Vitamin D3 and androgen receptors in testis and epididymal region of roosters (Gallus domesticus) as affected by epididymal lithiasis

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Abstract

Epididymal lithiasis is a dysfunction characterized by formation of calcium-rich stones in the epididymal region of roosters, associated with decreased serum testosterone and loss of fertility. The segment most affected by the lithiasis is the efferent ductules, which, in birds, are responsible for reabsorption of calcium and luminal fluid. Therefore, we postulated that epididymal lithiasis could result from local impairment of calcium or fluid homeostasis, culminating in initiation of stone formation. Transepithelial calcium transport depends on vitamin D3 and vitamin D3 receptor (VDR). Based on the fact that VDR are present in efferent ductules, possible changes in the pattern of VDR in roosters affected by the epididymal lithiasis was investigated, to start to gain an understanding of the molecular mechanisms involved in the development of calcium stones. To evaluate the potential impact of androgen reduction, changes in androgen receptor (AR) were also investigated. Both VDR and AR were increased in specific segments of the epididymal region, whereas no alterations were found in the testes of affected animals. The increase in VDR was most likely due to an increase in the number of VDR-positive mononuclear leukocyte infiltrates found in the connective tissue followed by an increase in epithelial receptors. The AR were increased, however, mainly in the epididymal duct epithelium. These results suggest that the vitamin D3 and androgen responsive system may be directly/indirectly involved in the development of the disease.

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1. Introduction

Epididymal lithiasis is a dysfunction described in diverse populations of roosters throughout the world, and is characterized by the formation of stones rich in calcium (Janssen et al., 2000; Mahecha et al., 2002; Jackson et al., 2006). The occurrence of epididymal stones appears restricted to roosters, because these stones were not found in several other avian species (Mahecha et al., 2002). This reproductive disorder results in an early loss of fertility, associated with decreases in daily sperm production and serum testosterone concentrations (Janssen et al., 2000; Boltz et al., 2004). Despite the potential negative economic impact, the cause of this anomaly and the molecular mechanisms involved in the formation of calcium stones and reduction in fertility are unknown. Several hypotheses have been proposed, including elevated dietary intake of calcium, avian infectious bronchitis virus vaccination or another infectious agent, as well as the intense genetic selection of roosters for rapid growth and egg production, which requires increased mobilization of calcium (Janssen et al., 2000; Mahecha et al., 2002; Boltz et al., 2004; Jackson et al., 2006). Nevertheless, none of these hypotheses have proven to be the primary cause of the epididymal lithiasis.

Within the epididymal region, formation of calcium stones is greatly restricted to the efferent ductules, resulting in severe epithelial injury in this segment (Mahecha et al., 2002; Boltz et al., 2004). In birds, efferent ductules are prominent, constituting up to 60% of the epididymal region (Aire, 1979; Oliveira et al., 2007), thus indicating the importance of these structures for avian reproduction. These ductules reabsorb most of the testicular fluid, an essential function to facilitate sperm concentration and maturation (Clulow and Jones, 1988). Differing from mammals, the avian efferent ductules also have an important role in the reabsorption of significant amounts of calcium (Clulow and Jones, 2004). Therefore, because calcium is a large component of the epididymal stones (Janssen et al., 2000; Mahecha et al., 2002), it is plausible to postulate that, regardless of its origin, epididymal lithiasis could result from local impairment of calcium or fluid homeostasis, culminating in an increased concentration of luminal calcium and consequent initiation of calcium stone formation.

Transepithelial calcium transport depends on 1,25-dihydroxyvitamin D3 [1,25-(OH)2D3], the active metabolite of vitamin D3, and its receptor VDR (vitamin D receptor) (Pike et al., 1978; Corradino et al., 1993). VDR is differentially expressed in the epididymal segments of roosters and efferent ductules exhibit greater amounts of the receptor (Dornas et al., 2007). These findings, associated with the knowledge that calcium stones are formed mainly in the efferent ductules of roosters (Janssen et al., 2000; Mahecha et al., 2002), raised the possibility that VDR would be a key molecule involved in the development of local calcium stones. Therefore, to investigate possible changes in the VDR in the components of the epididymal region of roosters affected by the anomaly was the aim of the present study. Additionally, testosterone concentrations are drastically reduced in animals affected by the lithiasis (Janssen et al., 2000; Boltz et al., 2004); therefore, the potential impact of androgen reduction on androgen receptor (AR) in the epididymis was also investigated.

2. Materials and methods

2.1. Animals

The investigation was performed on epididymal regions of 20 adult roosters (Gallus domesticus) obtained from commercial sources and housed at the Federal University of Minas Gerais.
facilities, under environmental temperature and light cycle. The animals received water and food *ad libitum*. The principles of research involving animals followed those advocated by the local ethical committee published by the Federal University of Minas Gerais (UFMG) (http://www.ufmg.br/coepbioetica/cetea/).

2.2. Tissue preparation

The roosters were weighed, anesthetized (i.p. sodium pentobarbital 50 mg/kg body weight), and perfused intracardially with 10% neutral buffered formalin (NBF) for histology or immunohistochemistry. After fixation, the epididymal regions were isolated from the testis and preserved in the same fixative until they were embedded in paraffin.

2.3. Diagnostics of epididymal lithiasis

For diagnosis of epididymal lithiasis, epididymal fragments from all roosters were made transparent by clearing in glycerin, as previously described (Mahecha et al., 2002). Briefly, fixed tissues were rinsed in phosphate buffer saline (PBS), transferred to 0.5% (w/v) sodium hydroxide for 24 h and then immersed in glycerin solutions (1:2, 1:1 and pure glycerin). The animals were classified as affected or non-affected according to the presence or absence of epididymal stones, respectively, as seen by transparency of the epididymal region viewed under stereomicroscopy (Fig. 1A and B). Macroscopical findings were validated by histopathological evaluation of fixed epididymal fragments that were stained with Periodic Acid Schiff (PAS) and counterstained with hematoxylin or hematoxylin and eosin (H&E) (Fig. 1C and D).

2.4. Western blotting

Protein analysis was performed by Western blot assay of isolated epididymal regions from non-affected and affected roosters (*n* = 6). Dissected tissues were frozen in liquid nitrogen, thawed and total protein was extracted by addition of sample buffer (1% SDS, 30 mM Tris–HCl pH 6.8, 2-mercaptoethanol, 12% (v/v) glycerol and bromophenol blue). Proteins were separated by electrophoresis on 10% polyacrylamide gels and transferred to nitrocellulose membranes for blocking with 10% normal rabbit serum (NRS) or 10% normal goat serum (NGS) for 1 h at room temperature. Then, the membranes were incubated for 1 h with rat anti-chicken monoclonal antibody against VDR (Labvision Co., Fremont, USA) or rabbit anti-rat polyclonal antibody against AR (Labvision Co., Fremont, USA), both at a dilution of 1:500. Biotinylated rabbit anti-rat (for VDR) and goat anti-rat (for AR) secondary antibodies (Dako, Carpinteria, CA) were diluted at 1:6000 or 1:2000, respectively. The membranes were incubated with an avidin–biotin complex (Vector Laboratories, Burlingame, CA) for 30 min and the reactivity was visualized using DAB/chloronaphtol chromogen. All protein assays were replicated and density of the VDR and AR bands was measured using the Scion Image software (www.scioncorp.com), as previously described (Picciarelli-Lima et al., 2006).

2.5. Immunohistochemistry

Fragments of NBF fixed testes and epididymal regions of non-affected and affected roosters (*n* = 4) embedded in paraffin were used for immunohistochemistry following standard methods for microwave antigen retrieval. For comparison between animals, staining was performed in parallel
Fig. 1. Diagnosis of epididymal lithiasis. (A) After clearing in glycerin, the epididymal region of non-affected animals was completely transparent and revealed absence of stones. (B) The epididymal region of affected animals presented a variable number of stones within the tissue (arrowheads). (C) Proximal efferent ductule of non-affected animal showing columnar and highly folded epithelium. (D) Proximal efferent ductule containing a stone (*) in the lumen. Mononuclear cell infiltrations (M) were frequently found around affected efferent ductules. T = testis; EP = epididymal region; PED = proximal efferent ductules. Bar in A–B = 0.5 mm; bar in C–D = 100 μm.

in triplicate sets. Sections were incubated in 10% NRS or 10% NGS and then with the primary antibodies (rat anti-chicken VDR or rabbit anti-rat AR, diluted 1:50 and 1:500, respectively; Labvision Co., Fremont, CA). Both antibodies were previously validated for use in bird tissues (Dornas et al., 2007, 2008; Oliveira et al., 2007). Negative controls were obtained by substituting the primary antibodies with PBS. After incubation with biotinylated secondary antibodies (rabbit anti-rat for VDR or goat anti-rat for AR) (Dako, Carpinteria, CA), the sections were incubated with an avidin–biotin complex (Vectastain Elite ABC kit—Vector Laboratories, Burlingame, CA) and visualized by immersion in 0.05% 3,3′-diaminobenzidine containing 0.01% H2O2 in 0.05 M Tris–HCl buffer, pH 7.6.

2.6. Semiquantitative immunohistochemical study

The intensity of VDR and AR immunostaining was quantified by computer-assisted image analysis, based on previously reported protocols (Dornas et al., 2007, 2008) using the Image-Tool software (version 3.00, University of Texas Health Sciences Center, San Antonio, TX). For this purpose, 25 nuclei of non-ciliated cells of both segments of the efferent ductules and principal
cells of the epididymal duct, all positive to VDR and AR immunostaining, were traced, measured and the pixel intensity was determined for the traced areas. Background intensity was determined by tracing an unlabeled area adjacent to the measured cells. Final pixel intensity was calculated by subtracting the values detected in labeled nuclei from the background.

2.7. Morphometry

Quantitative studies in the testis of affected and non-affected roosters were performed using classic stereological methodology (Weibel et al., 1969; Oliveira et al., 2007). The testicular epithelium and the interstitial spaces were evaluated using volumetric density (Vv%) to estimate the population of Sertoli and Leydig cells and the luminal areas of seminiferous tubules. For this purpose, 20 fields randomly chosen were scored for each animal (8000 points/animal) at 400× magnification (Almeida et al., 2006). The points intersecting on the seminiferous tubule lumen, epithelium and Sertoli cells, as well as interstitium and Leydig cells were scored. Each result was divided by the summation of all points scored to obtain the Vv% of these parameters (Oliveira et al., 2007). The morphometrical studies were performed in AR-stained tissue to facilitate cellular recognition.

In addition, the population of VDR-positive cells in connective tissue of the epididymal region was also analyzed. VDR-positive cells were counted in 15 randomly selected sections in constant areas (μm²) and then the proportion of cells/100 μm² was calculated. In order to facilitate the interpretation, the results were expressed in mm².

2.8. Statistical analysis

The variables valuated were statistically analyzed by the Student’s t-test (for the protein immuno-assessment and number of VDR-positive cells in the connective tissue) or the Mann–Whitney U-test (for the volumetric densities of the testis). Differences were considered significant at P ≤ 0.05.

3. Results

3.1. Testes

The testes of roosters were dominated by seminiferous tubules, lined by a seminiferous epithelium of germ cells and Sertoli cells. Leydig cells were found in the scarce interstitial tissue between the tubules. No significant differences were found in the proportion of interstitial tissue, seminiferous tubule lumen, epithelium or Sertoli cells (Fig. 2), when comparing affected and non-affected roosters. However, in the animals affected by lithiasis, a significant increase (about three fold) in the proportion of Leydig cells was observed (Figs. 2 and 3).

Within the testes, VDR was detected in spermatogonia, spermatocytes and Sertoli cells of the seminiferous epithelium, as well as in endothelium of blood vessels in the interstitium (Fig. 3A and B). Conversely, AR was in Sertoli cells, Leydig cells, some myoid cells and endothelial cells (Fig. 3C and D), which corroborates previous findings (Dornas et al., 2008). No detectable changes in the intensity and pattern of cell distribution of VDR and AR were observed in the testes between affected and non-affected animals.
3.2. Epididymal region

The epididymal region of roosters consisted of an extra-testicular rete testis, several proximal and distal efferent ductules, followed by connecting ducts and a single epididymal duct. In animals affected by epididymal lithiasis, variable numbers of stones were observed in the epididymal region, compared to unaffected animals (Fig. 1A and B). Histological evaluation showed that the stones were located primarily in the proximal efferent ductules (Fig. 1C and D). A concentrically distributed, PAS-positive material was observed within the stones. Also, sperm were found surrounding the stones or even inside them (Fig. 1D). Ductules that contained stones had a reduction in epithelial folds and cell height. Noteworthy was the presence of an increased number of mononuclear leukocytes within the affected epididymal regions (Fig. 1D), primarily localized in the connective tissue beneath the proximal efferent ductules and extra-testicular rete testis. Cellular infiltrations around the other extra-testicular ducts were rarely found. No evident histological alterations were observed in the extra-testicular rete testis, distal efferent ductules, connecting ducts or epididymal duct of affected animals.

3.3. VDR

Western blot analysis detected a 61 kDa protein band positive for VDR in the epididymal region of roosters (Fig. 4A), which is in agreement with previous studies in the chicken (Yoshimura et al., 1997; Dornas et al., 2007a). According to this assay, VDR was significantly increased in about 42% in the epididymal tissue of lithiasis-affected animals, compared to non-affected (Fig. 4B).
Fig. 3. Expression of VDR and AR in the testis of roosters non-affected (A and C) and affected (B and D) by epididymal lithiasis. (A and B) VDR was detected in spermatogonias, spermatocytes and Sertoli cells within the seminiferous tubules epithelium (SE). Compared to the non-affected animals (A), no differences in the intensity or localization of VDR was found in the testis of affected animals (B). (C and D) AR immunostaining was found in the Sertoli cells (S), some myoid cells (arrows) and in Leydig cells (arrowheads). Compared to non-affected animals (C), no differences in the immunostaining intensity were found in the testis of affected animals (D). However, an increase was observed in the proportion of Leydig cells in the interstitial tissue of affected animals (compare C with D). Insets in A and C = negative control. Bar in A = 20 μm.

Immunostaining for VDR was found in epithelial nuclei of all ducts in the epididymal region (Fig. 5A–C). In animals affected by epididymal lithiasis, there was a slight but significant increase in VDR staining intensity in non-ciliated cells of the distal efferent ductules epithelium, compared to non-affected animals (Figs. 5B, E, H and 6B). No significant alterations were observed in VDR either in ciliated cells of the distal efferent ductules or in epithelial cells of the proximal efferent ductules, connecting and epididymal ducts (Fig. 5A, D, G and Fig. 5C, F, I, respectively).

In the connective tissue of the epididymal region, VDR+ nuclei were observed in several unidentified cells (Fig. 6A and B). No differences were detected between non-affected and affected roosters in the intensity of VDR immunostaining among the connective tissue cells. However, the number of VDR+ connective tissue cells was increased nearly three fold in lithiasis-affected animals compared to non-affected (Fig. 6C). This drastic increase was due to the abundant infiltration of VDR+ mononuclear leukocytes (Fig. 6B).

3.4. AR

A positive AR band of about 100 kDa was detected by Western blot of the rooster epididymal region total protein (Fig. 4C), which is in agreement with the previously reported molecular weight of AR in avian tissue (Yoshimura et al., 1993; Oliveira et al., 2007). By Western blot, AR was
increased significantly (30%) in the epididymal region of lithiasis-affected animals, compared to non-affected (Fig. 4D).

AR was widely expressed in epithelial cell nuclei and in a few cells of the connective tissue of the epididymal region (Fig. 7A–C). Roosters affected by lithiasis showed an increase of 15% in AR in the epididymal duct, compared to non-affected animals (Fig. 7F and I). There was no difference in AR immunostaining in the efferent ductules (compare Fig. 7A-D-G and B-E-H) or rete testis (data not shown). There was no difference between affected and non-affected roosters in AR intensity or in the number of positive connective tissue cells. Mononuclear leukocytes seen in the peritubular infiltrations were negative for AR.

4. Discussion

The present study found that amounts of VDR and AR are altered in specific segments of the epididymal region of roosters affected by epididymal lithiasis, suggesting that both vitamin D and androgens may be involved in the development of this disease. The epididymal region of affected roosters contained luminal stones and showed structural alterations of the epithelium and connective tissue in the efferent ductules. Data presented here confirm and extend those previously reported (Janssen et al., 2000; Mahecha et al., 2002; Boltz et al., 2004, 2006; Jackson et al., 2006) and validate the use of tissue clearing for rapid diagnosis of epididymal lithiasis.

Efferent ductules of the rooster have the greatest amounts of VDR among the various epididymal regions (Dornas et al., 2007), which is consistent with a major physiological role of the ductal epithelium in the reabsorption of luminal calcium coming from the rete testis (Clulow...
Fig. 5. Vitamin D receptor (VDR) in the epididymal region of roosters non-affected (A–C) and affected (D–F) by epididymal lithiasis. (A–C) VDR was found in the non-ciliated cells (arrows) of the proximal (A) and distal (B) efferent ductules, whereas the non-ciliated cells (arrowheads) were weakly stained or negative. Epithelial cells lining the epididymal duct were also positive to VDR immunostaining (C). (D–F) When compared to non-affected animals, VDR was significantly increased in the non-ciliated cells of the distal efferent ductules but not in the proximal efferent ductules and epididymal duct of affected roosters. (G–I) Graphical representation of the immunohistochemistry image analysis. *$P \leq 0.05$; $n = 4$. PED = proximal efferent ductule; DED = distal efferent ductule; EP = epididymal duct; sp = sperm. Inset in B = negative control. Bar in A = 20 μm.

and Jones, 2004). It is not known whether there is a difference in the rate of calcium reabsorption between proximal and distal efferent ductules. In the present study, there was a slight but significant increase in VDR in non-ciliated cells of the efferent ductules of lithiasis-affected roosters. The distal ductules may have a slightly greater amount of VDR than the proximal efferent ductules, which would be consistent with the differential embryonic origin of the proximal and distal tubules. Unlike mammals, distal efferent ductules of birds originate from the mesonephric renal tubules, whereas the proximal efferent ductules are derived from glomerular tissue (Budras and Sauer, 1975; Budras and Meier, 1981). Thus, it is possible that the distal segment has a greater role in vitamin D3-dependent calcium transepithelial transport in the chicken epididymal region. In roosters with epididymal stones, an increase in VDR in the distal efferent ductules may be a compensatory mechanism in the maintenance of calcium homeostasis of luminal fluids.

The significant overall increase in VDR, found by Western blot analysis, in animals affected by lithiasis was due primarily to an approximately threefold increase in the number of VDR+ cells in the connective tissue, which paralleled an increase in mononuclear leukocytes surrounding the affected efferent ductules. Efferent ductule epithelium showed only a slight increase in
VDR staining; therefore, the inflammatory cells may account for the overall increase in VDR concentration observed by Western blotting analysis of affected epididymal tissue. This finding is consistent with previous studies showing that cells of the immune system, such as lymphocytes and macrophages have nuclear VDR and that vitamin D is an immuno-modulation hormone with a role in cell proliferation, differentiation and function (Deluca and Cantorna, 2001; Hayes et al., 2003; Dornas et al., 2007a).

The occurrence of mononuclear leukocyte infiltrations in connective tissue surrounding the efferent ductules appears to be a common feature among roosters affected by epididymal lithiasis (Janssen et al., 2000; Mahecha et al., 2002; Boltz et al., 2004, 2006; Jackson et al., 2006). Corroborating these findings, proximal efferent ductules of the chicken contain a greater population of antigen presenting cells, compared to other epididymal regions (Yoshimura et al., 2006). CD4+ and CD8+ T-cells are also abundant in these ductules (Yoshimura et al., 2005). It is noteworthy that the number or activity of these cells can be, respectively, regulated by sexual steroids, as
Fig. 7. Amount of androgen receptor (AR) in the epididymal region of roosters non-affected (A–C) and affected (D–F) by epididymal lithiasis. (A–C) Non-ciliated cells of the proximal efferent ductules (arrows) were weakly stained or negative for AR, whereas ciliated cells (arrowheads) were negative (A). (B) AR was found only in the non-ciliated cells of distal efferent ductules. (C) The epithelial cells lining the epididymal duct were strongly positive for AR. The intensity of AR staining was similar in the efferent ductules but increased in the epididymal duct of affected roosters when compared to the non-affected (compare A-C with D-F). (G–I) Graphical representation of the immunohistochemistry image analysis. *P ≤ 0.05; n = 4. PED = proximal efferent ductule; DED = distal efferent ductule; EP = epididymal duct; sp = sperm. Inset in C = negative control. Bar in A = 20 μm.

Androgens and estrogens, or by vitamin D3 (Hayes et al., 2003; Yoshimura et al., 2005, 2006). Although little is known regarding the immuno-defense system of the rooster epididymal region, these data suggest that the proximal efferent ductules may be a major site of antigen presentation and cell-mediated immune defense (Yoshimura et al., 2005, 2006). In mammals, these ductules are the primary site for leakage of antigens and a primary site for autoimmune response (Suzuki and Nagano, 1978; Tung and Alexander, 1980).

These findings support the hypothesis that the local chronic inflammation found in lithiasis-affected roosters may be involved in the formation of epididymal stones, possibly by sustaining a favorable micro-environment for calcification (Janssen et al., 2000; Boltz et al., 2006; Jackson et al., 2006). Others have already demonstrated an association of chronic inflammation with calcification in atherosclerotic lesions (Doherty et al., 2003) and urolithiasis (Lai et al., 1996). Therefore, further investigation is warranted to determine the role of immuno-modulation in the development and treatment of epididymal lithiasis.
In animals affected by the epididymal lithiasis, AR was significantly increased only in the epididymal region. Immunostaining revealed that the increase in AR detected by Western blot correlated with an equivalent increase in AR in the epididymal epithelium. The amount of protein in the other components of the epididymal region was not altered in the epithelium or in the connective tissue. Lack of correlation between circulating testosterone concentrations and AR in other avian tissue (Fusani et al., 2000; Nishizawa et al., 2002), including the epididymal region (Oliveira et al., 2007) has been previously described. This difference in AR regulation in reproductive organs suggests that the AR protein may be modulated by other factors than its cognate ligand in extra-testicular ducts, as proposed by others (Yoshimura and Kawai, 2002).

Among the variables analyzed in the testis, only the proportion of interstitial Leydig cells was altered, being three folds higher in the testes of affected roosters. This intriguing increase in Leydig cell population may be interpreted as an attempt to re-establish normal testosterone concentrations, which has been reduced in affected animals (Janssen et al., 2000). The lack of major evident morphological alterations in the seminiferous tubules of roosters affected by epididymal lithiasis corroborate previous studies showing that the testicular effects of epididymal lithiasis appear to be secondary to alterations found in the ducts composing the epididymal region (Janssen et al., 2000; Mahecha et al., 2002).

5. Conclusions

The VDR and AR proteins were found to be altered in different segments of the epididymal region of roosters affected by epididymal lithiasis, but not in the testis, suggesting that the vitamin D3 and androgen responsive system may be directly or indirectly involved in the development/progression of this intriguing reproductive tract anomaly.

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