Arterial diffuse intimal thickening associated with enzootic calcinosis of sheep[1]

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ABSTRACT.- Vasconcelos R.O., Barros S.S., Russowski D., Grando S.M. & Irigoyen L.F. 1998. **Arterial diffuse intimal thickening associated with enzootic calcinosis of sheep.** *Pesquisa Veterinária Brasileira 18(1):9-15.* Depto Patologia, Universidade Federal de Santa Maria, 97119-900 Santa Maria, RS, Brazil.

Morphometric, immunohistochemical and ultrastructural studies were carried out on the diffuse intimal thickening (DIT) in arteries of 7 sheep with clinical signs of naturally occurring enzootic calcinosis due to ingestion of the plant *Nierembergia veitchii*. Arterial lesions consisted of medial deposition of calcium salts and DIT. Calcification of the intima was rare, mild and located near the elastic lamina. By immunohistochemistry α-actin was detected in cells of the media and in cells forming the intimal thickening. Receptors for $1,25(OH)_2$ vitamin D_3 were detected in nuclei of intimal, medial and endothelial cells. DIT was irregularly distributed and was neither proportionally related to the intensity of the underlying mineralization area nor to the thickening of the remaining media. Ultrastructural morphometry in smooth muscle cells (SMCs) of the media and thickened intima revealed, in the latter, an increase of 318% in the volumetric fraction of those organelles involved in synthesis and a proportional decrease in contractile elements when compared to normal values of media cells. There were histological and ultrastructural evidences of modification of SMCs and their migration to the intima, where they proliferated causing DIT. It was concluded that DIT is a consistent component of arteriosclerotic lesions in *N. veitchii* induced calcinosis of sheep and that the predominant cell in this process is the SMCs originated from its predecessors of the media. It is suggested that the inducing factor for the arterial changes is $1,25(OH)_2$ D_3 present in *N. veitchii*.

INDEX TERMS: Enzootic calcinosis, *Nierembergia veitchii*, arterial lesions, sheep.

RESUMO.- [Espessamento intimal difuso das artérias na calcinose enzoótica dos ovinos.] Foram feitos estudos morfométrico, imunoistoquímico e ultra-estrutural do espessamento intimal difuso (DIT) das artérias de 7 ovinos com sinais clínicos de calcinose enzoótica espontânea causada pela ingestão da planta *Nierembergia veitchii*. As lesões caracterizavam-se por deposição de sais de cálcio na média como placas e estrias que, com frequência faziam saliência para a luz, criando rugosidades e irregularidades da íntima. Não foram observadas calcificações na artéria pulmonar e no sistema venoso. Microscopicamente, as calcificações das artérias restringiam-se quase que exclusivamente à média. Na imunoistoquimica, foi verificada α-actina nas células da média e nas do espessamento intimal. Receptores para $1,25(OH)_2D_3$ foram evidenciados nos núcleos das células musculares da média, da íntima e das células endoteliais. As análises morfométricas em microscopia ótica revelaram, nas artérias, DIT irregularmente distribuido sem relação com a intensidade dos processos de calcificação subjacente nem com a espessura da média remanescente. A morfometria das alterações ultra-estruturais das células musculares lisas da média e da íntima espessada, mostrou que nessas últimas foi verificado aumento de até 318% na fração volumétrica das organelas de síntese em detrimento dos elementos contráteis,

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quando comparados com os valores das células da média. Essas modificações indicam a transformação das células musculares lisas de um tipo contrátil para células de tipo sintético. Entre essses dois extremos foram evidenciadas diversas formas intermediárias. Foram observadas evidências histológicas e ultra-estruturais da transformação e migração de células da média através das fenestras da lâmina elástica interna, para a íntima, onde proliferam e formam o DIT. Foi concluído que o DIT é um componente constante nas lesões arterioscleróticas nas calcinoses dos ovinos induzidas pela planta *N. veitchii* e que as células predominantes são células musculares lisas provenientes de predecessoras da média. Foi constatado que o DIT só excepcionalmente apresenta calcificação, vascularização, presença de células espumosas e fendas de colesterol. Não foram vistas áreas de necrose. Sugere-se que o fator indutor das alterações arteriais é o $1,25(OH)_2D_3$ contido em *N. veitchii*.

TERMOS DE INDEXAÇÃO: Calcinose enzoótica, *Nierembergia veitchii*, lesão arterial, ovinos.

INTRODUCTION

Spontaneous calcinoses of domestic animals are chronic debilitating diseases characterized by mineralization of soft tissues, especially in the cardiovascular system and lungs (Carrillo & Worker 1967, Döbereiner et al. 1971, Dirksen et 1970, Riet-Correa et al. 1987). Most of these diseases are associated to calcinogenic plants, and $1,25(OH)_2D_3$ present in the plants is incriminated as the etiologic agent (Wasserman 1975, Boland 1986, Rambeck & Zucker 1982, Riet-Correa et al. 1987).

Enzootic calcinosis of sheep was described in several flocks of the State of Rio Grande do Sul, in southern Brazil, and the plant *N. veitchii* is the causative agent (Riet-Correa et al. 1987). Arterial lesions consist of diffuse calcification of the media with areas of cartilage and bone metaplasia and infiltration of macrophages and occasionally multinucleated giant cells. Diffuse intimal thickening (DIT) is very prominent and, in some areas, can reach several times the thickness of the media (Barros et al. 1970, Riet-Correa et al. 1987).

Although arterial lesions in calcinosis of domestic animals are well characterized, no description of the ultrastructure and the true cell population and extracellular matrix of DIT were reported. In this paper the cells and extracellular matrix of DIT in spontaneous calcinosis of sheep, induced by *N. veitchii*, are described.

MATERIALS AND METHODS

Seven 3-year-old sheep, 1 male and 6 females, with clinical signs of spontaneous enzootic calcinosis induced by *N. veitchii* were submitted to the following procedures. All animals were placed in a stall and received alfalfa and water *ad libitum*. Once a day, for approximately 5 hours, the sheep grazed in a natural pasture free of *N. veitchii*.

In 4 months three animals died spontaneously, due to heart failure and pulmonary edema and the remaining sheep were euthanatized by exsanguination on the 5th month. All sheep were necropsied, and fragments of the thoracic and abdominal aorta, carotid, femoral, renal, iliac, mesenteric, splenic, gastric and tongue arteries were collected in 10% buffered formalin and stained with hematoxylin-eosin (HE). Selected slides were stained with von Kossa.

Electron microscopy

Small fragments of both carotid arteries were collected immediately after death from 5 sheep; from 4 euthanatized and from one sheep 20 minutes after spontaneous death. The fragments were placed in 2% glutaraldehyde, 2% paraformaldehyde buffered solution in sodium cacodylate (0,3M and pH 7,4), post-fixed in osmium tetroxide and embedded in Epon. Thick sections were stained with methylene blue. From these sections, areas of intima and media were selected for thin sections and cut with a diamond knife. The sections were then stained with uranyl acetate and lead citrate, and observed in a transmission electron microscope.

Morphometry

Transversal sections of arteries stained with HE were used for optical morphometry. The thickness of the intima, calcified media, and non-calcified media, expressed in millimeters, was measured with a micrometric ocular adapted to a microscope. The data was analysed using an one-way analysis of correlation and variance and the Student-Newman-Keuls test.

Morphometry on electron microscopy was performed in random samples of electron micrographs of each section by photographing the field in one corner of each grid square (copper grid 200 mesh) in a primary magnification of x 4,000. Micrographs were enlarged photographically 2.7 times to obtain 17x23cm micrographs. Four micrographs were taken from each block.

The cells of the DIT were classified ultrastructurally as smooth muscle cells (SMCs) and non smooth muscle cells. The morphological criteria to characterize SMCs was the presence of pinocytotic vesicles, basal lamina, and bundles of myofilaments associated to dense bodies. Only those cell profiles unequivocally characterized as SMCs were computed. Cells and cell profiles that did not achieve the above criteria were discarded. A quadratic test lattice having spacing of 7mm, with a total of 621 points of intersections, was used. The lattice were placed over the micrographs, and the intersections were used for point counting volumetry. The volumetric fraction of synthetic organelles and myofibrils, expressed in percentage, was measured in each micrograph rather than for each cell, since this readily allowed the inclusion of small profiles of cells sectioned at some distance from the nucleus. This procedure avoided sampling bias since the organelles in SMCs are localized generally in the perinuclear region.

Immunohistochemistry

Tissue sections of carotid and femoral artery embedded in paraffin were sectioned at 3-4μm. The sections were deparaffinized at 58-59°C for 30 minutes, washed in xylene baths and rehydrated through a series of graded ethanols and treated with trypsin at 0.05% in $CaCl_2$ at 1% (pH 7.8) at 37°C for 10 minutes. The endogenous peroxidase activity was blocked in three 5-minutes-baths of H_2O_2 at 3% in distilled water. The sections were incubated with the primary antibody[4] for 15 hours at 4°C, with biotinilated secondary antibody[5] for 1 hour at 37°C, and with a conjugated streptavidin-biotin-peroxidase[6] for 30 minutes at 37°C. The 3,3-diaminobenzidine was used as chromogen and the slides were counterstained with Harris hematoxylin.

[4] Dako - smooth muscle α-actin, 1A4. - 1:300.

[5] Dako - 1:100.

[6] Dako - 1:100.

Other sections of the same block were deparaffinized and hydrated as described above and immersed in a solution of citrate buffer 0.01M, pH 6.0 and irradiated 6 times for 5 minutes each in a domestic microwave oven at 700 W. The activity of endogenous peroxidase was blocked by 3 baths in 3% H_2O_2 in distilled water. The sections were incubated with a monoclonal antibody anti-receptor of the $1,25(OH)_2D_3$[7] for 15 hours at 4°C and with biotinilated secondary antibody[5] for 1 hour at 37°C and with conjugate streptavidin-biotin-peroxidase[6] for 30 minutes at 37°C. The 3,3 diaminobenzidine was used as chromogen, and the slides were counterstained with Harris hematoxylin.

RESULTS

Gross lesions in all sheep were very similar and did not differ from those of spontaneous enzootic calcinosis induced by *N. veitchii* described elsewere. Microscopically, the aorta and muscular arteries had variable degrees of medial mineralization and intimal proliferation (Fig. 1). In the initial stages, monocytes, macrophages and swollen SMCs invaded the intima. Concurrently, calcium deposition was observed in the internal elastic lamina and between SMCs of the neighboring media. Macrophages, multinucleated giant cells and undifferenciated mesenchymal cells were generally seen in areas of mineralization of the media, frequently associated to plaques of cartilage and bone metaplasia. Thickening of the intima was observed in all cases and the predominant cells were modified SMCs, as evidenciated by the positive immunohistochemical reaction to α-actin of SMCs. The SMCs in the DIT had a fusiform, stellated or branched shape (Fig. 2), and were surrounded by dense collagen fibers and newly formed elastin. The nuclei of some but not all cells of DIT and the media reacted positively to VDR antibodies, and all endothelial cells reacted strongly.

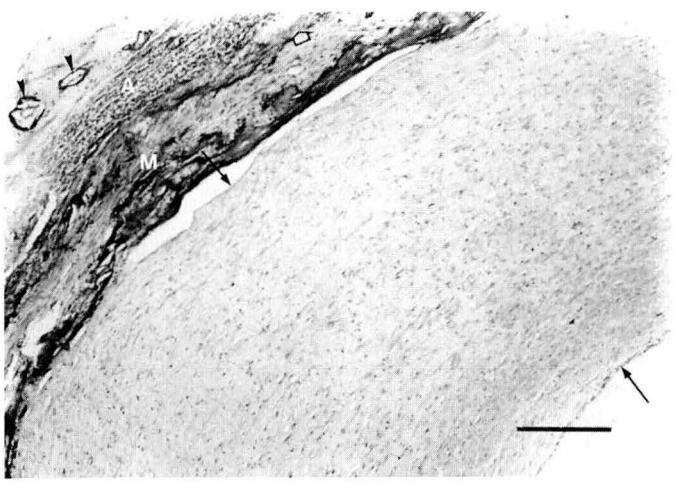

Fig. 1. Femoral artery - Prominent intimal thickening (arrows). Calcified Media (M). Adventitia (A). Calcified small arteries of adventitia (arrowheads). The non-calcified media is reduced to few layers of Smooth Muscle Cells-SMCs (empty arrow). HE. Bar = 250μm.

[7] MAB 1360, Chemicon International. - 1:2000.

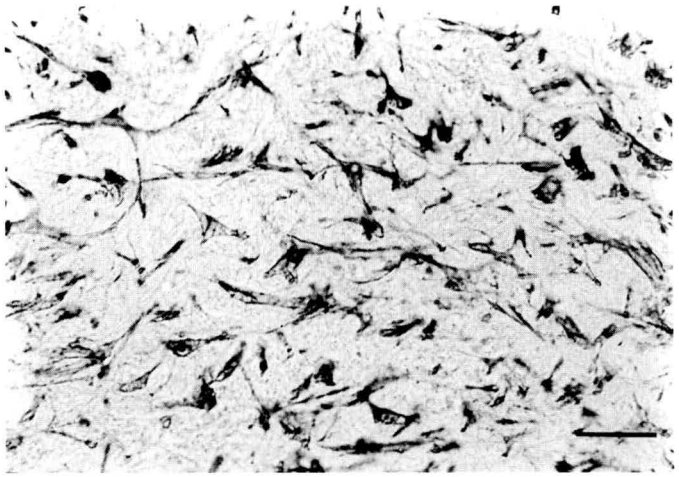

Fig. 2. Femoral artery - Intimal thickening. Note the stellated, fusiform and branched forms of modified SMC. Anti-α-actin of SMC - Streptavidin-biotin-peroxidase. Counterstained with hamatoxylin. Bar = 50μm.

The distribution of DIT was irregular in all arteries, and was particularly conspicuous in the muscular type. Neovascularization of the calcified media and within areas of cartilage and bone metaplasia was frequently seen. However, neovascularization of the intima was only observed in one carotid artery.

The mineralization process was restricted to the media and rarely involved the intima and, in those cases, calcium deposits were mild and in close contact with the internal elastic lamina. In the DIT of only one animal, there were cells with cytoplasmic lipid droplets and cholesterol clefts in the extracellular matrix of the femoral and carotid arteries. Necrotic foci were never seen in this location.

Ultrastructurally, endothelial cells were swollen and had dilatation of the rough endoplasmic reticulum. The basal lamina was replicated or fragmented and, in some areas, partially absent (Fig. 3). Edema and a flocculent material were seen in the sub-endothelial space. The SMCs of the DIT and of the media subjacent to the internal elastic lamina showed reduction of the myofibrils, and increase of the synthetic organelles (Fig. 4 and 5) and a continuum between a typical contractil and synthetic form was observed. The Golgi system was very prominent, associated with an increase and dilatation of the endoplasmic reticulum, and increase of free ribosomes and mitochondria. These modified cells exhibited reduction of the number of pinocytic vesicles, partial or complete loss of basal lamina, and in some cells the basal lamina was duplicated or fragmented (Fig. 3). Some cells of the media, located near the calcified areas, had a significant increase of glycogen granules sometimes forming large cytoplasmic aggregates. Occasionally, modified SMCs were seen migrating to the intima throughout internal elastic lamina gaps (Fig. 6).

Morphometry in light microscopy

There was no significant thickness variation between locations where the intima did not show thickening and where

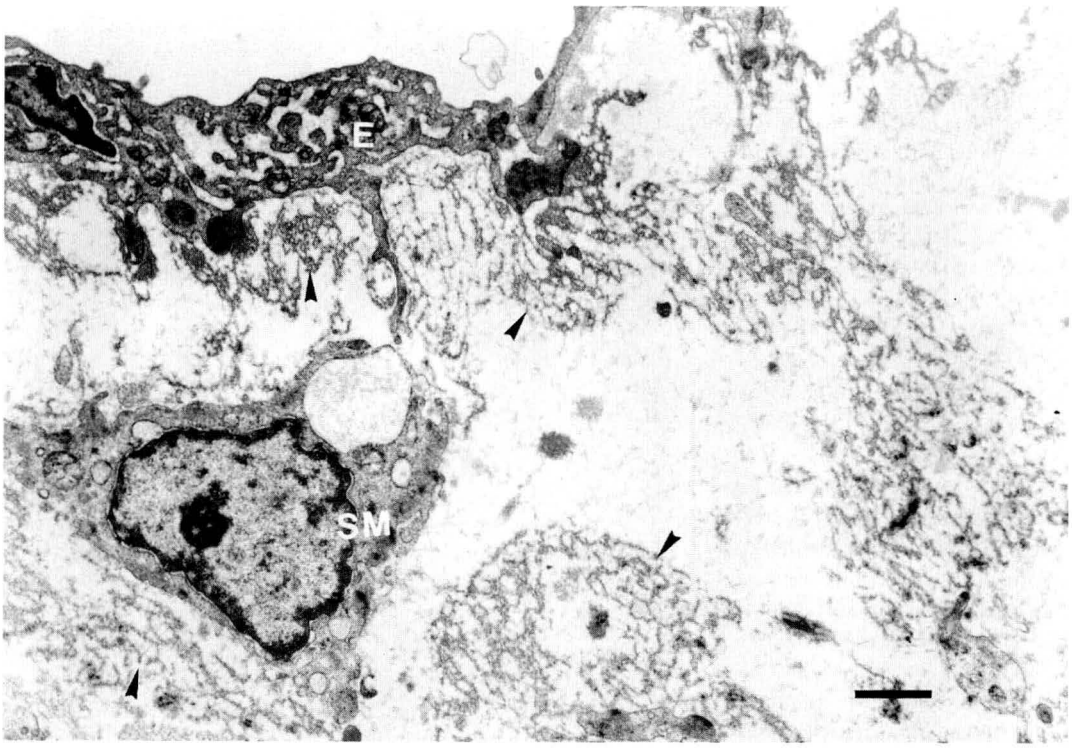

Fig. 3. Electron micrograph. Carotid artery. Swol-len endoplasmic reticulum of endothelial cells. Subendothelial edema and pronounced hyperplasia of the basal lamina-like material displaying several superimposed and partially anastomosed layers (arrowheads) on the endothelial cells (E) and modified SMC (SM). Uranyl acetate-lead citrate. Bar = 1mm.

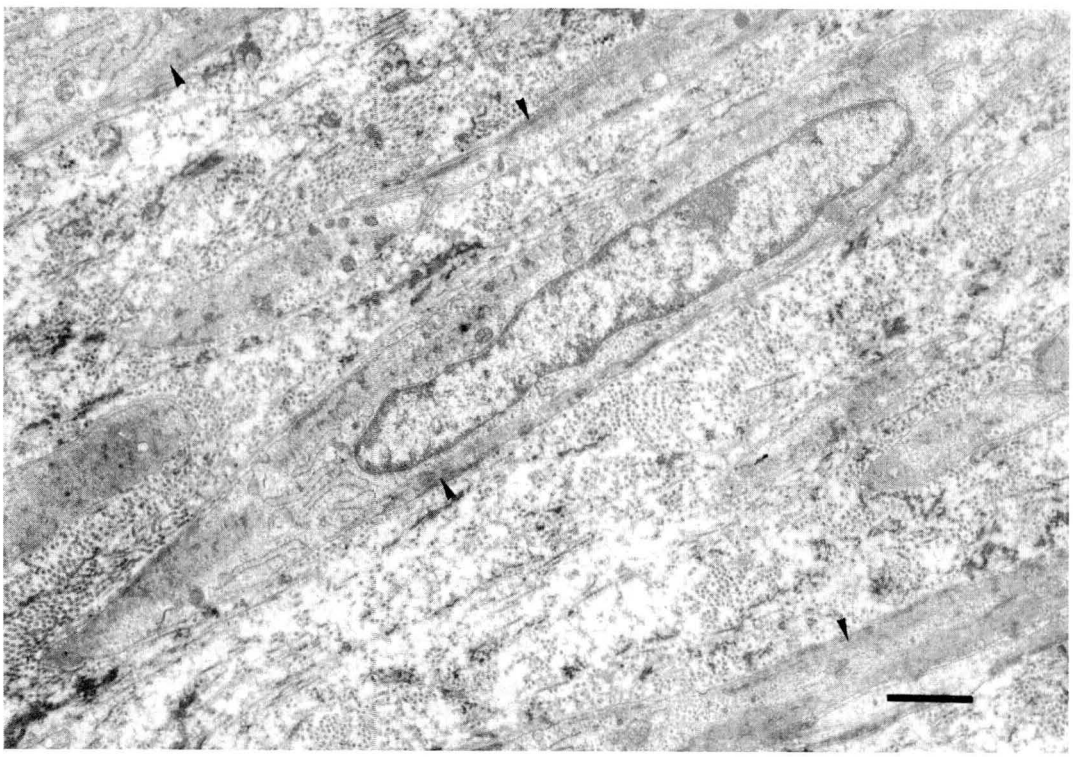

Fig. 4. Electron micrograph. Carotid artery. Typical modified SMCs of the synthetic type. Note the reduction and peripheric distribution of the myofilaments and dense bodies (arrowheads) and increase of synthetic organelles. Uranyl acetate-lead citrate. Bar = 1mm.

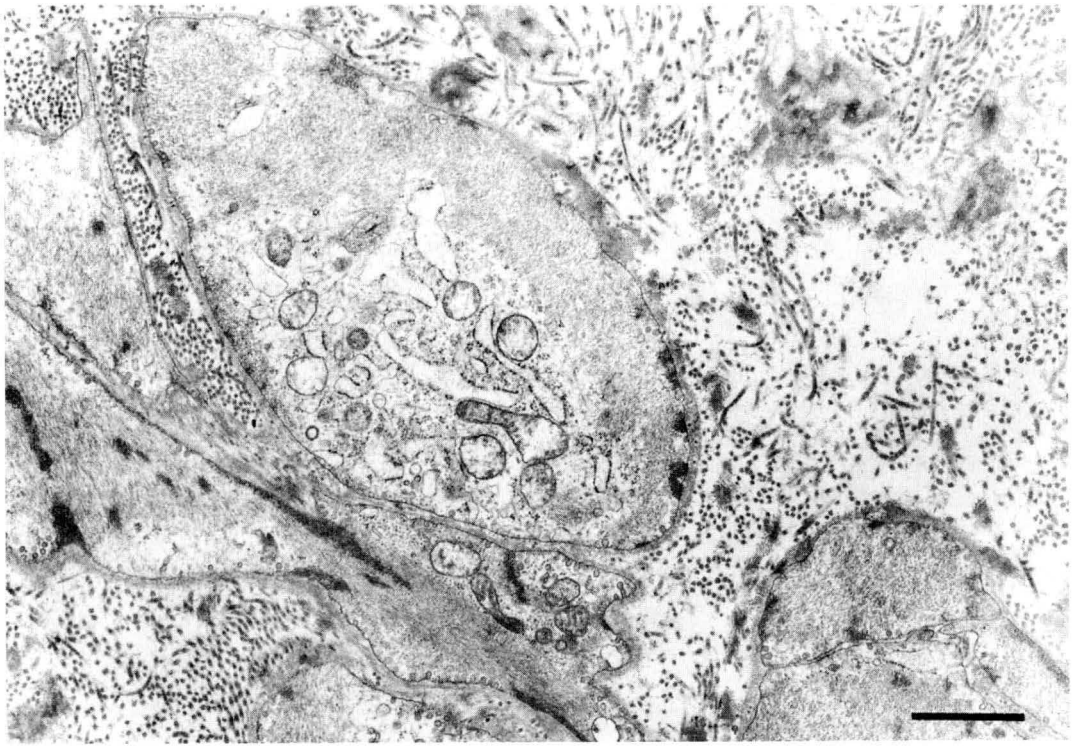

Fig. 5. Electron micrograph. Intimal thickening of carotid artery. Cross section of modified SMC. Bundles of colagem fobers and elastin in the extracellular matrix. Myofilaments are restricted to the periphery of the cell. Uranyl acetate-lead citrate. Bar = 1μm.

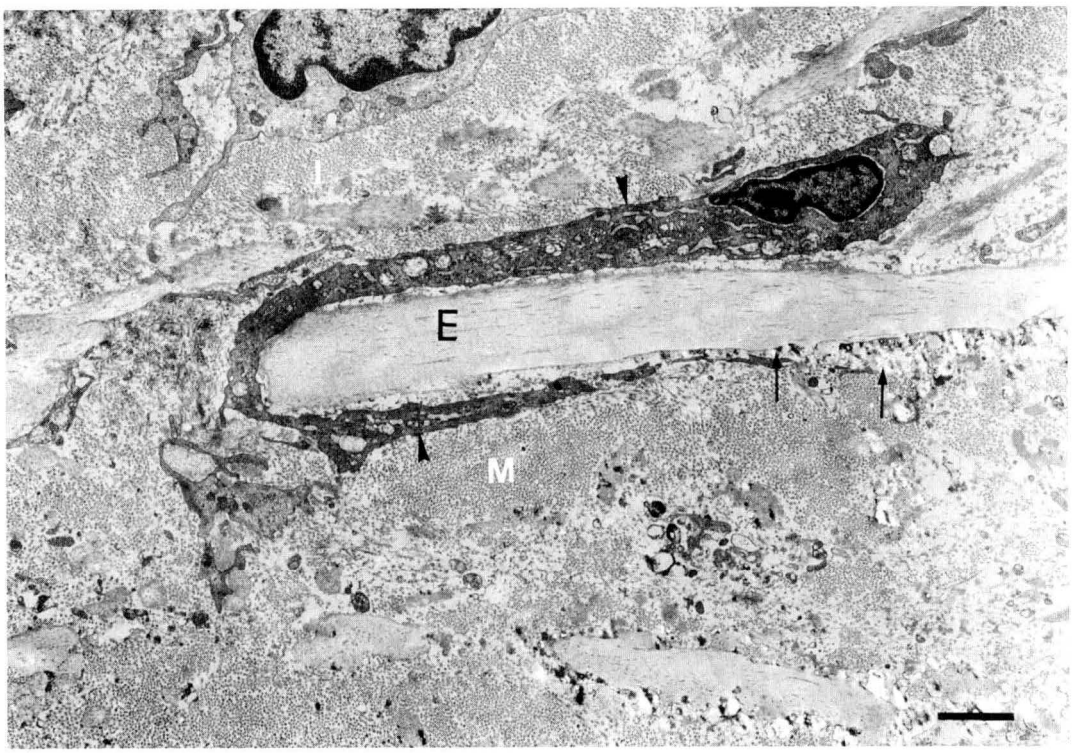

Fig. 6. Electron micrograph. Carotid artery. A modified SMC (arrowhead) migrate from the media (M) to the intima (I) through a gap in the Internal Elastic Lamina (IEL) (E). Note calcium precipitation on the medial side of the IEL (arrows). Uranyl acetate - lead citrate. Bar = 1μm.

the thickening was minimal. The femoral artery showed the highest thickening of the intima (1.33 mm) however did not differ significantly from other arteries that showed DIT. The abdominal aorta showed the most significant decrease in the thickness of the non-calcified media, sometimes limited to 2 or 3 layers of SMCs. The largest calcified areas were observed in the thoracic aorta.

The decrease or increase of the thickness of the intima, calcified media and non-calcified media did not show any correlation.

Morphometry in electron microscopy

There was a significant increase ($P<0.0006$) in cytoplasmic volume of synthetic organelles (44.26 ± 19.59%) in SMCs of the intima when compared to those of the media (13.88 ± 9.45%). Inversely, the cytoplasmic volume of myofibrils in cells of the DIT (55.74 ± 19.59%) decreased significantly ($P< 0.0001$) when compared to those of the media (86.12 ± 9.45%). In areas of DIT, the SMCs showed an increase of 318% in cytoplasmic volume of synthetic organelles when compared to those of the media.

DISCUSSION

Grosss and microscopic alterations observed in the arteries were similar to those described in natural and experimental calcinoses of domestic animals (Carrillo & Worker 1967, Barros et al. 1970, Dirksen et a.l 1970, Döbereiner et al. 1971, Riet-Correa et al.1981, Riet-Correa et al. 1987).

Calcification foci in thickened intima were rare, mild, and located near the calcified IEL. Similar findings were reported by Arnold & Brass (1956) and Döbereiner et al. (1971) in bovines with calcinosis. The lack of calcification of the intima may be related to the loss of capability of intimal cells to express the same phenotypic features as their predecessor cells of the media or to an unknown factor that may inhibit this expression.

The DIT was variable and irregular in all the examined arteries and was more pronounced in muscular and elastic-muscular arteries. Similar observations were reported by Hass et al. (1960) in experimental poisoning by vitamin D in rabbits and by Barros et al. (1970) and Riet Correa et al. (1987) in enzootic calcinosis in sheep. DIT was described in calcinosis of cattle (Arnold & Brass 1956, Lynd et al. 1965, Carrillo & Worker 1967, Köhler & Libiseller 1969, Dirksen et al. 1970, Krook et al. 1975) and calcinosis of sheep (Barros et al. 1970,Tustin et al.1973, Riet-Correa et al. 1987) as an increase of elastic and collagen fibers or subendothelial cell proliferation without a definitive identification of the cell types involved. In this report the cells present in the DIT were mostly SMCs as determined by immunohistochemistry and electron microscopy. These results are similar to what is described for DIT in human atherosclerosis (Orekhov et al.1984, Aquel et al. 1985, Mosse et al.1985, Katsuda et al. 1992) . Hass et al. (1960), studying hypervitaminosis D in rabbits, did not find correlation between the intensity of intimal proliferation and the morphologic abnormalities of the internal elastic lamina and media. In our study, similar observations were made, there was no correlation between the increase or decrease of the intimal thickening when compared to that of the media. The variation in thickness of the intima indicated the degree of irregularity of the DIT according to the marked variability of the standard deviation of the mean of each artery. Dead and dying SMCs seen in arteries by Toda et al. (1983) in experimental hypervitaminosis D in swine, were not observed in the calcinosis of sheep.

The SMCs present in areas of DIT had severe changes in phenotypic expression and it is likely that most of the undifferenciated mesenchymal cells found on these locations are derived from modified SMCs. In fact, the detection of the -isoform of actin in the majority of the cells of DIT and the media is an useful criterion to distinguish them from fibroblasts and other nonmuscle cells that do not contain the -isoform (Kocher et al. 1984). The ultrastructural morphometric analysis of the cells of DIT showed a significant increase in the volumetric fraction of synthetic organelles (44.26%) of intimal SMCs when compared to medial SMCs (13.88%). There was an increase of 318% in the cytoplasmic volume occupied by synthetic organelles of SMCs in areas of DIT, when compared to medial SMCs. Mosse et al. (1985), studying human carotids with atherosclerosis, reported a two-fold increase in the concentration of organelles of intimal SMCs and a great variability in the content of organelles of these cells when compared to medial SMCs.

In our study, receptors for $1,25(OH)_2D_3$ (VDR) were detected by immunohistochemistry in endothelial cells and SMCs of the media and the DIT. VDR's which are DNA-linked proteins (Mitsuhashi et al. 1991) were identified in SMCs *in vitro* (Kawashima 1987, Merke et al.1987, Koh et al. 1988). Stimulation of these receptors induce cell proliferation and differentiation in several tissues (Pike 1985, Pols et al. 1990, Walters 1992), and there is an increase in DNA synthesis and a decrease in heparan sulfate, a glycosaminoglycan responsible for the inhibition of the proliferation of these cells, allowing multiplication of the SMCs (Koh et al. 1990).

The process of intimal thickening in sheep calcinosis could be the result of the action of $1,25(OH)_2D_3$, contained in calcinogenic plants, on the medial SMCs, inducing cell differentiation, migration and proliferation in the intimal space as proposed by Barros et al. (1995)

In atherosclerosis, migration of SMCs results from the actio of chemical mediators released by macrophages and platelets (Ross & Glomset 1976, Ross 1986, Sanders 1994), endothelial cells (Guerrity 1981, Sanders 1994), injured SMCs (Sanders 1994) and fibroblasts (Jackson & Reidy 1993). Mediators can induce phenotypical modifications in SMCs which become synthetic and migrate throughout the internal elastic lamina to the intima (Sanders 1994). On the other hand, altered endothelium could stimulate adhesion and migration of monocytes to the subendothelial space (Simionescu et al. 1993). In the calcinosis of sheep, the induction factor of the endothelial and SMCs changes seems to be $1,25(OH)_2D_3$ contained in *N. veitchii*, as previously suggested by Moraña et al. (1994) for gastric SMCs of rabbits intoxicated with *Solanum malacoxylon* and by Barros et al. (1995) as the cause of vascular

SMCs alterations leading to DIT in calcinoses of domestic animals.

Comparing DIT of human atherosclerosis and of sheep calcinosis, the cells that initiate the DIT in both processes are apparently endothelial and SMCs. In the calcinosis, DIT is predominantly a fibromuscular process, there are neither atheromatous plaques nor thrombosis, as observed in atherosclerosis. Calcification of the intima, frequently observed in atherosclerosis, was mild and rarely observed in the calcinosis of sheep.

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