ANDRÉ GUSTAVO DE OLIVEIRA

EXPRESSÃO DE RECEPTORES DE VITAMINA D3 (VDR) E RECEPTORES DE ANDRÓGENOS (AR) NO TESTÍCULO E REGIÃO EPIDIDIMÁRIA DE GALOS (*Gallus domesticus*) AFETADOS PELA LITÍASE EPIDIDIMÁRIA

Instituto de Ciências Biológicas
Universidade Federal de Minas Gerais
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Dissertação apresentada ao Programa de Pós-Graduação em Biologia Celular do Departamento de Morfologia, do Instituto de Ciências Biológicas, da Universidade Federal de Minas Gerais, como requisito parcial para a obtenção do título de Mestre em Ciências.

Área de concentração: Biologia Celular

Orientadora: Dra. Cleida Aparecida de Oliveira

Instituto de Ciências Biológicas
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RESUMO

Recentemente foi descrita uma disfunção reprodutiva em galos domésticos denominada litíase epididimária, a qual está relacionada com drástica redução da fertilidade nos animais afetados. Essa anomalia é caracterizada pela formação de cálculos ricos em cálcio que acometem a região epididimária, principalmente no segmento dos dúctulos eferentes. Os dúctulos eferentes são responsáveis pela reabsorção de grande parte do fluido testicular e, nas aves, também de íons cálcio, funções importantes para a manutenção da fertilidade. Considerando essas funções dos dúctulos eferentes, associado ao fato dos cálculos epididimários serem formados principalmente neste local, hipotetizamos que o desenvolvimento da litíase epididimária poderia estar relacionado com alterações locais na homeostase de cálcio e/ou do fluido luminal. O transporte transepitelial de cálcio é regulado pela vitamina D3 e andrógenos e seus respectivos receptores VDR e AR. Nesse sentido, o presente estudo investigou possíveis alterações no padrão de expressão de VDR e AR em galos afetados pela litíase epididimária. Os resultados mostraram que tanto VDR quanto AR são amplamente expressos na região epididimária de galos, porém com distribuição diferenciada dependendo do tipo celular e do segmento considerado. Quando comparados com animais não-afetados, os galos afetados pela litíase epididimária apresentaram aumento na expressão de VDR e AR na região epididimária, enquanto nos testículos não foi detectada alteração nesses receptores. O aumento da expressão de VDR foi principalmente associado ao maior número de células VDR-positivas presentes em infiltrados mononucleares, embora um discreto aumento nos níveis de VDR também tenha sido observado no epitélio dos dúctulos eferentes distais. Por outro lado, a expressão de AR foi aumentada no epitélio do ducto epididimário. Esses resultados sugerem que os sistemas responsivos à vitamina D3 e andrógenos podem estar direta ou indiretamente relacionados com o desenvolvimento da litíase epididimária.

ABSTRACT

The epididymal lithiasis is a reproductive dysfunction recently described in roosters, which is related to a drastic decrease in fertility in affected animals. This anomaly is characterized by the formation of stones rich in calcium in the epididymal region, which were found especially in the efferent ductules. The efferent ductules are responsible for the reabsorption of a great amount of the testicular fluid and, in birds, they are also involved in the reabsorption of calcium; thus, playing an important role in the maintenance of fertility. Considering these functions of the efferent ductules, associated with the fact that epididymal stones are formed especially in these segments, we hypothesized that the development of epididymal lithiasis would be related to local disruption of fluid and/or calcium homeostasis. The transepithelial transport of calcium is regulated by vitamin D3 and androgens as well as their receptors VDR and AR, respectively. Therefore, this study investigated possible alterations in the expression of VDR and AR in roosters affected by epididymal lithiasis. The results showed that VDR and AR were widely expressed in the epididymal region of roosters; however, with distribution cell and segment specific. When compared to non-affected animals, roosters affected by epididymal lithiasis presented an increase in VDR and AR expression in the epididymal region, whereas no alterations in these receptors levels were detected in the testes. The increased VDR expression was mainly related to an increase in VDR-positive cells localized in the mononuclear cell infiltrations, although a slight increase in VDR level was found in the distal efferent ductule epithelium. On the other hand, AR was overexpressed in the epithelium of the epididymal duct. The results suggest that the responsive systems for VDR and AR may be directly or indirectly involved in the development of epididymal lithiasis.

INTRODUÇÃO E REVISÃO DE LITERATURA

I- INTRODUÇÃO E REVISÃO DE LITERATURA

1. SISTEMA GENITAL MASCULINO DE AVES

Os órgãos do sistema genital masculino de aves incluem o testículo, a região epididimária, o ducto deferente e, em algumas espécies, o fálus (Lake, 1981). Os testículos encontram-se situados no interior da cavidade corporal, sendo inseridos na região dorso-medial através do mesórquio. Os testículos relacionam-se cranialmente com os pulmões e as glândulas adrenais, medialmente com a aorta e veia cava caudal, e caudalmente com a veia ilíaca, além de serem envolvidos pelos sacos aéreos abdominais (Gray, 1937; Lake, 1957; King, 1975; Lake, 1981). Envolvendo os testículos, encontra-se uma delgada túnica albugínea ou cápsula testicular, formada por tecido conjuntivo denso e recoberta por peritônio. O parênquima testicular é formado por numerosos túbulos seminíferos flexuosos e anastomosados, entremeados por escasso tecido conjuntivo intertubular, amplamente vascularizado, o qual contém as células de Leydig (Lake, 1981). Diferente dos mamíferos eutérios, os testículos das aves não são separados em lóbulos e não apresentam mediastino (Gray, 1937; Lake, 1981).

A região epididimária encontra-se disposta ao longo da superfície dorso-medial do testículo e consiste da rede testicular, dos dúctulos eferentes proximais e distais, dos ductos de conexão e de um curto ducto epididimário, todos envolvidos por abundante tecido conjuntivo (Fig. 1) (King, 1975; Aire, 1979a; Aire, 2000).

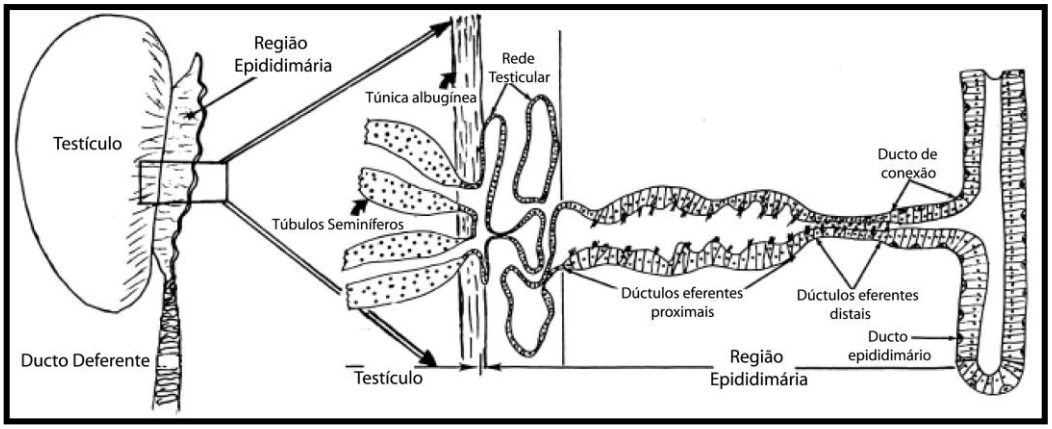

Figura 1: Desenho esquemático da região epididimária das aves. Baseado em Aire (2000).

A rede testicular conecta os túbulos seminíferos aos dúctulos eferentes (Aire, 1982). Nas aves, três regiões morfologicamente distintas são descritas na rede testicular: as porções intratesticular, intracapsular e extratesticular, sendo que apenas esta última é considerada parte da região epididimária. A rede testicular extratesticular ocupa cerca de 10% a 13% da região epididimária dependendo da espécie estudada (Aire, 1979b). O epitélio de revestimento é simples cúbico, com células apresentando curtas microvilosidades apicais. Na superfície lateral das células encontram-se complexos juncionais e interdigitações, respectivamente, nas porções apical e basal (Tingari, 1971; Lake, 1981; Aire, 1982).

Os dúctulos eferentes são encontrados entre a rede testicular e os ductos de conexão e são as estruturas mais abundantes da região epididimária, compreendendo cerca de 40% a 60% da mesma, dependendo da espécie considerada (Aire, 1979b; Oliveira et al., 2007). Os dúctulos eferentes subdividem-se em duas regiões contínuas, mas morfologicamente distintas, sendo a região proximal, com lúmen amplo e epitélio pregueado, e a região distal, com lúmen mais estreito e epitélio pouco pregueado (Lake, 1981). Ambas as regiões são revestidas por epitélio simples colunar, formado por dois tipos celulares: as células não-ciliadas e as células ciliadas, sendo as últimas mais abundantes na região distal dos dúctulos eferentes (Aire, 1979a). As células não-ciliadas apresentam abundantes microvilosidades, além de vesículas revestidas e grânulos densos, identificados como lisossomos, em seu citoplasma, características essas consistentes com a função reabsortiva atribuída a essas células (Aire, 1980; Oliveira et al., 2007). As células ciliadas são reconhecidas pelo núcleo em posição apical na célula, presença de cílios e de poucos e pequenos grânulos densos no citoplasma, além da aparência fracamente corada pela Hematoxilina/Eosina ou azul de toluidina, quando comparada com as células não-ciliadas (Aire, 1979a; Aire, 1980).

Semelhante aos mamíferos eutérios (Ilio & Hess, 1994), os dúctulos eferentes proximais das aves são os principais locais de reabsorção de fluido testicular (Clulow & Jones, 1988). Em codorna japonesa (*Coturnix japonica*), já foi demonstrado que os dúctulos eferentes são responsáveis pela reabsorção de aproximadamente 86% do fluido testicular (Clulow & Jones, 1988). Entretanto, em aves, foi demonstrado que os dúctulos eferentes também participam da reabsorção de uma grande quantidade de cálcio do fluido luminal (Clulow & Jones, 2004), podendo estar envolvidos na manutenção da homeostase

desse íon ao longo das vias extratesticulares. A função reabsortiva dos dúctulos eferentes tanto em relação ao fluido testicular, quanto ao cálcio, é essencial para a concentração e maturação dos espermatozóides (Clulow & Jones, 1988).

Os ductos de conexão unem os dúctulos eferentes distais ao ducto epididimário. Em conjunto, os ductos de conexão e o ducto epididimário constituem de 10% a 13% da região epididimária (Aire, 1979b; Oliveira et al., 2007). A diferenciação morfológica entre ambos é difícil, uma vez que os mesmos apresentam características histológicas semelhantes, sendo revestidos por epitélio pseudo-estratificado cilíndrico. No entanto, os ductos de conexão apresentam menor altura epitelial e diâmetro que o ducto epididimário (Aire, 1979a), sendo essa a base da diferenciação entre os dois segmentos. O ducto epididimário das aves é curto, flexuoso e localiza-se ao longo da superfície medial dos testículos, continuando-se caudalmente com o ducto deferente. A ausência de um ducto epididimário desenvolvido e regionalizado, como observado em mamíferos eutérios, levou a denominação de "região epididimária" ao invés de "epidídimo". O ducto epididimário caracteriza-se pela presença de lúmen amplo e regular, o qual geralmente encontra-se preenchido por espermatozóides (Aire, 1979a). O epitélio de revestimento é pseudo-estratificado cilíndrico, formado por células principais e células basais. As células principais são as mais abundantes do epitélio e apresentam numerosas microvilosidades, regulares e curtas. Estas células são unidas umas às outras por complexos juncionais localizados na porção apical. As células basais encontram-se apoiadas na lâmina basal, entre as células principais. Essas células apresentam forma cuboidal ou piramidal e núcleo irregular, oval ou triangular (Aire, 2000).

O ducto deferente é um ducto flexuoso, com lúmen amplo, o qual se apresenta túrgido e esbranquiçado quando preenchido por espermatozóides. Esse ducto estende-se caudalmente à região epididimária até desembocar na cloaca. Em sua porção distal, os ductos deferentes tornam-se retilíneos e apresentam uma expansão em seu diâmetro, originando uma estrutura fusiforme conhecida como receptáculo do ducto deferente. O receptáculo desemboca na cloaca através de uma papila cônica denominada papila do ducto deferente (Tingari, 1971; King, 1975; Lake, 1981; Oliveira & Mahecha, 1996). Os ductos deferentes são os principais locais de armazenamento de espermatozóides em aves (King, 1975; Clulow & Jones, 1982).

Em algumas espécies de aves, incluindo os da ordem Galliformes, está presente na cloaca um órgão copulador, ou fálus, que apresenta capacidade de ereção e desenvolve-se a partir da parede ventral do proctodeu (King, 1981). O fálus das aves pode ser intromitente ou não intromitente (King, 1981; Oliveira & Mahecha, 2000). Nas espécies que não apresentam fálus, a cópula normalmente envolve o contato da cloaca masculina com a feminina, evento comumente descrito como "beijo cloacal" (Gill, 1994).

2. LITÍASE EPIDIDIMÁRIA

2.1. Características gerais

A litíase epididimária é uma anomalia recentemente descrita e associada à região epididimária de galos, a qual é caracterizada pela formação de abundantes cálculos luminais ricos em cálcio, depositado em camadas concêntricas (Fig. 2A-C) (Janssen et al., 2000; Mahecha et al., 2002). Outros elementos, tais como oxigênio, carbono, enxofre, fósforo e matéria orgânica também são identificados nos cálculos, porém, em menor quantidade (Fig. 2C-F). A litíase epididimária foi primeiramente descrita em galos de diferentes linhagens nos Estados Unidos da América e no Japão, onde sua incidência foi de aproximadamente 75% (Janssen et al., 2000). Posteriormente, essa anomalia foi também diagnosticada no Brasil, em diversos estados como Minas Gerais, onde a proporção de animais afetados atinge cerca de 94% (Mahecha et al. 2002), bem como Santa Catarina, Espírito Santo, São Paulo, Goiás e mesmo em outros países da América do Sul como a Colômbia (dados não publicados), indicando que essa anomalia pode ter distribuição mundial ampla.

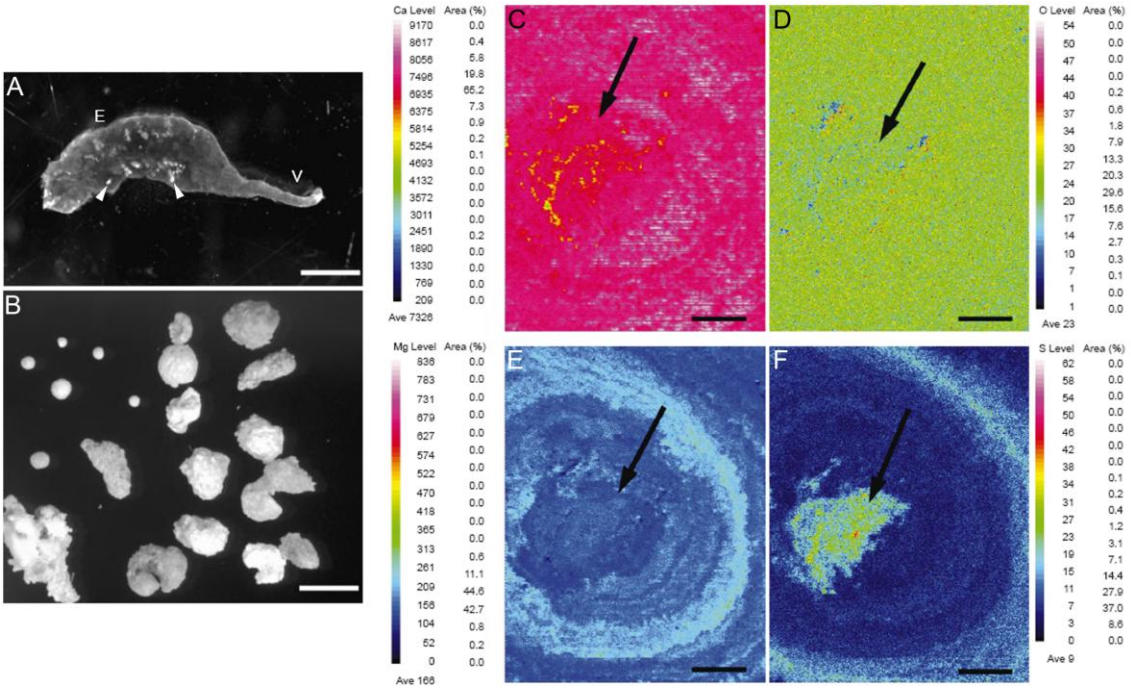

Figura 2: Aspecto geral e composição dos cálculos encontrados na litíase epididimária de galos. **(A)** Região epididimária (E) de galos afetados pela litíase com grande número de cálculos (cabeças de seta). V = ducto deferente. **(B)** Os cálculos apresentam forma e tamanho irregulares. **(C-F)** Microanálise dos cálculos epididimários para cálcio **(C)**, oxigênio **(D)**, magnésio **(E)** e enxofre **(F)**. A escala de cores ao lado de cada imagem representa as concentrações de cada elemento, sendo que os espectros do vermelho refletem as maiores concentrações enquanto os espectros do azul, as menores concentrações. Seta = centro do cálculo. Barra em A = 0,19mm; B = 350μm; C-F = 50μm. Modificado de Mahecha et al. (2002).

2.2. Alterações reprodutivas

Até o momento, sabe-se que dentre os componentes da região epididimária, os dúctulos eferentes são os segmentos mais afetados pela litíase epididimária, uma vez que constituem o principal local de formação e abrigo dos cálculos luminais (Janssen et al., 2000; Mahecha et al., 2002; Boltz et al., 2004; Boltz et al., 2006; Jackson et al., 2006). Os dúctulos eferentes afetados apresentam drásticas alterações morfológicas. Alguns dúctulos sofrem redução da altura e do pregueamento epitelial, além de discreta vacuolização citoplasmática supranuclear nas células não-ciliadas, enquanto, em outros, é observado aumento nos vacúolos, que passam a ocupar grande parte do citoplasma das células não-

ciliadas, além de apresentarem sinais de atrofia tubular e descamação epitelial (Janssen et al., 2000; Mahecha et al., 2002). Nenhuma alteração morfológica nas células ciliadas foi descrita até o momento. Outra característica da litíase epididimária é a presença de abundantes infiltrados de células mononucleares no tecido conjuntivo da região epididimária, especialmente nas adjacências dos dúctulos eferentes.

Alterações testiculares também foram identificadas em galos afetados pela litíase epididimária. Assim como os dúctulos eferentes, os testículos apresentam áreas menos afetadas, com descamação moderada do epitélio seminífero, e áreas mais afetadas, nas quais os túbulos seminíferos apresentam diâmetro reduzido e intensa descamação celular. Nesse caso, o epitélio seminífero é formado apenas por células de Sertoli e poucas espermatogônias, além de serem observados aumento de infiltrados de células mononucleares e perda da arquitetura testicular (Mahecha et al., 2002). Entretanto, nenhuma alteração na população de células de Leydig foi descrita, uma vez que a identificação das mesmas é dificultada pela presença dos infiltrados mononucleares.

As drásticas alterações nos dúctulos eferentes e nos testículos de galos afetados pela litíase epididimária sugerem fortemente que a anomalia afete consideravelmente a fertilidade dos animais. De fato, animais afetados apresentam redução significativa de cerca de 65% nos níveis séricos de testosterona e de aproximadamente 34% na produção diária de espermatozóides (Janssen et al., 2000). Em conjunto, todas as alterações ocasionadas pela litíase epididimária culminam em redução significativa da fertilidade dos animais afetados, como observado em experimentos de cruzamentos naturais, nos quais a produção de ovos embrionados foi drasticamente reduzida (62%) em comparação com galos não-afetados. Surpreendentemente, a inseminação artificial de galinhas com quantidade igual de espermatozóides obtidos do sêmen ejaculado de galos afetados e não-afetados pela litíase epididimária não foi capaz de restabelecer a fertilidade dos animais afetados pela anomalia, sendo nesse caso a produção de ovos embrionados 41% menor quando os dois grupos são comparados (Janssen et al., 2000). Esses achados indicam que as alterações na fertilidade possam ser ocasionadas não só pela menor produção de espermatozóides nos animais afetados, mas também por alterações na qualidade dessas células que podem refletir alterações funcionais na região epididimária.

2.3. Hipóteses sobre a causa da litíase epididimária

Apesar da litíase epididimária resultar em grave disfunção reprodutiva nos animais afetados, pouco se sabe sobre sua etiologia. Entretanto, algumas hipóteses foram propostas para explicar a etiopatogenia dos cálculos no lúmen dos dúctulos eferentes, como (1) infecção da região epididimária, (2) elevados níveis de cálcio e vitamina D3 (1,25 diidroxivitamina D3 – $1,25(OH)_2D_3$) presentes na dieta dos animais ou (3) resultado da seleção genética para o aumento da produtividade (Janssen et al., 2000; Mahecha et al., 2002).

A hipótese de infecção na região epididimária como causadora da litíase epididimária foi proposta devido à presença de abundantes infiltrados mononucleares nos animais afetados, que poderiam estar relacionados com a formação dos cálculos ricos em cálcio (Janssen et al., 2000). Uma das explicações para a origem desses infiltrados seria um possível agente infeccioso presente no local. Nesse sentido, foi proposto que esse agente poderia ser o vírus da bronquite infecciosa (IBV), uma vez que o mesmo apresenta tropismo por epitélios, especialmente aqueles com células ciliadas, como é o caso dos dúctulos eferentes (Dhinakar & Jones, 1997; Jackson et al., 2006). Experimentos foram realizados procurando correlacionar a infecção por IBV com o desenvolvimento da litíase epididimária; entretanto, o monitoramento sistemático da produção de anticorpos humorais contra IBV revelou que mesmo galos negativos para essa doença ainda desenvolveram a litíase epididimária (Mahecha et al., 2002). Em adição, estudos utilizando vacinação com IBV atenuado ou morto mostraram que, em galos vacinados, a incidência da litíase epididimária era maior quando comparados com animais não vacinados; porém, 25% dos animais não vacinados utilizados como controles do experimento também desenvolveram os cálculos ricos em cálcio (Boltz et al., 2006; Jackson et al., 2006). Em conjunto, esses dados indicam que, mesmo que a exposição ao IBV possa acelerar o desenvolvimento da litíase epididimária, ela não é o principal fator envolvido na sua etiologia.

Com relação à segunda hipótese proposta, não foi encontrada correlação entre o número de cálculos ou peso do testículo/região epididimária com a quantidade de cálcio ingerida (Mahecha et al., 2002). Correlação também não foi demonstrada entre os níveis de vitamina D3 na dieta e o desenvolvimento da litíase epididimária (Jackson et al., 2006). Desta forma, a ingestão de elevadas quantidades de cálcio e vitamina D3 não parece ser

responsável pelo desenvolvimento da litíase epididimária (Mahecha et al., 2002; Jackson et al., 2006).

A terceira hipótese proposta foi a da seleção genética. O galo doméstico constitui uma espécie que vem sendo selecionada geneticamente a mais de 3000 anos (Etches, 1993), com os objetivos principais de maior produção de ovos e rápido crescimento corporal para reduzir o tempo para o abate. Portanto, é possível que essa seleção de aves que mobilizam cálcio de forma mais eficiente para atingir tais objetivos tenha resultado na seleção de animais que expressam níveis mais elevados de proteínas de ligação e/ou transportadores de cálcio em diversos órgãos, inclusive nos dúctulos eferentes, que podem estar relacionadas com a formação dos cálculos de cálcio luminais (Mahecha et al., 2002).

3. TRANSPORTE DE CÁLCIO PELO EPITÉLIO

Os epitélios constituem os revestimentos de compartimentos biológicos, criando superfícies especializadas na proteção, secreção, absorção e/ou reabsorção de substâncias. A capacidade das células epiteliais de regular a (re)absorção e secreção de íons essenciais como o cálcio, por exemplo, é de suma importância na manutenção do balanço eletrolítico e conseqüentemente de funções vitais no organismo (Tang & Goodenough, 2003; Hoenderop et al., 2005).

A (re)absorção de cálcio no organismo ocorre através do epitélio de diversos órgãos como rins, intestinos, placenta e glândulas mamárias, num processo mediado por complexa seqüência de eventos regulados por vários fatores, por exemplo pH, concentrações extracelulares de cálcio e hormônios (Friedman & Gesek, 1995; Hoenderop et al., 2005). Duas vias são descritas para explicar o transporte de cálcio através do epitélio: a via paracelular, a qual envolve a movimentação de íons através dos espaços intercelulares; e a via transcelular, na qual os íons são transportados através do citoplasma das células epiteliais. Nessa via, o transporte de cálcio ocorre através de processo que envolve várias etapas, que compreendem a entrada de cálcio na célula pela membrana apical, a translocação do cálcio do citoplasma até a membrana basolateral e a liberação de cálcio para o sangue (Fig. 3) (Hoenderop et al., 2005). Cada etapa envolve a participação de

proteínas específicas. Acredita-se que a entrada de cálcio nas células epiteliais ocorra através de canais de cálcio localizados na membrana apical, de maneira dependente de gradiente eletroquímico favorável, direcionado no sentido do lúmen para o citoplasma. Esses canais são denominados TRPV5 ou TRPV6 (**T**ransient **R**eceptor **P**otencial **V**anilloid channel). Uma vez no citoplasma, os íons cálcio se ligam com alta afinidade à calbindina D9K ou calbindina D28K, as quais funcionam como transportadores que facilitam a difusão do cálcio entre o citoplasma apical e basolateral das células (Hoenderop et al., 2005). Finalmente, a extrusão do cálcio citoplasmático ocorre através de dois transportadores específicos localizados na membrana basolateral das células transportadoras de cálcio: o trocador de sódio e cálcio (NCX - Na^+/Ca^{2+} exchanger) e a ATPase de cálcio de membrana plasmática (PMCA - **P**lasma **M**embrane calmodulin-dependent **C**alcium **A**TPase) (Bindels et al., 1991; van Baal et al., 1996; Hoenderop et al., 2005).

3.1. Regulação hormonal do transporte de cálcio pelo epitélio

A homeostase de cálcio é mantida pela ação de diversos hormônios, sendo alguns clássicos, como paratormônio, calcitonina e vitamina D3 e outros cujos efeitos calciotrópicos são menos conhecidos, como estrógenos e andrógenos (Prince, 1994; Mundy & Guise, 1999; Hoenderop et al., 2005). Como o presente estudo focará nos sistemas responsivos para vitamina D3 e andrógenos, apenas o envolvimento desses hormônios na manutenção da homeostase de cálcio será abordado.

A vitamina D3 é comumente descrita como um dos principais hormônios envolvidos na regulação da homeostase de cálcio. Seu efeito é mediado pela ligação do hormônio com seu receptor nuclear VDR e, posteriormente, pela ligação do complexo vitamina D3/VDR em regiões específicas do DNA, conhecidas como elementos responsivos a vitamina D3 (VDRE), promovendo assim a transcrição de genes alvo (Walters, 1992; Hoenderop et al., 2005; Lips, 2006). Até o momento, a seqüência de VDRE já foi observada em diversos genes, dentre eles, alguns que codificam proteínas importantes para o transporte de cálcio (Hoenderop et al., 2001). Nesse sentido, estudos recentes mostram que as expressões de TRPV5, TRPV6, além da calbindina D9K e calbindina D28K (envolvidas na entrada e difusão do cálcio na célula, respectivamente) são intimamente reguladas por vitamina D3 em humanos e em diversos modelos animais

(Hoenderop et al., 2001; Hoenderop et al., 2005). Por outro lado, o efeito da vitamina D3 na expressão de proteínas relacionadas ao sistema de extrusão de cálcio (NCX e PMCA) é menos claro, mas já foi demonstrado efeito estimulatório de vitamina D3 na expressão de PMCA na membrana basolateral de células (re)absortivas dos intestinos e rins (Cai et al., 1993; Kip & Strehler, 2004).

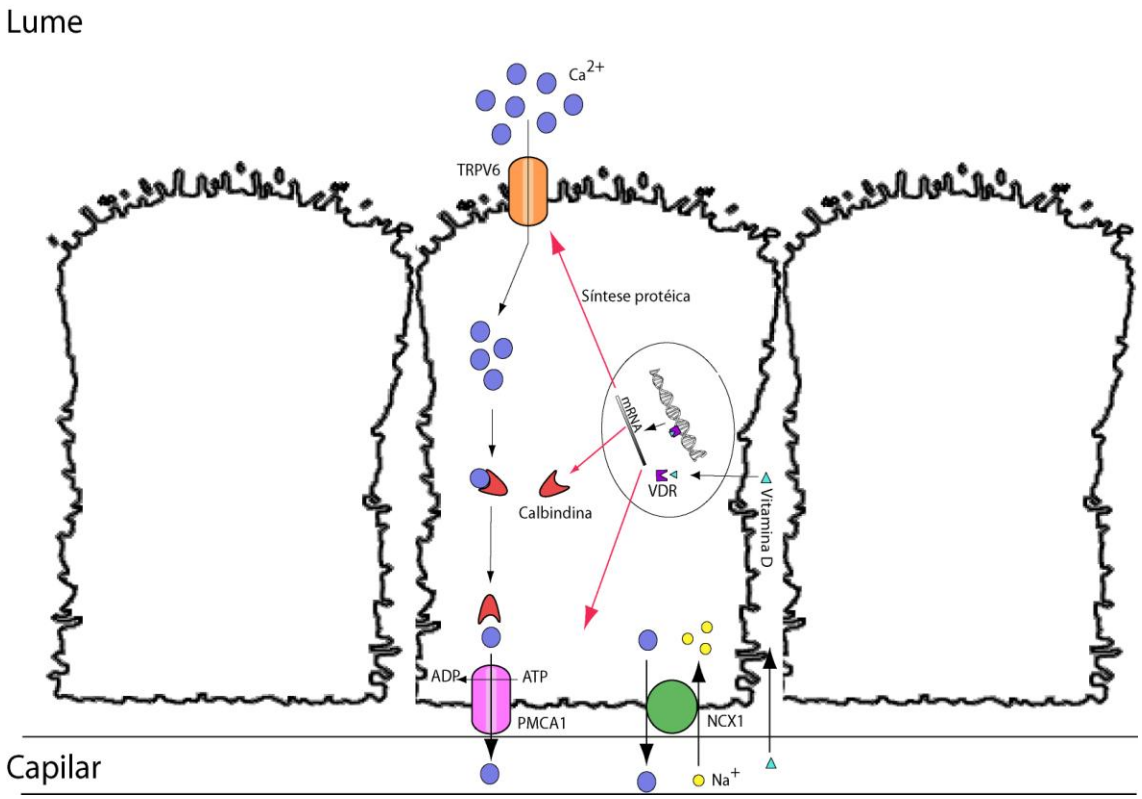

Figura 3: Desenho esquemático do transporte transepitelial de cálcio. Baseado em Hoenderop et al. (2005).

Os efeitos específicos de andrógenos no transporte de cálcio ainda são pouco conhecidos, embora a presença de receptores de andrógenos em tecidos envolvidos no metabolismo ou na (re)absorção de cálcio há muito tenha sugerido a participação deste esteróide na homeostase desse íon. Entretanto, estudo realizado por Mauras et al. (1999) demonstrou claramente a existência de correlação negativa entre níveis de testosterona e excreção de cálcio, onde redução nos níveis de testosterona culminava em maior excreção

de cálcio na urina. Corroborando esses resultados, investigações posteriores revelaram que a diidrotestosterona (DHT), um metabólito da testosterona, é potente indutor da atividade da proteína PMCA, envolvida na extrusão de cálcio do citoplasma das células reabsortivas renais (Dick et al., 2003).

4. DISTRIBUIÇÃO E IMPORTÂNCIA DOS RECEPTORES DE VITAMINA D3 E RECEPTORES DE ANDRÓGENOS NO SISTEMA GENITAL MASCULINO

Os receptores de Vitamina D3 são amplamente expressos no sistema genital masculino das poucas espécies de mamíferos estudadas até o momento (Merke et al., 1983; Levy et al., 1985; Schleicher et al., 1989; Johnson et al., 1996). Nos testículos de ratos, a presença desse receptor foi descrita nas células de Sertoli, espermatogônias e espermatócitos (Johnson et al., 1996). Por outro lado, nas vias excurrentes tanto de ratos quanto de camundongos, VDR foi localizado no epitélio dos dúctulos eferentes e na região da cabeça do epidídimo, enquanto as regiões do corpo e cauda do epidídimo apresentaram-se negativas (Schleicher et al., 1989; Johnson et al., 1996). Comprovando a importância da vitamina D3 na manutenção de funções reprodutivas, a ausência de expressão de VDR em camundongos *knockout* para esse receptor resultou em danos testiculares tais como aumento transitório do peso do órgão, dilatação do lúmen dos túbulos seminíferos e redução no epitélio seminífero, culminando na redução da espermatogênese (Kinuta et al., 2000). Em adição, nesse mesmo modelo experimental, foi observada redução na expressão da enzima aromatase (Kinuta et al., 2000), responsável pela síntese de estrógenos a partir da testosterona.

Apesar da importância da vitamina D3 na reprodução ser conhecida em mamíferos, sobretudo em roedores, são poucas as informações existentes para aves. Neste sentido, foi reportado que deficiência em vitamina D3 em galos resulta em degeneração testicular e espermatogênese incompleta (Kurtul, 2002). O papel da vitamina D3 nos testículos de galos também já foi demonstrado pelo seu efeito na modulação da expressão da proteína calbindina D28K, um dos principais marcadores moleculares da ação da vitamina D3

(Inpanbutur et al., 1996). No entanto, dados sobre a distribuição tecidual deste receptor em aves não foram encontrados na literatura consultada.

No sistema genital masculino de mamíferos eutérios, os receptores de andrógenos (AR) são conhecidos pela regulação de fatores importantes para o processo espermatogênico e para a manutenção de microambiente favorável à maturação dos espermatozóides nos epidídimos. Nos testículos de diferentes espécies de mamíferos, AR foi localizado nos elementos somáticos, como as células de Sertoli, células mióides e células de Leydig (Suárez-Quian et al., 1999; Zhu et al., 2000; Zhou et al., 2002). Em adição, AR é expresso em todos os segmentos das vias excurrentes, sendo a maior expressão encontrada no ducto epididimário, seguido pelos dúctulos eferentes, em diferentes espécies (Roselli et al., 1991; Goyal et al., 1997; Zhou et al., 2002).

Nas aves, as informações sobre a ocorrência e distribuição de AR em órgãos do sistema genital masculino são escassas e controversas. Em galos, a proteína AR foi encontrada nas células de Leydig (Shanbhag & Sharp, 1996), enquanto no canário seu transcrito foi identificado apenas nas células de Sertoli (Nastiuk & Clayton, 1994). As informações sobre a distribuição desse receptor nas vias excurrentes são ainda mais escassas e incompletas, sendo que apenas um trabalho foi encontrado (Shanbhag & Sharp, 1996); entretanto, os autores não detalharam as distribuições celulares e regionais dessa proteína na região epididimária, nem qualitativa nem quantitativamente.

JUSTIFICATIVA E OBJETIVOS

II – JUSTIFICATIVA E OBJETIVOS

1. JUSTIFICATIVA

A litíase epididimária é uma disfunção reprodutiva caracterizada pela presença de cálculos ricos em cálcio na região epididimária de galos domésticos. Essa anomalia foi primeiramente descrita em galos domésticos dos Estados Unidos da América e Japão (Janssen et al, 2000) e mais recentemente também em outros países como o Brasil. Somente no estado de Minas Gerais, sua ocorrência mostrou-se alta, sendo que aproximadamente 94% dos galos são afetados (Mahecha et al., 2002). A litíase epididimária resulta em drástica redução na fertilidade dos animais afetados (Janssen et al., 2000). Esse prejuízo na função reprodutiva é concomitante com drásticas alterações morfológicas no testículo e região epididimária, bem como com a redução nos níveis séricos de testosterona. Esses efeitos prejudiciais da litíase epididimária na fertilidade dos animais, associados com sua alta incidência, tornam essa disfunção reprodutiva um potencial fator de impacto negativo na indústria avícola do país, principalmente se considerarmos que o Brasil, atualmente, é o primeiro produtor e exportador mundial de frangos e que Minas Gerais ocupa papel de destaque nessa produção. Dessa forma, estudos que forneçam conhecimentos sobre essa anomalia são plenamente justificáveis, ainda mais quando se considera a existência de poucos estudos relacionados à litíase epididimária. Até o momento, foi demonstrado que essa disfunção afeta principalmente os dúctulos eferentes (Janssen et al., 2000; Mahecha et al., 2002), que representam o segmento das vias excurrentes das aves responsáveis pela reabsorção do fluido testicular e também de íons cálcio (Clulow & Jones, 1988; Clulow & Jones, 2004). Considerando estas funções dos dúctulos eferentes, associado ao fato dos cálculos ricos em cálcio, característicos da litíase epididimária, serem formados principalmente neste local, hipotetizamos que o desenvolvimento da litíase epididimária poderia advir de alterações na homeostase do transporte transepitelial de cálcio nesses dúctulos, resultando em sua maior concentração local. O acúmulo de cálcio luminal poderia servir como centro de nucleação para a formação dos cálculos epididimários.

Já está bem estabelecido que o transporte de cálcio é dependente de vitamina D3 e que os andrógenos também participam no processo. Esses hormônios atuam como moduladores da síntese ou atividade de proteínas importantes no transporte de cálcio,

tais como a calbindina, que se liga ao cálcio, e a PMCA, que bombeia o cálcio através das membranas (Pike et al., 1978; Kinuta et al., 2000; Dick et al., 2003). Sendo assim, investigar a região epididimária de galos quanto às possíveis alterações na expressão dos receptores de vitamina D3 e receptores de andrógenos, que são componentes envolvidos na homeostase de cálcio na aves, poderá servir como ponto de partida para a identificação de mecanismos moleculares envolvidos no desenvolvimento dos cálculos epididimários e para o esclarecimento das conseqüências da redução dos níveis séricos de testosterona nos órgãos em estudo.

2. OBJETIVOS

2.1. Objetivos Gerais

O presente estudo tem como objetivo determinar eventuais alterações moleculares na expressão de proteínas envolvidas na manutenção da homeostase de cálcio na região epididimária de galos domésticos afetados pela litíase epididimária.

2.2. Objetivos Específicos

1) Determinar a ocorrência e distribuição celular de receptores de vitamina D3 (VDR) e receptores de andrógenos (AR) na região epididimária de galos;

2) Investigar alterações na expressão de VDR e AR na região epididimária de galos afetados pela litíase, quando comparados com espécimes não-afetados, utilizando ensaios de Western Blotting;

3) Confirmar as alterações na expressão tecidual e distribuição subcelular das proteínas investigadas utilizando imunohistoquímica;

4) Realizar estudo semi-quantitativo das alterações imunohistoquímicas detectadas, utilizando análise de imagem com auxílio de computador.

ARTIGOS PUBLICADOS

ARTIGO 1

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Distribution of vitamin D3 receptor in the epididymal region of roosters (*Gallus domesticus*) is cell and segment specific

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Abstract

Vitamin D3 is a steroid hormone well known by its role in maintaining calcium homeostasis, however this hormone may also participate in other biological functions, including control of reproductive processes. The vitamin D3 action is mediated by the vitamin D3 receptor (VDR). VDR is widely distributed in the rodent reproductive tract, however the occurrence of VDR and the role of the vitamin D3 in the avian reproductive tract remain unknown. The aim of the present study was to investigate the expression and cellular distribution of VDR in the epididymal region of roosters. VDR expression was investigated by Western blotting analysis and the tissue distribution of the receptor was determined by immunohistochemistry. The Western blotting assay revealed a major VDR protein band of 61 kDa in the epididymal region of rooster. Nuclear VDR expression was found in all segments of the epididymal region, namely rete testis, efferent ductules, connecting ducts and epididymal ducts. Nonciliated cells of the distal efferent ductules showed the highest levels of VDR expression, followed by the proximal efferent ductules and rete testis. The connecting and epididymal ducts showed less intense VDR immunostaining. The differential VDR expression in the epididymal region segments reveals that several extratesticular ducts may be target for vitamin D3 action and suggests that vitamin D3 may have a regional-specific function, such as calcium transport, that is modulated through VDR activity.
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Keywords: Vitamin D3 Receptor; Efferent ductules; Epididymis; Roosters

1. Introduction

Vitamin D receptor (VDR) is a member of the steroid/thyroid hormone super-family of nuclear receptor, which mediates the effects of the vitamin D3 (1,25-$(OH)_2D_3$). Vitamin D3 is a steroid hormone well known by its role in maintaining calcium homeostasis (Bennett et al., 2006; Hoenderop et al., 2005; Lips, 2006; Walters, 1992). However, this hormone also participates in other important biological functions, including modulation of cell proliferation and cell differentiation (Walters, 1992; Lips, 2006), as well as control of reproductive processes (Kwiecinski et al., 1989; Stumpf and Denny, 1989).

VDR is widely distributed in the female and male reproductive tissue of rodents, indicating that this hormone likely plays an important local role (Johnson et al., 1996; Kinuta et al., 2000; Levy et al. (1985a,b); Merke et al., 1983; Schleicher et al., 1989). Indeed, deficiency in vitamin D3 has been shown to cause reduction in male and female fertility (Kwiecinski et al., 1989; Stumpf and Denny, 1989). In the male, genetic disruption of VDR (VDR-KO) resulted in testicular effects, as seen by a transient increase in testicular weight, dilation of seminiferous tubules lumen and reduction in epithelium, resulting in decreased spermatogenesis

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(Kinuta et al., 2000). These effects culminated in atrophy of the tubules in older animals. Similar to rodents, lack of vitamin D3 resulted in testicular degeneration and incomplete spermatogenesis in chicken (Kurtul, 2002). A role for vitamin D3 in the avian testis has also been indirectly demonstrated by its effect on the modulation of the expression of Calbindin-D28k, a molecular marker for vitamin D action (Inpanbutr et al., 1996). However, information about vitamin D effects in extratesticular organs as well as information about the occurrence of VDR in the testis and all other segments of the male genital system of birds is still lacking.

Contrasting to mammals, the male genital tract of avian species is characterized by the occurrence of prominent efferent ductules and a short and non-differentiated epididymal duct (Aire, 1979; Clulow and Jones, 2004), which in conjunction with the extratesticular rete testis composes the epididymal region. The prominence of the efferent ductules points out that this segment may be of high importance for avian reproduction. The avian efferent ductules, similar to those of mammals, play an important role reabsorbing more than 90% of the fluid coming from the testis (Clulow and Jones, 1988). On the other hand, differing from mammals, avian efferent ductules are also responsible for significant reabsorption of calcium (Clulow and Jones, 2004). The molecular mechanism underlying the fluid and calcium reabsorption in the bird male tract is still not determined. Considering that transcellular calcium movement in other calcium-transporting epithelia, such as intestine and kidney, depends on vitamin D3 and a functional VDR (Hoenderop et al., 2000; Li et al., 2001; Van Cromphaut et al., 2001), the presence of VDR in the avian efferent ductules would be predicted. Therefore, the aim of the present study was to investigate the cellular distribution of the VDR in the extratesticular ducts of the rooster, emphasizing the efferent ductules and epididymal duct.

2. Materials and methods

2.1. Animals

The investigation was performed on epididymal region of 08 crossbreed roosters (*Gallus domesticus*), obtained from domestic and commercial sources and housed in the facilities of the Federal University of Minas Gerais, Brazil. The principles of research involving animals followed those expressed in the 'Princípios éticos para o uso de animais em experimentação', advocated by the 'Comitê de ética em pesquisa', published by the Federal University of Minas Gerais—UFMG (http://www.ufmg.br/coep/cetea.html).

2.2. Tissue preparation

The roosters were weighted, anesthetized (i.p. sodium pentobarbital 50 mg/kg body weight), and perfused intracardially with 10% neutral buffer formalin. After fixation, the epididymal regions were isolated from the testis and fragments of tissue were embedded in paraffin, sectioned at 5.0 μm and used for immunohistochemistry.

2.3. Immunohistochemistry

Vitamin D receptor (VDR) expression was localized in the epididymal region by using immunohistochemistry, following previous protocol (Oliveira et al., 2004). Staining was performed in two different sets to confirm the results. Sections were dewaxed in xylene, rehydrated through a graded series of ethanol, washed in distilled water and phosphate buffer saline (PBS) and then blocked for endogenous peroxidase by incubation with 0.6% H_2O_2 in methanol for 30 min. The sections were subjected to antigen retrieval procedure by microwaving in 0.01 M sodium citrate buffer pH 6.0. After washing in PBS, the avidin–biotin non-specific binding was blocked using the Vector blocking kit (Vector Laboratories, Burlingame, CA). Additional washing in PBS was performed before the next 1 h incubation in 10% normal rabbit serum. The sections were incubated for 40 h at 4 °C with the diluted (1:50) primary rat anti-chicken VDR antibody (Labvision Co., Fremont, CA). For negative control, the sections received PBS in place of the primary antibody. After washing in PBS, the sections were exposed to 10% normal rabbit serum for 1 h before incubation with a rabbit anti-rat biotinylated secondary antibody (Dako, Carpinteria, CA), used at 1:50 dilution. The sections were then incubated with avidin–biotin complex (Vectastain Elite ABC kit—Vector Laboratories, Burlingame, CA) for 30 min. To visualize the immunoreaction, sections were immersed in 0.05% of 3,3′ diaminobenzidine containing 0.01% H_2O_2 in 0.05 M Tris–HCl buffer, pH 7.6. The reaction was monitored microscopically and stopped by immersion in distilled water, as soon as a brown color staining was visualized. Sections were lightly counterstained with Mayer's hematoxylin, dehydrated in ethanol, cleared in xylene and mounted.

2.4. Image analysis

VDR immunostaining intensity was quantified by computer-assisted image analysis, based on previously reported protocols (Oliveira et al., 2003; Zhu et al., 2000). Images from 05 different areas of the proximal and distal efferent ductules as well as connecting and epididymal ducts of each animal were taken by using a Nikon Eclipse E600 microscope (Nikon Co., Melville, NY). Digital images were processed with Adobe Photoshop (Adobe Systems, Mountai View, CA), converted to the grayscale mode and inverted. The images were then exported to Image-Tool software (University of Texas Health Sciences Center, San Antonio, TX), for quantitative analysis. For this proposal, 25 nuclei of nonciliated cells of the efferent ductules and principal cells of the connecting and epididymal ducts, all positive to VDR immunostaining, were traced, measured and pixel intensity was determined for the traced areas. Due to difficulties to clearly delimitate the nuclei of the epithelial cells of the rete testis after image processing, the quantification of the immunostaining in this segment was not performed. 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. Data were expressed as mean ± SEM.

2.5. Statistical analysis

Differences in VDR expression among segments of the epididymal region were analyzed by using the multiple variance analyses (ANOVA). The post-hoc Tukey test was used for multiple comparisons between segments. Differences were considered significant at $P \leqslant 0.05$.

2.6. Western blotting

Western blotting analyses were performed to confirm the specificity of the antibody used. Rooster duodenum was used as a positive control. For this purpose, duodenum and epididymal regions from roosters perfused with a 0.75% saline solution were dissected out and rinsed vigorously in PBS before freeze in liquid nitrogen. Frozen fragments (200 mg) were macerated in dry ice and resuspended in 1.5 ml of sample buffer under reducing conditions. After boiling for 5 min, the samples were subjected to continuous electrophoresis using 10% SDS–PAGE (sodium dodecyl sulphate–polyacrylamide gel electrophoresis). The separated proteins were transferred to nitrocellulose membrane and blocked with 10% normal rabbit serum for 1 h at room temperature. The membrane was incubated with rat anti-chicken monoclonal antibody against VDR (Labvision Co., Fremont, CA) diluted 1:500 for 1 h. After washing with PBS–tween 0.05%, the

blot was incubated in a biotinylated secondary antibody rabbit anti-rat (Dako, Carpinteria, CA), used at 1:6000 dilution. The membrane was then incubated with the avidin–biotin complex (Vectastain Elite ABC kit—Vector Laboratories, Burlingame, CA) for 30 min. After several washes, the reaction was developed by the addition of 0.1% 3,3'diaminobenzidine in PBS containing 0.05% chloronaphtol, 16.6% methanol and 0.04% H_2O_2. The reaction was stopped with deionized water.

3. Results

The rooster epididymal region contains the rete testis, proximal and distal efferent ductules, connecting ducts and epididymal duct. All these segments showed positivity for VDR immunostaining (Fig. 1). The immunoreactivity was noted as a nuclear staining seen in the epithelium and some cells of the connective tissue. The intensity of the epithelial staining was different depending on the segment considered: stronger positivity (measured in pixels) was found in the distal efferent ductules followed by the proximal efferent ductules and epididymal duct as well as the connecting ducts (Table 1 and Fig. 2). The intensity of staining for the connecting ducts was not statistically different when compared to the epididymal ducts. In contrast to the epithelium, the staining of the stromal cells showed similar intensity throughout the excurrent ducts. Vascular endothelium also showed positivity for VDR. No immunolabeling was observed in control sections treated with PBS in place of the primary antibody (Fig. 1).

3.1. Rete testis

The cuboidal epithelial cells lining the rete testis were moderately immunopositive for VDR (Table 1). Cells morphologically similar to macrophages (Aire and Malmqvist, 1979), which were present in the lumen of the rete, were also positive for VDR (Fig. 1B).

3.2. Efferent ductules

In both proximal and distal segments of the efferent ductules, nuclei of the epithelial nonciliated cells were always positive for VDR (Fig. 1C and D). However, the nonciliated cells of the distal ductules exhibited more intense staining for the receptor (Fig. 1D and Table 1). On the other

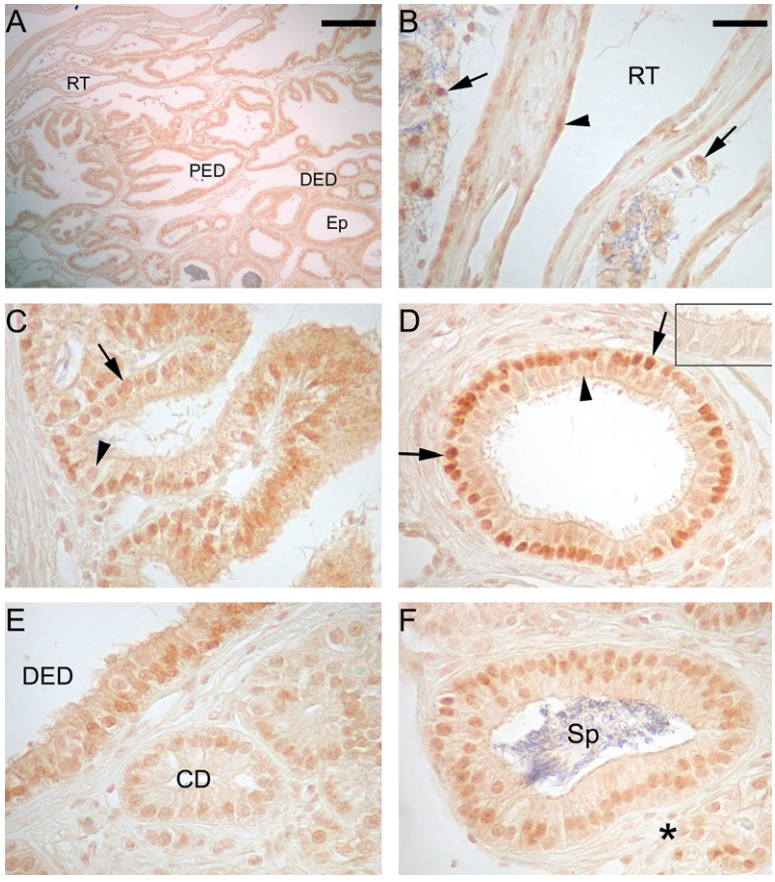

Fig. 1. Expression of VDR in the epididymal region of roosters. (A) General view of the epididymal region showing positive immunostaining for VDR in different segments of the excurrent ducts. RT; rete testis, PED; proximal efferent ductule; DED; distal efferent ductule, Ep; epididymal duct. (B) Extratesticular rete testis (RT) showing moderate immunostaining in the nuclei of epithelial cells (arrowhead), as well as positive macrophage-like cells (arrows) in the lumen. (C) Proximal efferent ductule has moderately positive staining in the nonciliated cells (arrow), in contrast to ciliated cells (arrowhead), which were negative or weakly stained. (D) Distal efferent ductule has the strongest nuclear immunostaining in the nonciliated cells (arrow). Ciliated cells (arrowhead) are negative. The smaller insert represents the negative control. (E) Connecting ducts (CD) showed discrete reactivity to VDR. (F) In the epididymal duct, weak VDR immunostaining was detected in the epithelium as well as connective tissue (∗). Spermatozoa (Sp) were found in the lumen of the duct. Bar in A = 200 μm. Bar in B (the same for C–F) = 20 μm.

Table 1
Immunostaining intensity for VDR in the ducts composing the epididymal region of the roosters

	VDR
Rete testis	
Epithelial cells	++
Luminal macrophages	+++
Stromal cells	+/–
Proximal efferent ductules	
Epithelial nonciliated cells	++
Epithelial ciliated cells	+/–
Stromal cells	+/–
Distal efferent ductules	
Epithelial nonciliated cells	+++
Epithelial ciliated cells	+/–
Stromal cells	+/–
Connecting and epididymal ducts	
Epithelial principal cells	+
Epithelial basal cells	+
Stromal cells	+/–

Score were as follows: –, negative; +/–, intermittent staining; +, weak staining; ++, moderate staining; +++, strong staining.

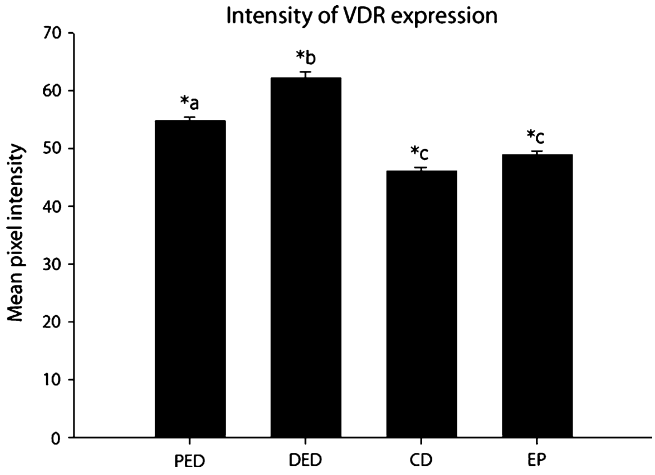

Fig. 2. Quantification of immunohistochemistry for VDR in the extratesticular ducts of roosters. *a, *b, and *c means difference statistically significant ($P \leqslant 0.05$) between the segments analyzed; Values represent mean ± SEM; $n = 04$. PED, proximal efferent ductules; DED, distal efferent ductules, CD, connecting duct; EP, epididymal duct.

hand, the ciliated cells were weakly stained or negative at all (Fig. 1C and D, Table 1).

3.3. Connecting duct and epididymal duct

In the connecting and epididymal ducts, the basal and principal cells appeared weakly stained for VDR (Fig. 1E and F). There appeared to be no difference in staining intensity between both epithelial cell types (Table 1).

3.4. Western blotting analysis

In the epididymal region, it was detected a major band of approximately 61 kDa (Fig. 3), while the duodenum presented

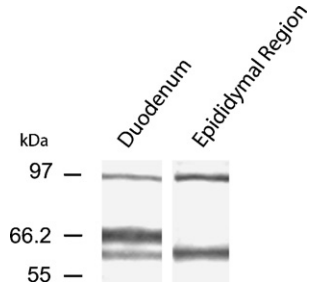

Fig. 3. Western blotting analysis of VDR expression in duodenum and epididymal region of roosters; $n = 04$.

other positive band of about 65 kDa. In both duodenum and epididymis, distinct minor band of 97 kDa was also observed.

4. Discussion

VDR was shown to be widely distributed in the epididymal region of roosters, with specific regional and cellular expressions along the reproductive tract.

The Western blotting pattern observed for VDR in this study is in agreement with previous data in hen's oviduct (Yoshimura et al., 1997). In the duodenum it was detected a doublet band of 61 and 65 kDa, which may correspond to the 58.6 and 60.3 kDa isoforms previously described in avian tissues (Elaroussi et al., 1994; Lu et al., 1997). However, only a 61 kDa protein was detected in the epididymal region, suggesting that only the low molecular weight isoform may be expressed and/or active in the epididymal region. Since VDR performs a wide variety of functions, it has been speculated that VDR isoforms may exist in different tissues with different roles (Lu et al., 1997). This possibility warrants further investigation.

The most intense immunostaining for VDR was found in the efferent ductule epithelium. These data are in broad agreement with those previously described for the rat, in which testicular Sertoli cells and efferent ductules were found to have the most intense binding sites for [^{3}H]-1,25-$(OH)_2D_3$ (Stumpf and O'Brien, 1987). The exact role of vitamin D3 in the efferent ductules is not known, but it has been suggested that it may participate in the modulation of fluid reabsorption (Johnson et al., 1996). It was already demonstrated that avian efferent ductules are also involved in fluid reabsorption (Bahr et al., 2006; Clulow and Jones (1988, 2004); Zaniboni et al., 2004), with the rate of reabsorption being even greater in birds than in rats (Clulow and Jones, 1988). So, it is possible that vitamin D acting through VDR is participating in the regulation of this important physiological function that increases sperm concentration for storage in the avian epididymal and deferens ducts. On the other hand, differing from rats, there is significant reabsorption of calcium along the avian efferent ductules (Clulow and Jones, 2004). Therefore, other possible explanation for higher VDR expression in these ductules may be related to modulation of the transepithelial calcium transport.

VDR expression was less abundant in the rooster epididymal duct. This finding is in agreement with data obtained in

rat in which the epididymis also presented low level of binding to [^{3}H]-1,25-$(OH)_2D_3$ (Stumpf and O'Brien, 1987; Walters, 1984). Connecting ducts presented similar expression of VDR when compared to the epididymal duct, corroborating previous interpretation that together they form one functional unit, as shown by several parameters, including estimative of fluid reabsorption and spermatozoa transit times (Clulow and Jones, 1988), as well as expression of some ion transporters (Bahr et al., 2006). There is little net fluid transport in the epididymal duct, but this region appears to be a site where the spermatozoa acquire their mature pattern of motility (Clulow and Jones, 1988). The exact physiological role of vitamin D3 in this segment remains to be established.

In conclusion, VDR is widely but differentially expressed along the male genital tract of roosters, with higher levels of the receptor being found in the efferent ductules. This data suggest that several extratesticular ducts may be target for vitamin D3 action and that this hormone may have a regional-specific function, such as calcium transport, that is modulated through VDR activity.

Acknowledgments

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Comparative expression of androgen receptor in the testis and epididymal region of roosters (*Gallus domesticus*) and drakes (*Anas platyrhynchos*)

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Abstract

The androgen receptor (AR) mediates the physiological actions of androgens, which play a crucial role in the maintenance of male reproductive function and fertility. Although the AR distribution pattern is well established in mammalian reproductive organs, information about the AR expression in the testes and epididymal region of birds is still scarce. To better clarify the pattern of AR expression in the avian male tract, we investigated the expression and precise cellular distribution of AR in the testis and epididymal region of roosters and drakes. AR expression was investigated by immunohistochemistry and Western blotting. In the testis, AR was found restricted to the nuclei of Sertoli cells, Leydig cells and some myoid cells in both species. Within the epididymal region, AR was widely expressed in the epithelia of all segments, although with segment specific differences in intensity and cellular distribution. Stronger positivity for AR was found in the principal cells of the epididymal duct, followed by the rete testis epithelium and non-ciliated cells of the distal efferent ductules. Non-ciliated cells of the proximal efferent ductules epithelium showed the lowest immunostaining. Ciliated cells of both segments of the efferent ductules were negative for AR. The connective tissue of roosters presented fewer AR-positive cells when compared with drakes; despite the similar total number of cells in both species. In conclusion, cellular and segment specific differences in AR expression suggest difference in sensitivity to androgens among the ducts composing the epididymal region of roosters and drakes.
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Keywords: Androgen receptor; Efferent ductules; Epididymis; Roosters; Drakes

1. Introduction

The androgen receptor (AR) is a member of the nuclear steroid hormone family, which modulates the expression of androgen-dependent target genes (Hiipakka and Liao, 1998). This protein mediates the physiological actions of androgens, i.e., testosterone and dihydrotestosterone, which are essential for maintenance of male reproductive functions and fertility (McLachlan et al., 2002; Dohle et al., 2003). In mammals, androgen is critical for maintenance of the spermatogenesis and regulation of the luminal microenvironment to guarantee the sperm transport, maturation and storage (Vornberger et al., 1994; Robaire and Viger, 1995; Dohle et al., 2003; Hill et al., 2004). In birds, studies concerning the role of androgens in the male reproductive organs are still scarce. It has been shown that in some seasonal breeding avian species there is a positive association between the testosterone plasma levels and testis size (Garamszegi et al., 2005; Denk and Kempenaers, 2006). Nevertheless, direct or indirect inhibitory effect of testosterone to the growth of testicular components has also been described (Purcell and Wilson, 1975).

Comparing with the testis, lesser is known about the androgen response in other segments of the avian male genital tract. Actually, it was not until 2006 that the full-length androgen receptor cDNA of chicken, one of the avian species most extensively used for commercial and experimental

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purposes, was cloned (Katoh et al., 2006). Similarly, it was just recently that the pattern of testosterone secretion was determined in this species (Vizcarra et al., 2004). Therefore, it is not surprising that the distribution, function and mechanism of regulation of the androgen responsive system in male reproductive organs of chickens as well as of other avian species remain poorly understood. To address these issues, it becomes important first to clearly determine the androgen-target cells within the testis and genital tract. In this regard, the cellular expression of AR in the avian testis is still a matter of debate (Nastiuk and Clayton, 1994; Shanbhag and Sharp, 1996). In chicken, AR protein has been found in Leydig cells (Shanbhag and Sharp, 1996), whereas in the seasonal breeding canary the AR transcript has been described in Sertoli cells but not in Leydig cells (Nastiuk and Clayton, 1994). These results point out for possible differences in the pattern of AR cell distribution among species and/or breeding pattern.

Differing from mammals, the male excurrent ducts of avian species are characterized by the occurrence of prominent efferent ductules, which composes approximately 50% of the epididymal region, contrasting with a short and non-differentiated epididymal duct (Lake, 1957; Aire, 1979; Oliveira et al., 2007). The epididymal region also includes the extratesticular rete testis and a short connecting duct, which connect the distal efferent ductules with the epididymal duct. Concerning the AR expression in the rooster epididymal region, one report has been published; however, the authors have not detailed the cellular and regional expression of this receptor (Shanbhag and Sharp, 1996). Furthermore, to the best of our knowledge, complete information about the AR distribution in the epididymal region of drakes is not available. Therefore, owing to better clarify the pattern of AR expression in the avian male tract, in the present study we aimed to investigate the expression and precise cellular distribution of AR in the testis and each segment composing the epididymal region of two species, roosters and drakes.

2. Materials and methods

2.1. Animals

The investigation was performed on the epididymal region of adult crossbreed roosters (*Gallus domesticus*) and mallard drakes (*Anas platyrhynchos*), during breeding phase. The animals were obtained from domestic and commercial sources and housed at the facilities of the Federal University of Minas Gerais, MG, Brazil. The animals were maintained in natural conditions of light, humidity and temperature, and were allowed free access to water and commercial food (Socil-III/Guyomarc'H; Belo Horizonte, MG, Brazil). The principles of research involving animals followed those expressed in the 'Princípios éticos para o uso de animais em experimentação', advocated by the local ethical committee (Comitê de ética em pesquisa), published by the Federal University of Minas Gerais—UFMG (http://www.ufmg.br/bioetica/cetea/).

2.2. Tissue preparation

The roosters and drakes were weighted, anesthetized (i.p. sodium pentobarbital 50 mg/kg body weight), and perfused intracardially with 10% neutral buffer formalin. After fixation, the epididymal regions were isolated from the testis and fragments of tissue were embedded in paraffin, sectioned at 5.0 μm and used for immunohistochemistry.

2.3. Immunohistochemistry

Androgen receptor (AR) expression was localized in the epididymal region by using immunohistochemistry, following previous protocol (Oliveira et al., 2007). Staining was performed in two different sets to confirm the results. Sections were dewaxed in xylene, rehydrated through a graded series of ethanol, washed in distilled water and phosphate buffer saline (PBS) and then blocked for endogenous peroxidase by incubation with 0.6% H_2O_2 in methanol for 30 min. The sections were subjected to antigen retrieval procedure by microwaving in 0.01 M sodium citrate buffer pH 6.0. After washing in PBS, the avidin–biotin non-specific binding was blocked using the Vector blocking kit (Vector Laboratories, Burlingame, CA, USA). Additional washing in PBS was performed before the next 1 h incubation in 10% normal goat serum. The sections were incubated overnight at 4 °C with the diluted (1:500) primary rabbit anti-rat polyclonal AR antibody (AR Ab-2—Labvision Co., Fremont, CA, USA) raised against a synthetic peptide derived from the N-terminus of rat androgen receptor. For negative control, the sections received PBS in place of the primary antibody. After washing in PBS, the sections were exposed to 10% normal goat serum for 1 h before incubation with a goat anti-rabbit biotinylated secondary antibody (Dako, Carpinteria, CA, USA), used at 1:50 dilution. The sections were then incubated with avidin–biotin complex (Vectastain Elite ABC kit—Vector Laboratories, Burlingame, CA, USA) for 30 min. To visualize the immunoreaction, sections were immersed in 0.05% 3,3′-diaminobenzidine containing 0.01% H_2O_2 in 0.05 M Tris–HCl buffer, pH 7.6. The reaction was monitored microscopically and stopped by immersion in distilled water, as soon as a brown color staining was visualized. Sections were lightly counterstained with Mayer's hematoxylin, dehydrated in ethanol, cleared in xylene and mounted.

2.4. Scoring of immunostaining intensity

AR immunostaining intensity was quantified by computer-assisted image analysis, based on previously reported protocol (Dornas et al., 2007). Images from five different areas of the rete testis, proximal and distal efferent ductules as well as connecting/epididymal ducts of each animal were taken by using a Nikon Eclipse E600 microscope (Nikon Co. Melville, NY, USA). Due to difficulties to distinguish the connecting ducts from the epididymal duct, these segments were considered together. Digital images were processed by using Adobe Photoshop (Adobe Systems, Mountain View, CA, USA). After conversion to the grayscale mode and inversion, the images were exported to Image-Tool software (University of Texas Health Sciences Center, San Antonio, TX, USA), for quantitative analysis. For this purpose, 25 nuclei of epithelial non-ciliated cells of the proximal and distal efferent ductules as well as principal cells of the epididymal duct were measured. The nuclei were traced 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.

Considering that the staining intensity was not measured in all cell types composing the duct of the epididymal region, a qualitative scoring of immunostaining was also performed on the basis of nuclear staining, which was classified as negative (no staining), weak (+), moderate (++) or strong (+++). Nuclei were reported as negative when the staining did not differ from the negative control sections. Staining for AR in epididymal duct was defined as a baseline strong staining.

2.5. Western blotting

The antibody used in this study has already been shown to be specific to drake androgen receptor (Oliveira et al., 2007), but its specificity against rooster androgen receptor has not been reported. Therefore, Western blotting analyzes were performed based on previously described protocol

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(Oliveira et al., 2007), to confirm the specificity of the antibody used. In summary, epididymal regions from rooster and drakes perfused with a 0.75% saline solution were dissected out, rinsed vigorously in PBS and frozen in liquid nitrogen. Frozen fragments (100 mg) were macerated in dry ice and resuspended in 750 μl of sample buffer under reducing conditions. After boiling for 5 min, the samples were subjected to continuous electrophoresis using 10% SDS–PAGE (sodium dodecyl sulphate–polyacrylamide gel electrophoresis). The separated proteins were transferred to nitrocellulose membrane and blocked with 10% normal goat serum for 1 h at room temperature. The membrane was incubated with rabbit anti-rat polyclonal antibody against AR (AR Ab-2—Labvision Co., Fremont, CA, USA) diluted 1:500 for 1 h. After washing with PBS–Tween 0.05%, the blot was incubated in a biotinylated secondary antibody goat anti-rabbit (Dako, Carpinteria, CA, USA), used at 1:2000 dilution. The membrane was then incubated with the avidin–biotin complex (Vectastain Elite ABC kit—Vector Laboratories, Burlingame, CA, USA) for 30 min. After several washes, the reaction was developed by the addition of 0.1% 3,3′-diaminobenzidine in PBS containing 0.05% chloronaphtol, 16.6% methanol and 0.04% H_2O_2. The reaction was stopped with deionized water.

2.6. Morphometry

Quantitative studies of the total cells and those immunolabeled to AR in the connective tissue of the epididymal region of roosters and drakes were performed by using classic stereological methodology (Weibel et al., 1969). For each case, the number of cells was obtained by counting all the stromal cells as well as the cells positive to AR, in 15 randomly selected sections of constant areas (μm^2), using a grid of 100 intersections. Then, the proportion of cells/mm^2 was calculated.

2.7. Statistical analysis

Differences in AR expression among segments of the epididymal region of roosters and drakes were analyzed by Kruskal–Wallis one-way analysis of variance. The post-hoc Tukey test was used for multiple comparisons between segments. In the case of the morphometry of the connective tissue, differences in the number of AR-positive cells as well as the total cells in the stromal tissue were compared using the Student's *t*-test. Differences were considered significant at $P \leqslant 0.05$.

3. Results

3.1. Western blotting

The specificity of the androgen receptor antibody for use in avian tissue was confirmed by Western blotting assay. The AR-antibody recognized a single band of about 100 kDa in the epididymal region of both species studied (Fig. 1), which is in agreement with the AR molecular weight previously described for chickens and drakes (Yoshimura et al., 1993; Oliveira et al., 2007).

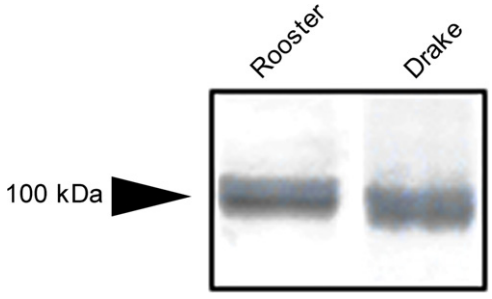

Fig. 1. Western blotting analysis of androgen receptor (AR) expression in the epididymal region of roosters and drakes. The antibody to AR detected a single protein band of about 100 kDa in the epididymal region tissue of both species.

3.2. Regional distribution of AR protein

The testis and the epididymal region of both roosters and drakes showed positivity to androgen receptor protein, which was cell and segment specifics. The immunoreactivity to AR was noted as a nuclear staining seen in somatic cells of the testis (Fig. 2), as well as epithelial cells and some connective tissue cells surrounding the ducts composing the epididymal region (Figs. 3–5). The intensity of the epithelial staining in the epididymal region varied depending on the segment considered: stronger positivity was found in the epididymal duct, followed by the rete testis and distal efferent ductules (Table 1, Figs. 3 and 4). Proximal efferent ductules showed the lowest immunoreactivity among the excurrent ducts. No immunolabeling was observed in negative control sections (Figs. 2 and 3 inserts).

3.2.1. Testis

The testes of roosters and drakes were formed by numerous convoluted seminiferous tubules, which contained the spermatogenic and Sertoli cells, surrounded by a thin tunica propria. The interstitial tissue was scarce and contained the Leydig cells and blood vessels. AR was expressed in the Sertoli cells, Leydig cells and some myoid cells (Fig. 2), as well as in the vascular endothelial cells of both species analyzed. Immunostaining was not detected in spermatogenic cells.

3.2.2. Epididymal region

The avian epididymal region consisted of the extratesticular rete testis, proximal and distal efferent ductules, followed by the connecting and epididymal ducts, all involved by abundant connective tissue. All these segments showed positivity for AR in both rooster and drake (Table 1, Figs. 3 and 4), as following:

Rete testis. The cuboidal epithelial cells lining the rete testis of both rooster and drake were moderately positive for AR (Fig. 3a and b). Macrophages found at the lumen of this segment were weakly immunoreactive (inserts Fig. 3a and b).

Proximal efferent ductules. Non-ciliated cells of the rooster efferent ductules showed intermittent positivity for AR, being negative in some cells (Fig. 3c). In drakes, the epithelial non-ciliated cells of the efferent ductules were weakly positive for AR (Fig. 3d). Ciliated cells were negative for AR in both species.

Distal efferent ductules. Similar to proximal efferent ductules, ciliated cells of the distal ductules were not stained for AR. In both species, the non-ciliated cells were moderately positive for this receptor (Fig. 3e and f).

Connecting and epididymal duct. In roosters and drakes, the principal cells were strongly positive for AR. Conversely, the basal cells were weakly and intermittently

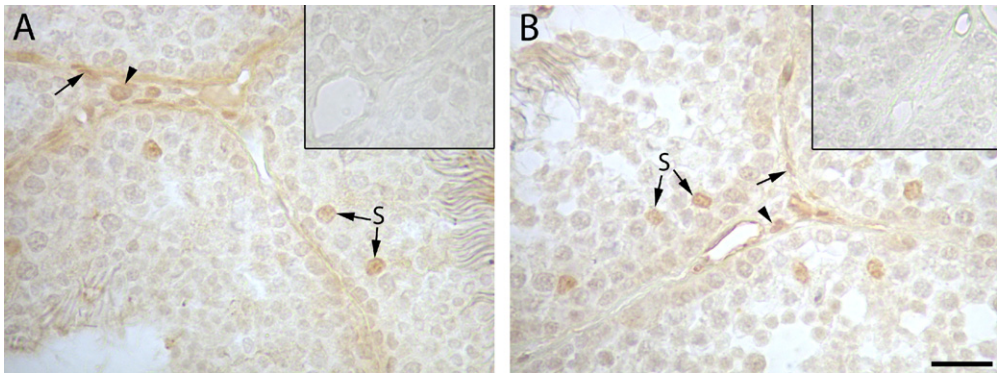

Fig. 2. Androgen receptor (AR) expression in the testis of roosters (A) and drakes (B). AR staining was found in the Sertoli cells (S), Leydig cells (arrowheads) and myoid cells (arrows) in both species. The inserts in (A) and (B) show the negative controls. Bar = 20 μm.

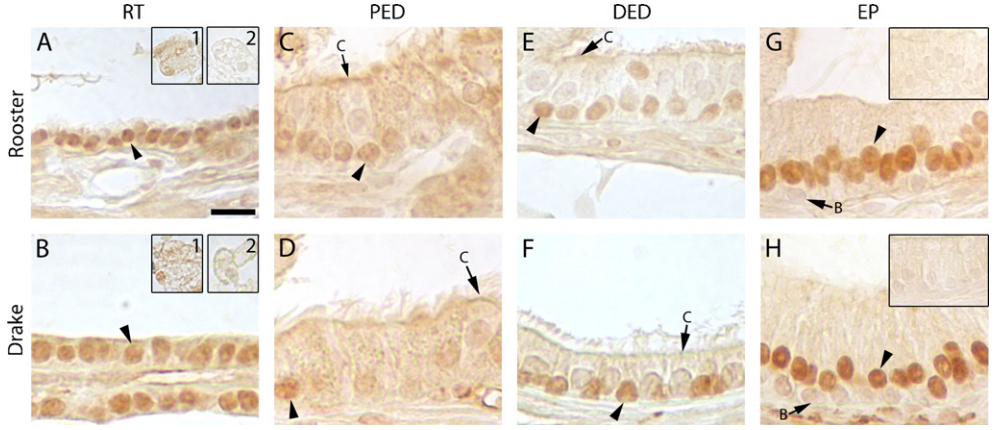

Fig. 3. Androgen receptor (AR) expression in the epididymal region of roosters (A, C, E and G) and drakes (B, D, F and H). (A and B) Extratesticular rete testis (RT) presented AR-positive epithelial cells (arrowheads). Inserts 1 show macrophages positive to AR, whereas inserts 2 show the negative controls. (C–D) Proximal efferent ductule (PED) showed positivity for AR in the non-ciliated cells (arrowheads) which contrasted with negative ciliated cells (C). (E–F) Distal efferent ductule (DED) with moderately immunostained non-ciliated cells (arrowheads). Ciliated cells were negative (C). (G–H) Epididymal duct (EP) showed strong positivity to AR in the principal cells (arrowheads) whereas basal cells (B) were weakly positive or negative. Inserts show the negative controls. Bar in $A = 20$ μm.

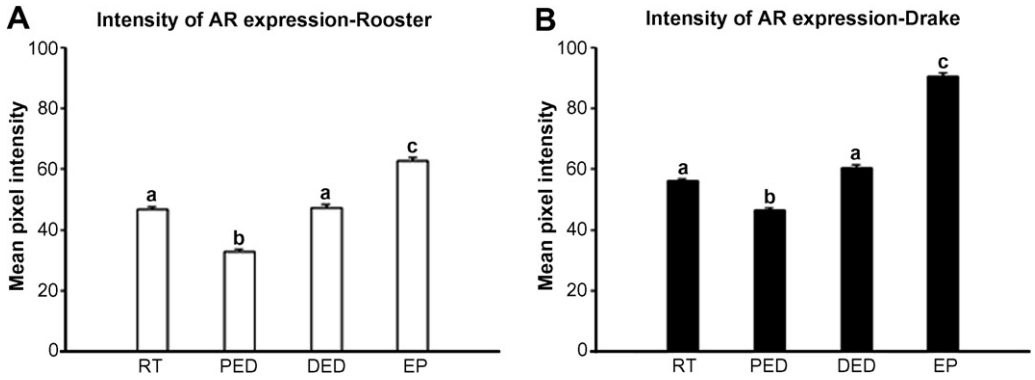

Fig. 4. Quantification of immunohistochemistry for androgen receptor in the epithelium of extratesticular ducts of roosters (A) and drakes (B). a, b, and c indicate differences statistically significant ($P \leqslant 0.05$) among the segments analyzed. Values are represented as means ± SEM; $n = 04$. RT, rete testis; PED, proximal efferent ductules; DED, distal efferent ductules; EP, epididymal duct.

immunostained in drakes, and negative at all in rooster connecting and epididymal ducts (Fig. 3g and h).

Connective tissue. The connective tissue cells were just weakly stained throughout the excurrent ducts of roosters, whereas they were moderately positive for AR in drakes. Compared to drakes, fewer cells of the connective tissue were positive for AR in the epididymal region of roosters. As shown by the morphometrical study, the number of

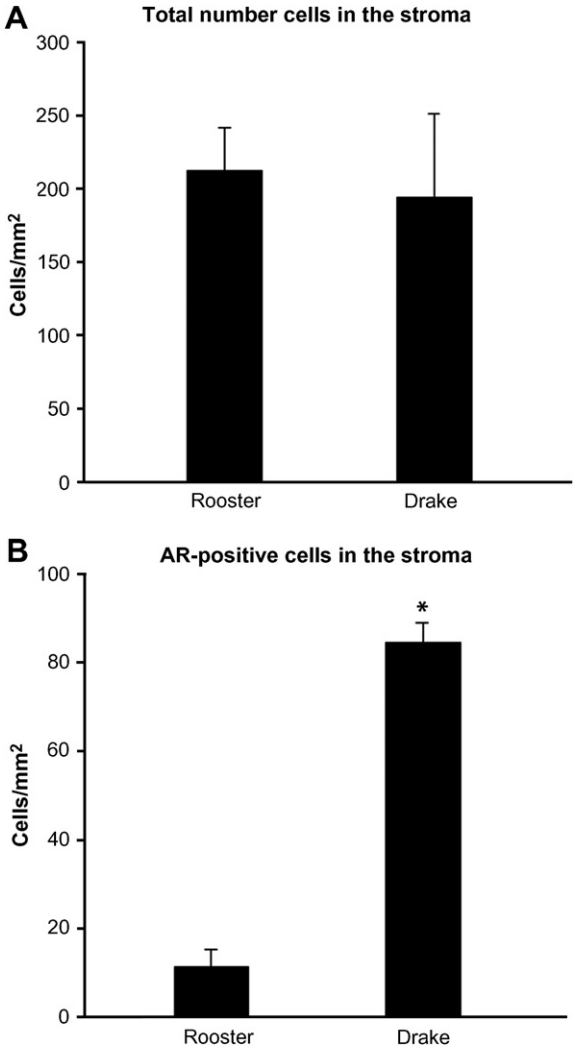

Fig. 5. Quantification of total cells (A) and AR-positive cells (B) in the connective tissue of the epididymal region of roosters and drakes. Values represent mean ± SEM; $^{*}P \leqslant 0.05$. $n = 04$.

Table 1
Scoring of the immunostaining intensity for androgen receptor (AR) in the ducts composing the epididymal region of roosters and drakes

	AR	
	Rooster	Drake
Rete testis		
Epithelial cells	++	++
Macrophage-like cells	+	+
Proximal efferent ductules		
Epithelial non-ciliated cells	+/−	+
Epithelial ciliated cells	−	−
Distal efferent ductules		
Epithelial non-ciliated cells	++	++
Epithelial ciliated cells	−	−
Connecting and epididymal ducts		
Epithelial principal cells	+++	+++
Epithelial basal cells	−	+/−
Connective tissue	+/−	++

Score were as follows: −, negative; +/−, intermittent staining; +, weak staining; ++, moderate staining; +++, strong staining.

cells positive to AR was about 8-fold greater in drakes in comparison to roosters, despite both animals presented similar number of total cells in the stroma (Fig. 5). Vascular endothelium showed positivity for AR in both species.

4. Discussion

In this study we compared the immunolocalization of androgen receptor protein in the testis and epididymal region of roosters and drakes. AR was shown to be restricted to the somatic cells in the testis but widely distributed in the segments composing the epididymal region, although with specific regional pattern of expression along the ducts. Great differences related to the amount of AR-positive cells in the connective tissue of the epididymal region were found between both species analyzed.

Testicular distribution of AR was found in the somatic cells, namely Sertoli cells, Leydig cells and myoid cells, in both roosters and drakes. Previous study in chicken has described AR protein just in Leydig cells (Shanbhag and Sharp, 1996). Conversely, in the canary, the AR transcript has been described in Sertoli cells but not in Leydig cells (Nastiuk and Clayton, 1994). Unlike these previous findings, our result shows a wider distribution of AR in the testis of both species investigated, which is in complete agreement with those found for mammals (Ruizeveld de Winter et al., 1991; Suárez-Quian et al., 1999; Zhu et al., 2000; Zhou et al., 2002). Little is known about spermatogenesis in birds, especially in regard of hormonal regulation of this process. In terms of androgen responsivity, our findings suggest that the regulation of spermatogenesis in roosters and drakes may share similarity with mammals as this hormone appear to influence testicular somatic cells, rather than acting directly on the germ cells (Johnston et al., 2001; Tan et al., 2005).

The occurrence of AR in the efferent ductules and epididymal ducts of roosters and drakes is in agreement with previous findings for avian species (Shanbhag and Sharp, 1996; Nishizawa et al., 2002; Yoshimura and Kawai, 2002), as well as mammals (Roselli et al., 1991; Goyal et al., 1997; Zhu et al., 2000). However, the present result adds to past studies in birds by showing that the rete testis is positive for AR and may also be a target for androgens. Moreover, our data shows for the first time that there is a clear difference in expression of AR along the ducts, suggesting that the proximal efferent ductules may be the least sensitive, whereas the epididymal duct is potentially the most sensitive to androgen among the segments of the epididymal region in both species analyzed. Thus, specific regional functions, as protein expression, fluid reabsorption (Bahr et al., 2006), as well as sperm concentration and maturation (Esponda and Bedford, 1985; Zaniboni et al., 2004) in the avian epididymal region may be differentially regulated by androgens.

The difference in AR expression found between the proximal and distal segments of the efferent ductules favors previous data showing that, despite the common name, these avian male tract segments arise from different embry-

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onic origins (Budras and Sauer, 1975; Budras and Meier, 1981) and presents remarkable morphological and functional differences (Aire, 1980, 2000; Holsberger et al., 2002; Aire et al., 2004; Clulow and Jones, 2004). Based on the weak to negative expression of AR in the proximal efferent ductules, it is possible to speculate that instead of androgen this segment may be regulated by other factors. On this sense, there are convincing data showing that in mammal species the efferent ductule is a segment of the male tract more sensitive to estrogen than androgen (Hess et al., 1997; Oliveira et al., 2002; Hess, 2003). Therefore, estrogens would be one reasonable candidate to regulate the avian proximal efferent ductules as well. In line with this interpretation, the proximal efferent ductules of roosters exhibited strongest positivity for P450 aromatase, the enzyme responsible for conversing testosterone to estradiol (Kwon et al., 1995). Similarly, this segment showed high expression of estrogen receptor ERα (Kwon et al., 1997). It is also noteworthy that the proximal efferent ductules were more sensitive to exposure to Roundup, an herbicide with activity of aromatase inhibition (Richard et al., 2005; Oliveira et al., 2007). Further investigation addressing regional differences in the distribution of estrogen receptors, especially ERβ, in the avian epididymal region would be helpful to substantiate the assumption that estrogen may be the major regulator of the proximal efferent ductules.

Principal cells of the epididymal duct were shown to have the greatest expression of AR, compared to the other segments of the epididymal region, both in roosters and drakes. This result is in agreement with previous findings for the counterpart mammalian epididymis (Roselli et al., 1991; Goyal et al., 1997; Zhu et al., 2000; Yamashita, 2004), indicating that this male tract segment is a major androgen target across species. There was difference among positivity of epididymal basal cells to AR when roosters and drakes were compared. In drakes, the epididymal basal cells were weakly immunostained for AR, which is in agreement with the pattern found for mammals (Goyal et al., 1997; Zhu et al., 2000). The epididymal basal cells of roosters appeared predominantly negative for AR. Previous studies in avian epididymal ducts have not addressed differences in the AR expression between principal and basal cells (Shanbhag and Sharp, 1996; Nishizawa et al., 2002; Yoshimura and Kawai, 2002). However, favoring our results, a weaker and sometimes intermittent staining for AR has been found in basal cells of the epididymis of several mammal species (Zhu et al., 2000; Parlevliet et al., 2006; Pearl et al., 2006).

The connective tissue of the epididymal region of drakes and roosters showed an unexpected and significant difference in number of cells positive for AR. We do not have a clear explanation for this fact. However, one possibility is that the difference may be related to divergences in the pattern of breeding cycle, as drakes have marked breeding periods along the year (Donham, 1979; Haase, 1983), whereas domestic fowl presents a less conspicuous annual breeding cycle (Lofts and Murton, 1968; Sexton, 1983). Testosterone is a potent physiological factor controlling seasonal plasticity in avian tissue (Tramontin et al., 2003; Garamszegi et al., 2005). Therefore, it is possible that the great number of positive AR cells found in the stroma of the epididymal region of the drakes may be mediating and guaranteeing the main functions of the components of this region during breeding phase.

In conclusion, besides the testis, AR was found widely expressed along the segments of the epididymal region of roosters and drakes; however, with cell and segment specific differences in intensity of receptor expression, suggesting different sensitivity to androgens.

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ARTIGO 3

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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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Keywords: Epididymal lithiasis; Vitamin D receptor; Androgen receptor; Epididymis; Efferent ductules; Rooster

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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., 2007a). 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

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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

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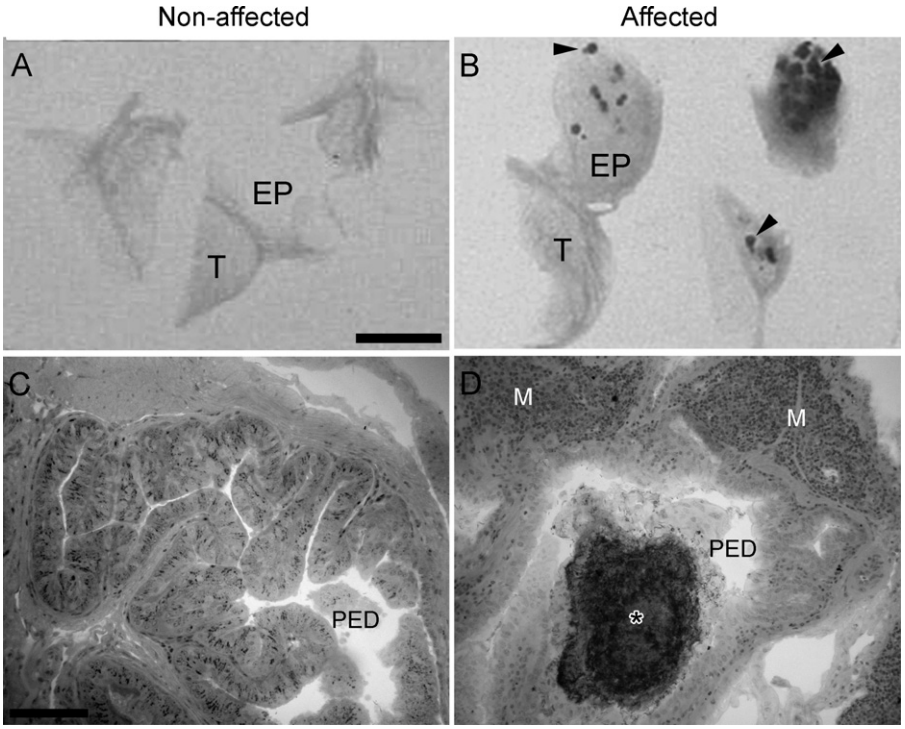

Fig. 1. Diagnosis of epididymal lithiasis. (A) After clearing in glycerin, the epididymal region of non-affected animals was completely transparent and revealed absence of stones. (B) The epididymal region of affected animals presented a variable number of stones within the tissue (arrowheads). (C) Proximal 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., 2007a,b; 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., 2007a,b) 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

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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 (μm^2) and then the proportion of cells/100 μm^2 was calculated. In order to facilitate the interpretation, the results were expressed in mm^2.

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 $P \leq 0.05$.

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., 2007b). 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.

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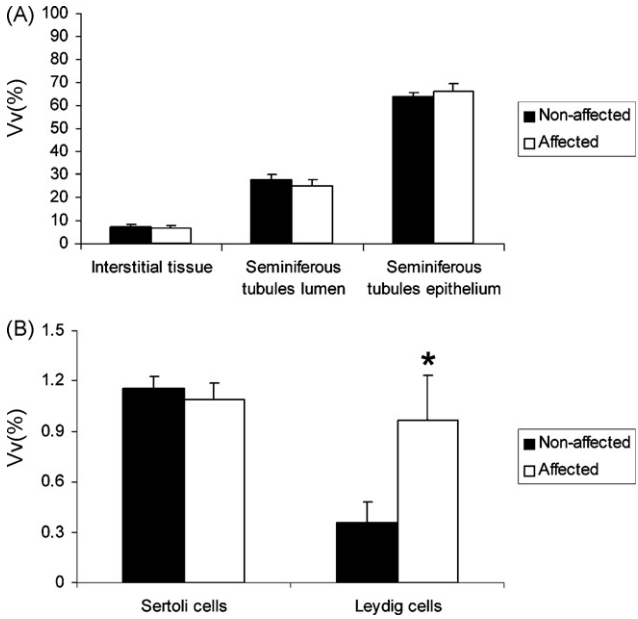

Fig. 2. Volumetric proportion (Vv%) of the interstitial tissue, seminiferous tubules lumen and epithelium (A) as well as Sertoli cells and Leydig cell populations (B) in the testes of animals non-affected and affected by epididymal lithiasis. *$P \leq 0.05$; $n = 4$.

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).

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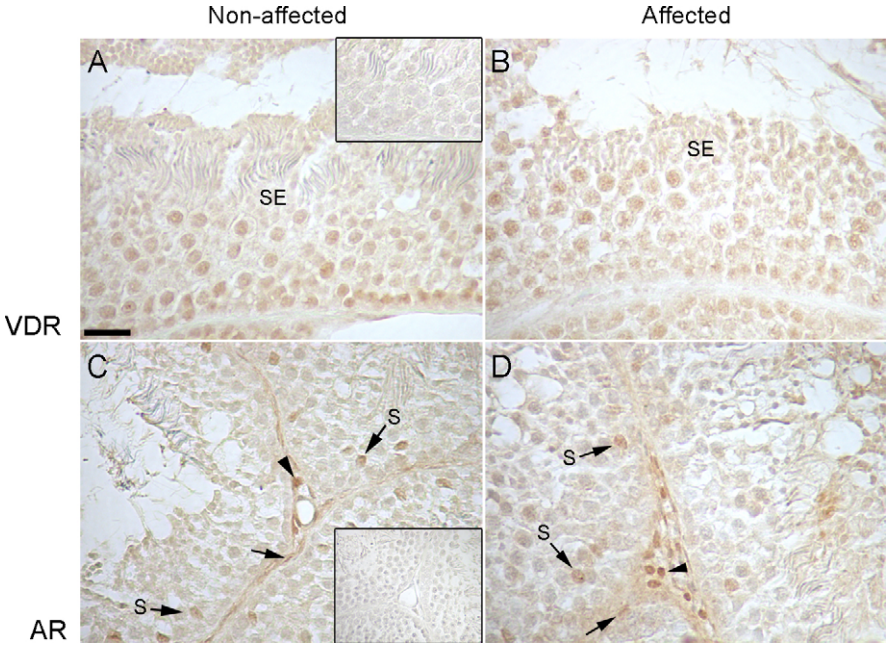

Fig. 3. Expression of VDR and AR in the testis of roosters non-affected (A and C) and affected (B and D) by epididymal lithiasis. (A and B) VDR was detected in spermatogonias, spermatocytes and Sertoli cells within the seminiferous tubules epithelium (SE). Compared to the non-affected animals (A), no 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

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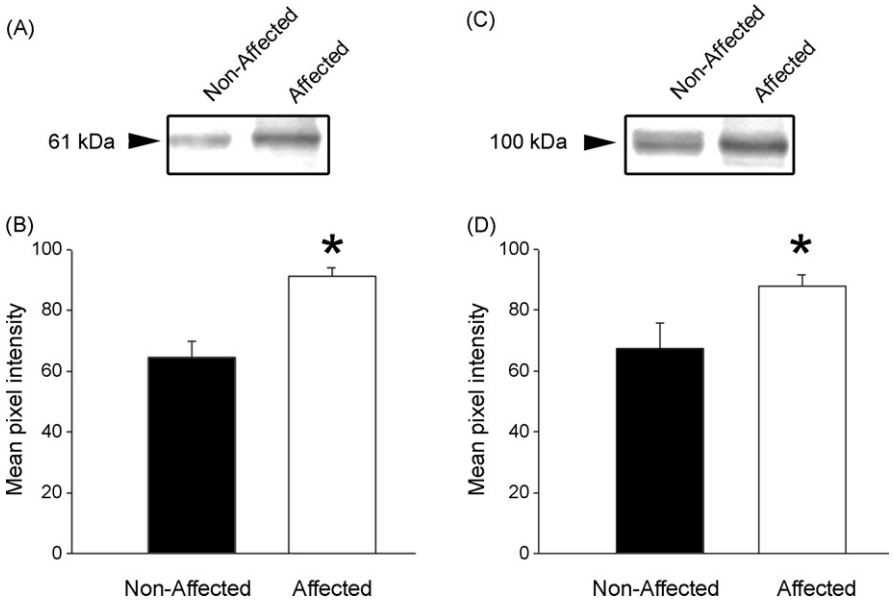

Fig. 4. Western blotting analyses of VDR (A and B) and AR (C and D) in the epididymal region of roosters. (A) Compared to non-affected animals, amounts of VDR were greater in roosters affected by epididymal lithiasis. (B) Graphical representation of image analysis of the VDR western blots. (C) Amounts of AR were also greater in the epididymal region of affected animals when compared to those non-affected. (D) Graphical representation of image analysis of the AR western blots. *$P \leq 0.05$; $n = 6$.

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., 2007a), which is consistent with a major physiological role of the ductal epithelium in the reabsorption of luminal calcium coming from the rete testis (Clulow

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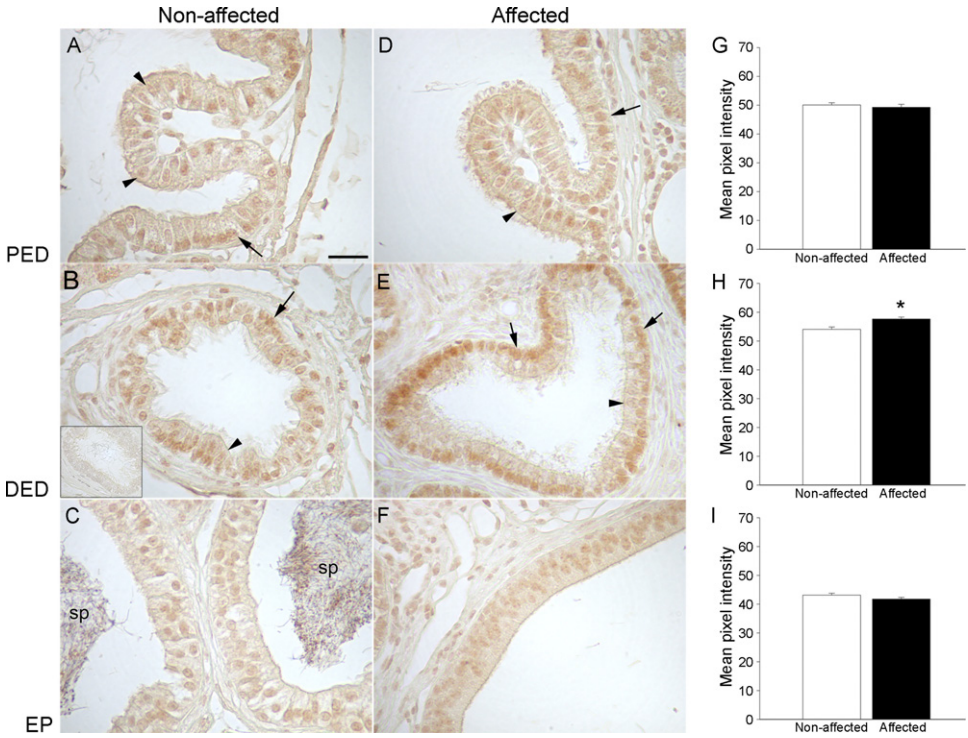

Fig. 5. Vitamin D receptor (VDR) in the epididymal region of roosters non-affected (A–C) and affected (D–F) by epididymal lithiasis. (A–C) VDR was found in the non-ciliated cells (arrows) of the proximal (A) and distal (B) efferent ductules, whereas the non-ciliated cells (arrowheads) were weakly stained 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 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 slighter 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

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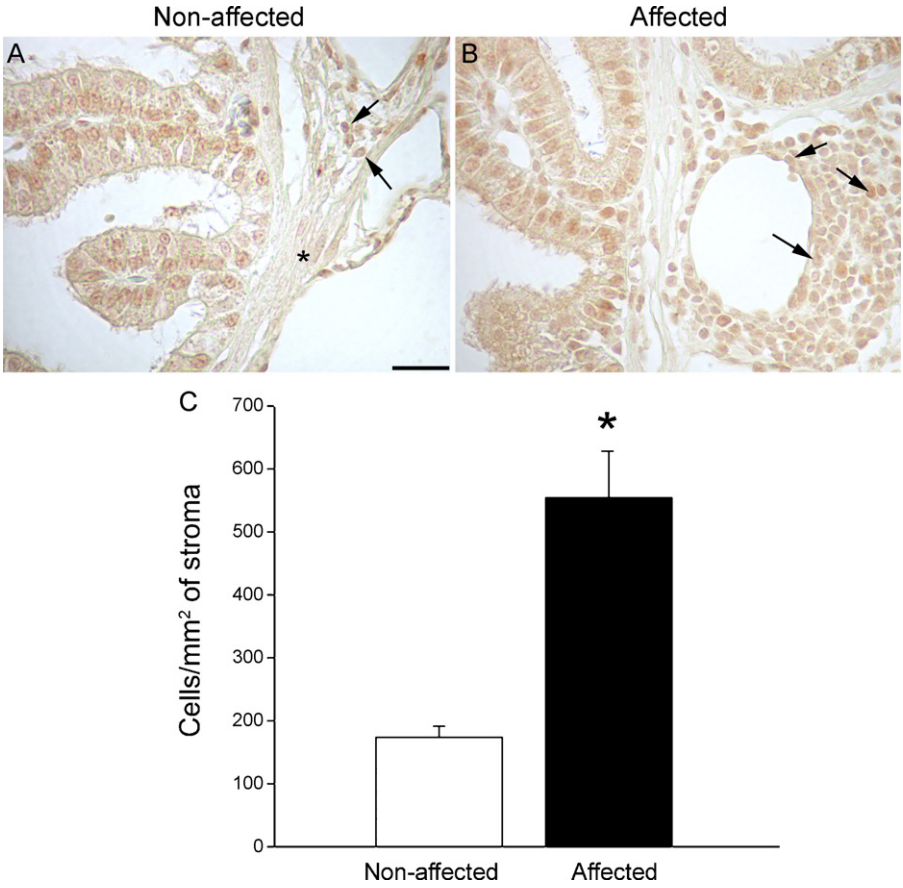

Fig. 6. VDR-positive cells in the epididymal region connective tissue. (A) Non-affected animals presented a small number of connective tissue cells positive to VDR (arrows). (B) Roosters affected by epididymal lithiasis showed numerous VDR-positive cells (arrows) in the connective tissue, which were localized especially in the mononuclear cell infiltrates. (C) Frequency of VDR-positive cells in the epididymal region connective tissue of non-affected and affected roosters. *$P \leq 0.05$; $n = 4$.

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

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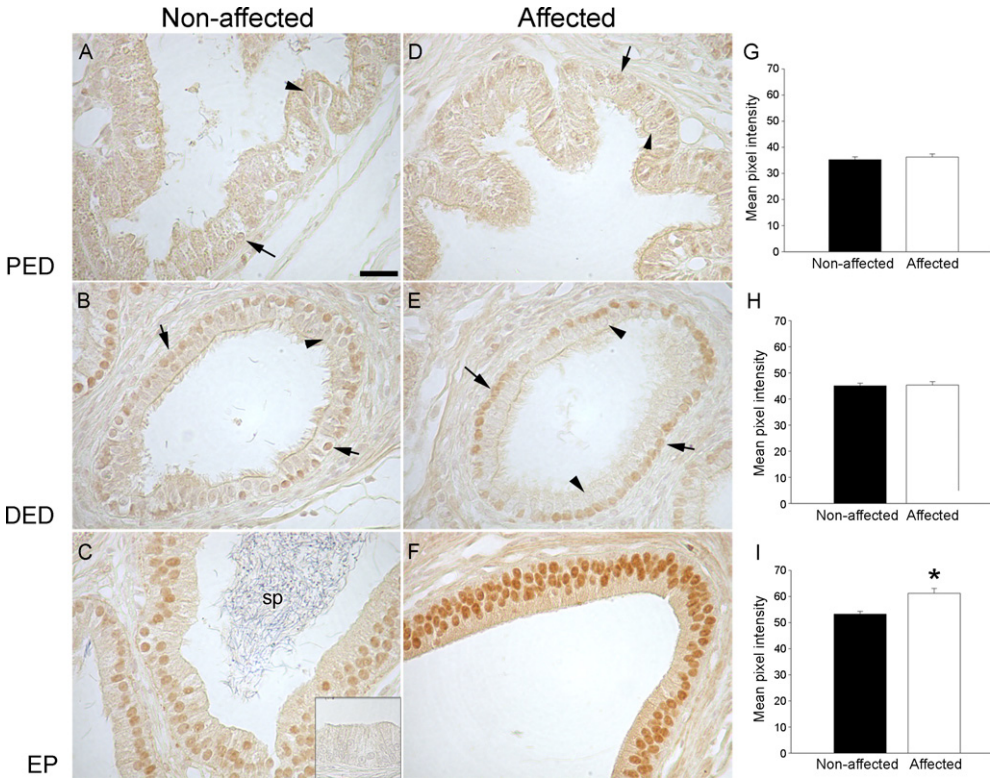

Fig. 7. Amount of androgen receptor (AR) in the epididymal region of roosters non-affected (A–C) and affected (D–F) by epididymal lithiasis. (A–C) Non-ciliated cells of the proximal efferent ductules (arrows) were weakly stained or negative for AR, whereas ciliated cells (arrowheads) were negative (A). (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. $^{*}P \leq 0.05$; $n = 4$. 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.

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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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DISCUSSÃO E CONCLUSÃO

IV – DISCUSSÃO E CONCLUSÃO

O presente estudo demonstrou que os receptores de vitamina D3 (VDR) e andrógenos (AR) são amplamente expressos na região epididimária de galos domésticos, porém com diferenças específicas quanto ao tipo celular e segmento analisado, sugerindo diferente sensibilidade a esses hormônios entre os constituintes desta região. Os maiores níveis de VDR foram observados na região dos dúctulos eferentes. O papel exato da vitamina D3 nesses dúctulos não é conhecido, mas é sugerido que esse hormônio possa participar da modulação da reabsorção de fluido luminal (Johnson et al., 1996). Considerando que já foi demonstrado que os dúctulos eferentes das aves também estão envolvidos na reabsorção de fluido (Clulow & Jones, 1988; Zaniboni et al., 2004; Clulow & Jones, 2004; Bahr et al., 2006), inclusive com taxas de reabsorção maiores que em mamíferos eutérios (Clulow & Jones, 1988), é possível que a vitamina D3 através de VDR possa regular essa função também em aves. Em adição, os dúctulos eferentes das aves são responsáveis pela reabsorção de grandes quantidades de cálcio (Clulow & Jones, 2004), sendo que a participação da vitamina D3 no transporte transepitelial de cálcio é outra possível explicação para a maior expressão de VDR nos dúctulos eferentes. Por outro lado, a expressão de AR foi maior no ducto epididimário, indicando que esse segmento possa ser, dentre os componentes da região epididimária, o mais dependente de andrógenos para a manutenção de sua morfofisiologia.

Em animais afetados pela litíase epididimária, foi detectado aumento na expressão tanto de VDR quanto de AR. O aumento de AR foi coincidente com a maior imunomarcação detectada no ducto epididimário, enquanto o aumento de VDR relacionou-se mais com a presença de um grande número de células VDR-positivas, localizadas, sobretudo, em infiltrados de células mononucleares próximos aos dúctulos eferentes, do que com o discreto aumento na imunoreação detectada nos dúctulos eferentes distais. A presença de abundantes células positivas para VDR nos infiltrados mononucleares é interessante, uma vez que esse dado corrobora a hipótese de que a inflamação encontrada nos animais afetados pela litíase epididimária possa estar envolvida na formação dos cálculos epididimários, muito provavelmente por criar um microambiente favorável à

calcificação. De fato, associação entre inflamação crônica e calcificação já foi demonstrada em modelos patológicos como aterosclerose (Doherty et al., 2003) e urolitíase (Lai et al., 1996). Com relação à expressão de AR, os níveis desse receptor foram maiores no ducto epididimário, indicando que o sistema responsivo para andrógenos possa estar mais associado a prováveis alterações na qualidade dos espermatozóides, sugeridas em trabalhos anteriores e que culminam na drástica redução da fertilidade dos animais afetados (Janssen et al., 2000), do que primariamente com a formação dos cálculos epididimários.

Em suma, nossos dados sugerem que os sistemas responsivos a vitamina D3 e andrógenos podem estar direta ou indiretamente envolvidos com o desenvolvimento da litíase epididimária de galos.

PERSPECTIVAS

V - PERSPECTIVAS

Os complexos vitaminaD3/VDR e andrógenos/AR são capazes de modular a homeostase de cálcio alterando a expressão ou atividade de suas proteínas transportadoras (Dick et al., 2003; Hoenderop et al., 2005). Entretanto, o transporte transepitelial de cálcio também depende de estrógenos e seus receptores, que podem ser de dois subtipos: ERα e ERβ (Enmark & Gustafsson, 1999). A associação entre estrógenos e a homeostase de cálcio vem sendo demonstrada nos últimos anos, sendo um excelente exemplo, o importante papel exercido por estrógenos na prevenção da perda óssea ou osteoporose em mulheres submetidas à reposição hormonal após a menopausa (Prince, 1994). Também já foi demonstrada correlação entre os níveis de ER e calcificação em condições patológicas, como a aterosclerose (Christian et al., 2006). Baseado nesta íntima relação entre estrógenos e homeostase de cálcio e sabendo-se que a formação dos cálculos epididimários ocorre nos dúctulos eferentes, um segmento das vias genitais reconhecido pela elevada expressão de receptores de estrógenos (Hess et al., 1997b; Kwon et al., 1997), torna-se importante estudar também o padrão e níveis de expressão de ERα e ERβ em animais afetados pela litíase epididimária. Também é necessário investigar a expressão de outras proteínas envolvidas no transporte transepitelial de cálcio, como as proteínas TRPV, calbindina e PMCA, cujas expressões e/ou atividade são intimamente dependentes de VDR, AR e ER.

Uma outra necessidade iminente surgida dos resultados deste estudo é a investigação do papel dos infiltrados mononucleares na litíase epididimária. Nossos dados mostraram abundantes células VDR+ nesses infiltrados em animais afetados. Esse resultado, inédito, é interessante, uma vez que o número e atividade de diversas células inflamatórias são regulados por esteróides sexuais, como andrógenos e estrógenos, além da vitamina D3 (Hayes et al., 2003; Yoshimura et al., 2005; Yoshimura et al., 2006). Estes dados confluem e reforçam a possibilidade de que a inflamação na região dos dúctulos eferentes possa estar envolvida com a formação dos cálculos epididimários por criar um ambiente favorável à calcificação (Janssen et al., 2000; Boltz et al., 2006; Jackson et al., 2006). Dessa forma, torna-se interessante estudar o papel da regulação imunológica no desenvolvimento e tratamento da litíase epididimária.

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