron microscopy (TEM) and describe some difficulties encountered during samples' processing. Fifteen Nelore cattle, managed under intensive grazing, were used in the present study, of which three samples from the coronary band measuring 1mm were collected after slaughter. Samples were evaluated using a transmission electron microscope and imaged using Megaview GII Image Capture system/Olympus Soft Imaging Solutions. Ultrastructural analysis of the coronary band was descriptive and allowed identification of keratinocytes and desmosomes tonofilaments. The long time frame between collecting and analyzing tissue samples were associated with dryness, which interfered with material quality, as well as some issues encountered during samples' process.

Key words: Bovine, hoof, electron microscopy, keratin, Nelore breed

INTRODUCTION

Nelore cattle have genetic and phenotypic characteristics which sustains their good performance and adaptability in tropical environments. These characteristics were described by previous studies which aimed at evaluating population growth, reproductive performance and carcass quality[1]. However, to adequately develop in any environment, animals must still be healthy, well managed and fed. Digital illnesses can influence production, reproductive performances and depending on either disease's progression or costs of treatment can even cause the animal to be discarded. Despite breed's resistance, occurrence of foot lesions in Nelore cattle has been described even in extensive farming systems.

Identification of most important foot lesions in cattle has motivated numerous studies, nevertheless it is still not possible to define the pathophysiology of major diseases. An alternative that may help understanding pathogenesis is the ultraestutural examination of the hoof. The coronary band is a connective structure between the hoof wall and skin, therefore is closely related to the horny sheath growth[4]. Studies concerning the characteristics of coronary band and its layers, such as coronary epidermis, coronary subcutaneous and coronary dermis or coronary corium, can be critical when establishing the relation between different etiology factors involved in a disease process.

Keratinization and formation of the protein matrix, which is responsible for keratin resistance and thus for the hoof's, are initiated in the coronary band[5]. Therefore, as hoof's strength is closely linked to coronary corium microstructure, the importance of this structure can't be denied when studying pathogenesis, but there are still little information on cattle coronary corium ultrastructural composition.

Thus, the present study aimed to describe by transmission electron microscopy (TEM) method the coronary band ultrastructure of Nelore cattle hooves, with emphasis on coronary corium. Concomitantly, will reveal difficulties encountered during samples' analysis process.

MATERIALS AND METHODS

This study was approved by the Ethics Committee of Goiás Federal University (UFG), under protocol number 189/11. The first stage was held at Tomé Pinto Farm, which belongs to School of Veterinary Medicine and Animal Science (EVZ/UFG), in the city of San Francisco, Goiás, Brazil, and began in 2012. During this stage, fifteen Nelore bulls were managed under intensive grazing and, after 120 days, were slaughtered in a slaughterhouse under Federal Inspection. A fragment containing epidermis, underlying dermis and connective tissue measuring 1 cm^3 was excised from the coronary band region, after slaughter. Later, fragments were sectioned until achieving 1 mm^3.

During the second stage, samples were immersed in 2 ml cryogenic plastic tubes with Karnovsky fixative reagent which contains 3% glutaraldehyde and 2% formaldehyde in 0.1M cacodylate buffer at pH 7.4, and paraformaldehyde. After 24 hours, samples were fixed in phosphate-buffered saline (PBS). After chemical fixation process, they were sent to Electron Microscopy Laboratory of the Institute of Biomedical Sciences, Uberlândia Federal University (ICBM/UFU), Minas Gerais, Brazil, where remained until analysis. This stage began in 2012, when samples were collected, and finalized in 2014, when they were analyzed.

At ICBM/UFU tissue samples were cut into 1 mm fragments and fixed in PBS 1%. The first bath, lasted 24 hours and was followed by a second bath, which lasted only 5 minutes. After PBS buffer baths, the material was immersed in a 1:1 solution of osmium tetroxide 2%, this step was carried out in a laminar flow hood under sterile conditions. Thirty minutes later, fixed samples were dehydrated through the following series of acetone concentrations: 50%, 70%, 80%, 90% and 95% for five minutes each. Tissue fragments were washed in 100% propylene oxide twice for ten minutes each and then deposited in a 1:2 solution propylene oxide:Epon for 12 hours at 37°C. The procedure was repeated with a 1:1 volume ratio solution.

On the following day, infiltration process was continued by exchanging solution to 100% resin. Ependorfs were left opened between two and four hours, at 37°C. This step aimed to evaporate remaining propylene oxide. Blocks were then embedded in resin molds and stored for 48 hours at 60°C. After this process, an ultramicrotome was used to cut blocks in sections of 60nm. These ultra thin slices were contrasted with 3% uranyl acetate for 30 minutes at 37ºC, and then with lead nitrate (and sodium hydroxide) for 20 minutes at room temperature. The preparation of biological specimens technique used in this study was adapted from Bozzola and Russell[6]. Samples were analyzed using a transmission electron microscope (Zeiss Electron Microscope EM 109) and imaged using Megaview GII Image Capture system/Olympus Soft Imaging Solutions. Images were taken at 3000, 4400 and 7000x magnification for dermis and epidermis evaluation of the coronary corium.

During samples' processing some adjustments were made to minimize tissue damage that could compromise identification of coronary corium ultrastructures. In case of problems that could not be addressed, especially those concerning long storage period, results were not considered. Structures visualized after ultrastructural analysis were identified and described in order to provide a better understanding of ultrastructural anatomy of Nelore cattle coronary corium.

RESULTS

During TEM analysis, the main findings were: tissue areas rich in collagen fibrils and highly vascularized (Figures 1-A and 1-B); prolongations of basal lamina which enabled epidermal-dermal communication through membrane basement, making them a single layer

characterized by rounded cells joined by desmosomes (Figure 1-C); significant amounts of tonofilaments in the spinous layer, adhered to epithelial cells' desmosomes; keratinocytes and extracellular matrix containing expressive quantities of melanin granules; at some tissue areas where cells were far from basal lamina, concentration of tonofilaments became more evident and keratinocytes acquired flattened shape (Figure 1-D).

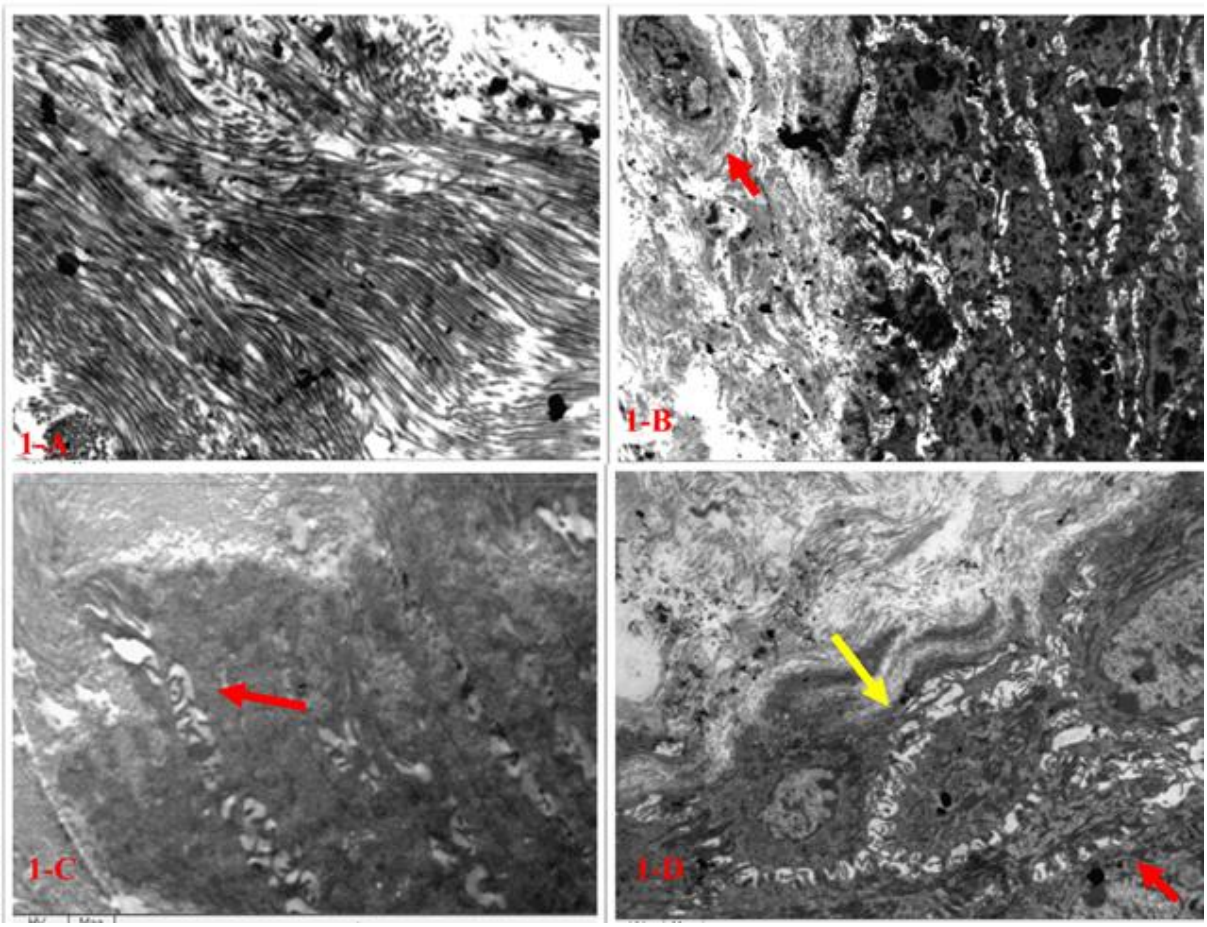

Figure 1. Coronary corium ultra micrograph sections of Nelore's hooves. **A)** Collagen fibrils; **B)** Vascularized tissue, blood vessel (red arrow); **C)** Desmosomes (red arrow); **D)** Keratinocytes, rounded shape (yellow arrow) and flattened shape (red arrow).

Some difficulties were faced during samples' processing and analysis which may have damaged tissue and compromised final results. Damage to the material was not assigned to sample gathering, as it happened immediately after slaughter in a synchronized and rapid action. Therefore, previous staff training resulted in agility in order to avoid any post-mortem alterations that could interfere with study's results. However, benefits obtained from effective samples' collection were counterbalanced by the long time frame between gathering and analyzing data. Material awaited processing for longer than one year which resulted in dryness, a condition known to compromise tissue's quality.

Different infiltration degrees of propylene oxide:epon solution found in cuts from the border areas between coronary corium and skin, interfered with final samples' quality as

well. In this case, there was no penetration of resin into coronary corium at perioplic horn probably due to increased rigidity which resulted in detachment of epidermis during cutting.

Cut standardization to allow simultaneous evaluation of all skin layers and appendages was another issue encountered. Where epidermis and dermis meet, both are thrown into a series of folds that interdigitate, which may be the main cause of this problem. Trying to minimize this, some samples were cut longitudinally in order to include all layers.

Due to tissue increased rigidity at perioplic horn, diamond blades were damaged during cutting process. Discontinued micro anfractuosities in the blade were reproduced in tissue sections after cut, leading to irregularities that diminished quality of some samples as well.

DISCUSSION

Among ultrastructures identified, keratinocytes and keratin filaments were present in all samples. However, it was not possible to quantify these filaments as they were aligned parallel to long axis and organized in "tufts" of different densities. Similar findings were reported by Tomlinson et al[5]. Even though, comparative cell analysis suggests that this organization pattern as well as higher concentration of these structures provides increased rigidity which results in higher mechanical strength. These findings may indicate greater strength of Nelore cattle hoof's tissues which suggests sufficient resistance to occurrence of digital diseases, according to Mulling and Budras[7].

Different degrees of hardness encountered in hoof structures can be determined by the presence or absence of epidermal-dermal communications through basal lamina extensions, composed by round cells joined by desmosomes and high concentration of keratin tonofilaments. Previous studies analyzed equine hoof's structure and observed clear differences in these cells' types and orientation among different areas and their relation to strength. In addition, Anthauer et al. also described some particularities of desmosomes, keratinocytes and tonofilaments found in equine's sole and hoof wall[10]. In cattle, studies involving the coronary band and coronary corium structures are important to correlate different factors involved in foot diseases' etiology. According to Dellman and Brown[11], the coronary band is a modified prolongation of epidermis which after keratinization process will compose the hoof wall being distinguished by the absence of stratum granulosum. According to Stump JE[4], stratum spinosum composition is mainly of cells that migrated from stratum basale after keratinization process. Stratum basale, the deepest epidermis' layer, has a single layer of cells at the coronary band. Knowing this anatomic features, further studies are necessary to determine how those structures and cells are found in different species and how they behave during any a pathologic process.

An interesting finding was the significant amount of tonofilaments arranged in "tufts" and adhered to desmosomes of stratum spinosum epithelial cells, which may suggest that they are important in keratinization process of the horny sheath as well as in keratinocytes'

stability through desmosomes. Mulling CH[12] also described by transmission electron microscopy, the occurrence of keratinocytes at different stages of differentiation and also reported the importance of desmosomes and intercellular cement to hoof's resistance. Rabelo et al.[13] analyzed pigmented and depigmented hooves in Holstein cattle and showed that darker hooves have longer length and thicker epidermis papillae, which may indicate greater capacity to produce keratin and thus greater resistance to injuries, whether of an infectious or traumatic cause. Different melanin granules' concentrations in keratinocytes' cytoplasm, were also observed in the present study, which may infer that this finding can also contribute to resistance. Further studies involving pigmentation are still needed to clarify the role of melanin in hoof's stiffness.

Not only the presence of melanin in keratinocytes and extracellular matrix, but also different cell shapes observed either in basal lamina or in spinous layer, needs to be further analyzed. The final step in the keratinization/cornification process is the secretion of a lipid-rich extracellular matrix, the so-called intercellular cementing substance, by which mature keratinocytes become glued together. Aggregation of keratin filaments increases, leading to epidermal differentiation with consequent formation of intermolecular bonds and keratinocytes' apoptosis, as reported by Tomlinson et al[5]. The protein matrix is responsible for alignment, adequate stabilization of filaments into cytoplasm and keratin resistance. In this case, keratinocytes acquired more flattened shape when into spinous layer which may be directly related to cell cluster capacity which also increase hoof's resistance, as described. Perhaps, this flattening and accumulation of cells is greater in certain breeds but more studies are needed to confirm this supposition.

Evidence showed that time frame between material collection and analysis, which was over a year, resulted in important changes that compromised results and consequently their interpretation. Thus, even after all efforts to avoid any postmortem tissue alteration during material collection, several samples were affected by dryness, which usually happens over time. Other studies, such as the one conducted by Mulling and Budras involving electron microscopy analysis of similar tissue areas, did not report major issues concerning samples' processing[7]. Although several factors, as described previously, have compromised material's quality and consequently limited analysis, other tissue samples remained ideal and enabled identification and description of ultrastructures in the coronary corium of coronary band region.

An important issue observed during cutting process was material hardiness which made it difficult to accomplish. The ultra microtome diamond knifes acquired micro anfractuosities after cutting samples and ended up damaging tissue. Even though the digits' coronary band were less hard than hoof's wall it didn't prevent diamond knifes from damaging. The main area where complications may have occurred during cutting is the perioplic horn due to its hard keratin composition. However, problems concerning diamond blades' damage have

not been reported in other studies involving process of rigid materials, such as hooves and horns[8,9].

Knowing the complexity of the present subject, it can be inferred that hoof's strength may be closely linked to the microstructure of coronary band epidermis and dermis, hoof wall and other areas. Thus, studies concerning anatomical structures and their role in hoof's composition are important to clarify diseases' pathogenesis. More detailed information, especially concerning quantification and role of keratinocytes, desmosomes, keratin filaments and melanin in horny sheath's resistance are needed. TEM method showed to be an important tool to allow identification of structures that cannot be identified by optical microscopy. Therefore, it is believed that the present study provided useful information on Nelore cattle hoof's ultrastructure and may stimulate future research on hoof's anatomy of different cattle breeds in order to soon understand some foot diseases' pathogenesis.

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