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Histomorphological Changes by Epididymal Lithiasis in Roosters

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ABSTRACT

Epididymal lithiasis (EL) histopathology is described using light and electronic microscopy in roosters (*Gallus gallus domesticus*) naturally affected by EL in Minas Gerais, Brazil. The histologic and morphological changes by EL in roosters was performed regarding cellular and subcellular details through light and electron microscopy. Efferent ductules epithelium lysosomal increase in size and numbers, membrane rupture, cellular vacuolation, ciliary loss, basal membrane degeneration, inflammatory reaction with mononuclear infiltrations, edema, epithelial and vascular endothelium losses were described. All industrial and free-range chickens showed EL in varying degrees in the efferent ductules (ED). However, ED altered areas did not correlate with the presence of luminal stones. Non-ciliated ED epithelium cells presented several atypically large lysosomes. Plicae loss and basal vacuoles were observed in the epithelium of dilated regions. Cellular cilia loss and apical cytoplasmic membrane rupture resulted in leakage of the cytoplasmic contents to the ED lumen, and ED epithelium desquamation occurred with or without lesion to the basal membrane. Basal membrane alterations were associated with profound sub-epithelial connective tissue damage. Aggregations of desquamated epithelium and spermatozoa were seen in the lumen of ED and compact aggregates were considered the basis for calculi formation. The widespread occurrence and high severity of EL lesions are indicative of the importance of EL as a cause of infertility in male chickens.

1. INTRODUCTION

After domestication, female chickens have developed continued egg production throughout the year. However, for breeding stocks, males are retired from reproduction sooner than females, mainly due to infertility, with reduced spermatogenesis, in a condition characterized by the precocious decline in fertility in roosters (Muncher *et al.*, 1995). Infertile roosters exhibit an almost complete loss in fertility by 110 weeks of age and the pathogenesis involves the formation of calcium carbonate calculi in the testicular efferent ductules (Rosenstrauch *et al.*, 1994, Muncher *et al.*, 1995).

Epididymal lithiasis (EL) was previously described in 75% of sexually mature 18-26 weeks old domestic roosters in the USA, with smooth spherical cystic formations containing a clear fluid. In roosters older than 26 weeks of age, the spherical formations may be solid and irregular with a yellow core, and the number and size of calculi, varying from 9 to 160μm, increased with age, being widespread in old roosters. The chemical analysis revealed calcium (48%) as the major component and only 7.7% organic compounds. Reduced spermatogenesis and lowered blood testosterone levels were found (Janssen, *et al.*, 2000). Mahecha

et al. (2002) studied the occurrence of EL in commercial and subsistence roosters from different regions of Minas Gerais State, Brazil. and found in 94,3% of chickens with calculi. The number of calculi varied from 4 to 245 per male and no lesion was observed in other organs. This study was proposed due the lack of detailed histomorphometry on EL, a condition of potentially high economic significance for the poultry industry. An investigation of EL in 100% of different breeds and from different geographical regions of Brazil the pathology mainly after 55 weeks of age, with more severe testicular dysfunction at 100 weeks of age. EL seemed higher and more severe in intensively-reared chickens, as compared to backyard roosters, in which 50% presented calculi. The demonstration of EL in infectious bronchitis virus (IBV) negative SPF flocks, although aggravating, virtually eliminated IBV as a primary cause (Rocha Jr *et al.*, 2009).

2. MATERIALS AND METHODS

2.1 – Chickens

Fifty-eight adult domestic roosters, all showing EL, obtained from different regions of Minas Gerais state were studied. Roosters were euthanized with intravenous penthobarbital (50mg/kg), perfused intravenously with saline (Nacl 0,75%) and glutaraldehyde 2,5% in phosphate buffer (0,1M pH 7.4; 4°C). Testes were removed and the epididymis was dissected at binocular microscopy. The diagnosis of EL was based on visualization, palpation and light microscopy, and positive and negative epididymides were examined by transmission electronic microscopy.

2.2 – Light microscopy

Epididymal fragments were decalcified in Perenji solution, dehydrated in methanol increasing concentration series, embedded in glycol methacrylate and cross-sectioned in a glass blade microtome (Reichert-Jung, Germany). Cross-sections were stained by toluidine-blue 1% in sodium borate 0.5%/30s. Photo documentations were performed in an Olympus camera microscope with Kodak 100 ASA film.

2.3 – Electron microscopy

Selected efferent ductules fragments were transferred to glutaraldehyde 2.5% in phosphate buffer 0,1M, pH 7,4/4°C/1h and washed three times in the same buffer. Phosphate buffer tissue fragments were post fixed with osmium tetroxide 2.5% in phosphate buffer as above for 3 hours. Tissues were dehydrated in increasing ethanol concentrations, followed by three washes in acetone, preinfiltrated in resin (Araldite and Epon 812 at equal concentrations in acetone), infiltrated with pure resin and embedded in plastic capsules. Thin sections (0,5 a 1,5µm) were performed with 6mm glass blade, mounted in glass slides and stained with toluidine blue 1% in sodium borate 0,5% for preparing ultra-thin sections (60 a 100 nm) with a diamond blade on a ultramicrotome. Ultra-thin sections were mounted onto a 200/300 pore/inch copper mesh and counterstained with 2% uranile acetate and lead citrate, examined in a transmission electron microscope Zeiss EM-10 and photographies were printed on F3 Kodak paper.

3. RESULTS AND DISCUSSION

3.1- Epithelial tissue lesions

The examination of macroscopically affected epididymis at optical microscopy revealed efferent ductules with areas of normal and altered epithelium, in which the ciliated and non-ciliated cells were not affected. However, areas with affected epithelium could not be associated to luminal calculi, although all roosters presented a certain degree of alteration. The spatial distribution of epithelial alterations was not continuous or sequential and did impose difficulty for the analysis of the chronology of events. However, the semi-serial cross-sections analyses enabled the assumption of relationships between epithelial alterations and luminal calculi, which could be supported by the analyses of the electron microscopy (EM) photomicropraphs. Considering EL pathogenesis, the initially mild changes in a few epididymis cells and structures, evolved to cellular membrane alterations and culminate to epithelial degeneration.

The first detected change in the epididymis efferent ductules epithelium was the non-ciliated cells increase in numbers of supranuclear region lysosomes (Fig. 1-A) and sizes (Fig. 1-B), and despite no quantification was performed, the alteration was visible by optical microscopy. In normal roosters, an average of eight lysosomes would be visible per epithelial cell, and larger numbers were observed in the EL. In ductules epithelium affected by EL, the lysosomes showed rupture and disorganization, increased in size apparently through fusion, and in numbers, by the formation of secondary lysosomes with internal varying EM density vesicular bodies (Fig. 1-B). At sites with dilation and plicae loss

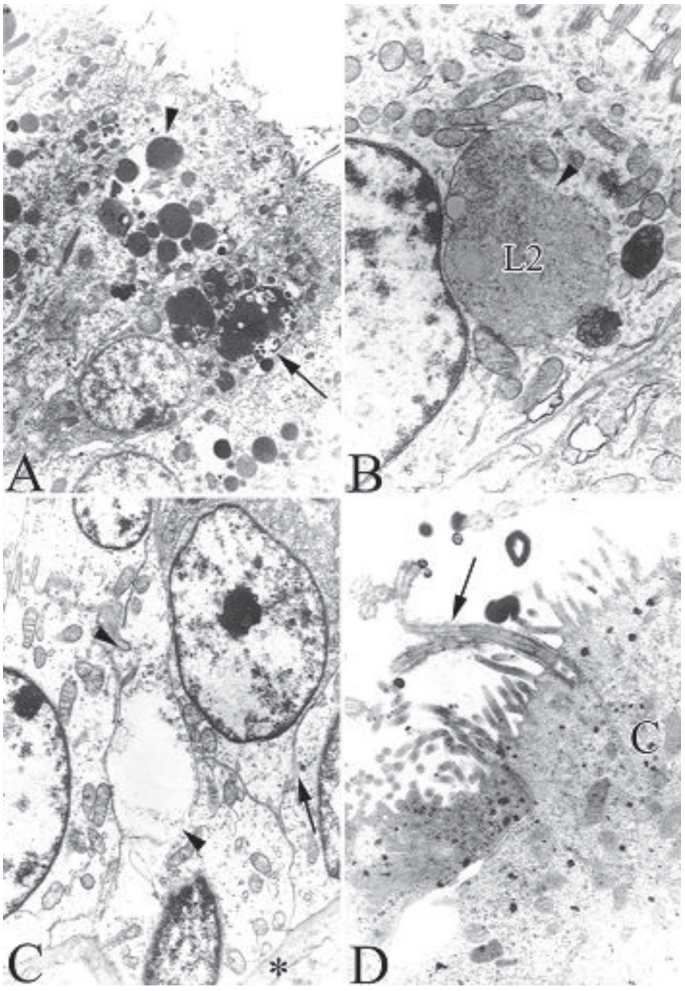

Figure 1 – Transmission Electron Microscopy (TEM). Efferent ductule epithelium ultrastructural alterations of roosters affected by epididymal lithiasis.

A – Primary (arrow head) and secondary (arrow) lysosomal numbers increase in the supranuclear region of cells. The secondary lysosomes show vesicular bodies of different electronic densities. (14.000X)

B – Secondary lysosome increase in size (L2), with ruptured membrane and leakage into the cytoplasm (arrow head). (30.000X)

C – Basal epithelial cell vacuolation starting with tumefaction of electron dense internal and external plasmatic membrane (arrow) and rupture of cellular membranes (arrowheads) at basal and lateral sides. The basal membrane (*) presents a normal aspect. (30.000X)

D – Ciliated cell (C) with cilia loss. Remaining cilium with apical fragmentation (arrow). (30.000X).

in efferent ductules epithelium, basal vacuoles were observed, which varied from discrete to involving the entire cytoplasm (Fig. 1-A, B, C and D), in agreement with previous findings (Mahecha *et al.*, 2002). The vacuolization of ciliated and non-ciliated cells of the efferent ductules epithelium was possibly caused by the rupture of the cytoplasm membrane, initiating by the tumefaction of the electron dense internal and external layers of the membrane of basal and lateral cells (Fig. 1-C and 4-C). The tumefaction was evident by the loss in sharpness of the electron-dense layers contours, layers which may rupture partially or totally, maintaining single-layer images (Fig. 1-C). The continuity of cytoplasmatic membranes loss was followed by the formation of clear content vacuoles with organelle debris at the periphery (Fig. 2-A, B, C and D; 4-A and B). The coalescing contiguous vacuoles

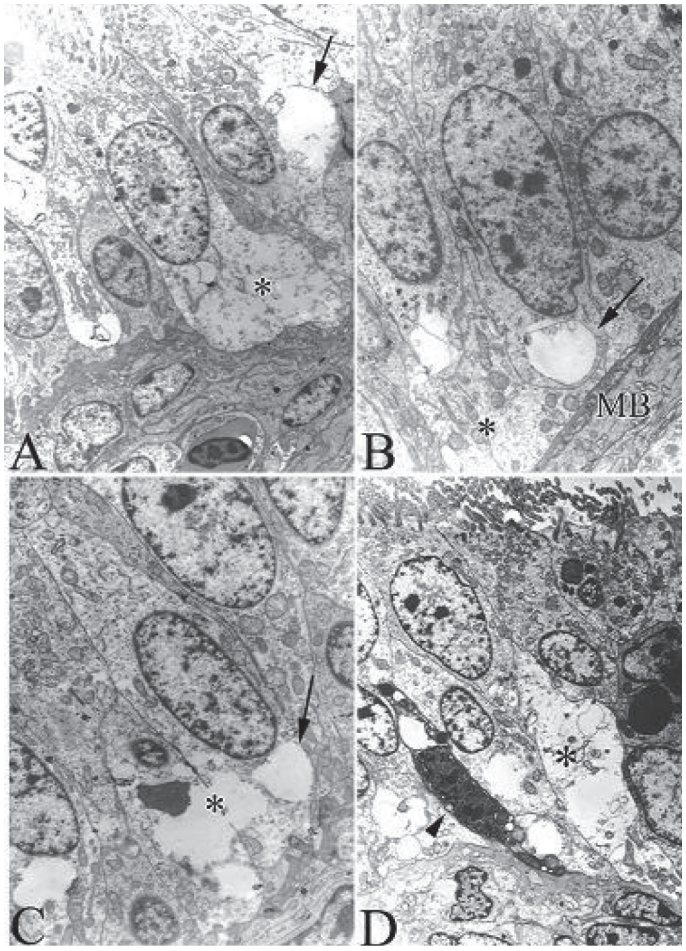

Figure 2 – Ultrastructural aspects of cellular vacuolation (TEM).

A, B, C and D - Vacuoles containing residues of organelles in lysis (*). Rupture of cellular membrane and formation of vacuole (arrow). Basal vacuolation (arrow) with preserved basal membrane (MB). (14.000X)

may evolve to the complete hydrolisis of the cell. The rough and smooth endoplasmic reticula membranes showed also rupture and disorganization, similar to that of the cytoplasmic and lysosomic membranes, releasing enzymes to the cytoplasm, possibly associated to cellular vacuolation, and would culminate in cellular apoptosis (Fig. 3-B). However, the mitochondrial and nuclear membranes did not show fragmentation, even in advanced cellular degradation processes. The microvilli of epithelial cells apical region were also degraded. The rupture apical membranes enabled cytoplasmic contents to leak into the tubular lumen (Fig. 1-B and 3-A). Ciliated cells showed cilia loss, the remaining cilia with apical fragmentation (Fig. 1-D).

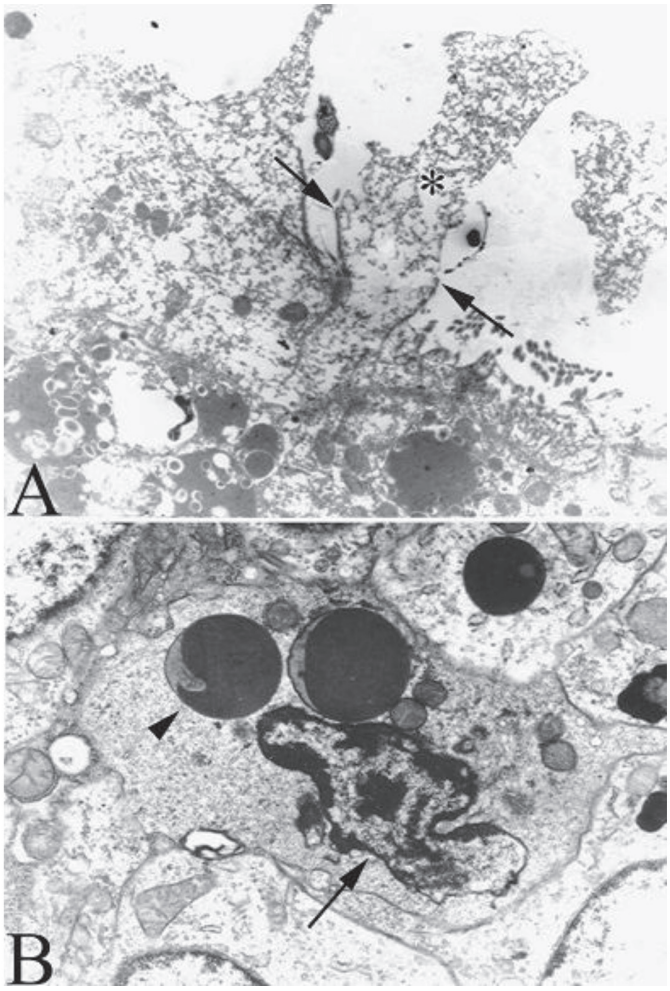

Figure 3 – Efferent ductules ultrastructural aspects of cellular vacuolation and other alterations (TEM).

A – Epithelial cell apical membrane rupture with leakage of cytoplasmic content into the efferent ductule lumen. (30.000X)

B – Epithelial cell with apoptotic bodies (arrow head). The nucleus is going through apoptosis (arrow). (30.000X)

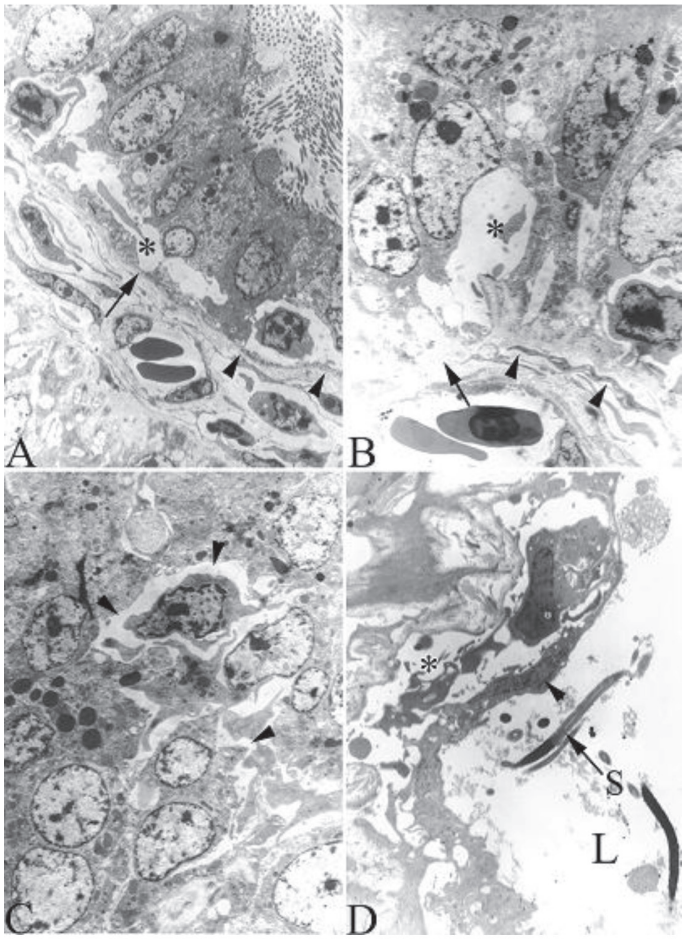

Figure 4 – Ultrastructural aspects of the efferent ductule epitelial desquamation (TEM)

A, B – Epithelium under vacuolation (*). Basal membrane (arrowheads) with fragmented portion (arrow). (14.000X)

C – Tangential section of epithelium with edema shown by increased intercellular space (arrowheads). Cells present nuclear condensation. (14.000X)

D – Epithelium loss with fibroblast (arrowhead) in direct contact with the lumen. Edema of the subepithelial connective tissue (*). Spermatozoon (S) in the ductule lumen (L). (14.000X)

Efferent ductules epithelial necrosis was also observed, together with edema, retraction, liquefaction and apoptosis (Fig. 3-A and B), alterations which lead to desquamation, with or without basal membrane lesion (Fig. 1-C). Desquamating cells may be also with normal trilaminar cytoplasmic membrane, nucleus and organelles. Basal membrane fragmentation and rupture (Fig. 4-A and B) is involved in the desquamation of efferent ductules epithelium and lead to profound subepithelial connective tissue changes. Epithelial desquamation did not seem to represent a final event for the efferent ductules, as recanalization was observed (Fig. 5-D). Masses of desquamated cells (calculi) were surrounded by connective tissue (5-E), due to the epithelial loss. However, other cells which undergo desquamation may be found in the lumen (5-B and F) with the pathology described above.

In advanced cases, the inflammatory process was chronic and characterized by fibroplasia up to total epithelial substitution by fibrous connective tissue (Fig. 5-F). With the total epithelial loss, the connective tissue was also affected by calculi.

3.2- Connective tissue lesions

Connective tissue changes are initiated by infiltrating mononuclear inflammatory cells, primarily macrophages and lymphocytes, as described previously (Janssen *et al.*, 2000; Mahecha *et al.*, 2002). Infiltrates were diffuse or multifocal (not shown).

Endothelial changes with vascular clotting formation and endothelium desquamation (not shown) were observed. Sub-endothelium edema was observed only in areas where the adjacent epithelium was degenerated (not shown).

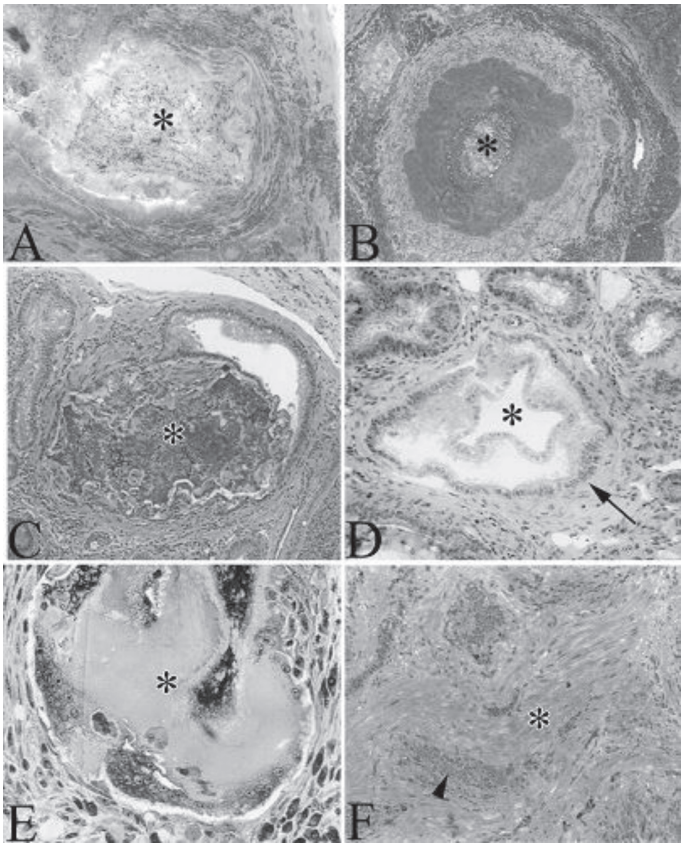

Figure 5 – Light microscopy of efferent ductules. Formation of epididymal calculi stained by sodium borate toluidine blue 1%.

A – Luminal accumulation of spermatozoa (*) (600X)

B – Luminal accumulation of desquamated cells and spermatozoa, organized as a central mass (*). (300X)

C – Luminal calculus (calcified) (*). Calcium stain (alizarin). (300X)

D – Recanalization (arrow). (600X)

E – Epididymal calculus (*) surrounded by connective tissue. (1000X)

F – Epithelial substitution by fibrous connective tissue (*). Epithelial residues (arrowhead) are within fibrous areas. (600X)

3.3- Calculi formation

Efferent ductules spermatozoa accumulations were observed (Fig. 5-A), possibly associated to epithelial degeneration and fluid reabsorption loss, with reduced epithelium plicae. Desquamation of lining and tubular epithelia formed aggregates with spermatozoa (Fig. 5-B). Dislocated calculi appeared in the epididymal ductule. Mixed cellular aggregates may organize into compact masses, as described previously (Mahecha *et al.*, 2002), may calcify, forming the efferent ductules calculi (Fig. 5-C), which may be lined by ductule epithelium, and may induce an inflammatory reaction (not shown). Calcified calculi were observed in the lumen of the efferent ductule (6-A and B), in the epididymal duct (6-C) and deferent duct (6-D). Calculi (6-D) were surrounded by several layers of mucus and the deferent duct indicating the elimination route of calculi (6-D).

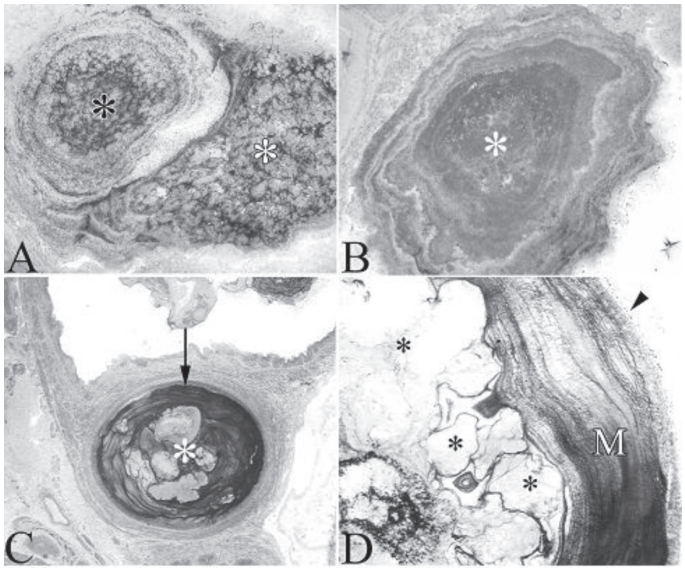

Figure 6 – Aspects of epididymal calculi stained by sodium borate toluidine blue 1%. A, B, C and D - Calculus (*) well organized and calcified in the lumen of the efferent ductule (A and B), in the epididymal duct (C) and deferent duct (D). Calculi (D) are surrounded by several layers of mucus (M). Epididymal duct epithelium (arrow) (C). Deferent duct (arrowhead) (D) indicating the elimination route of calculi (*). (300X)

4. CONCLUSIONS

The study of cellular and subcellular details by light and electron microscopy of the histologic and morphological changes caused by EL in roosters was performed. Major changes were efferent ductules epithelium lysosomal increase in size and numbers, membranes rupture, cellular vacuolation, ciliary loss, basal membrane degeneration, inflammatory reaction with mononuclear infiltrations, edema, epithelial and vascular endothelium losses. The severity of lesions here described, of high significance for fertility, and its widespread occurrence, as previously reported, justify the recommendation for preventive measures against EL, possibly changes in the biosecurity strategy, although research is still required on the elucidation of the etiology and pathogenesis.

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