ANDRÉ GUSTAVO DE OLIVEIRA

RECEPTORES DE ESTRÓGENOS (ERα E ERβ) E PROTEÍNAS ENVOLVIDAS NO TRANSPORTE TRANSEPITELIAL DE CÁLCIO NA LITÍASE EPIDIDIMÁRIA DE GALOS DOMÉSTICOS

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

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LISTA DE ABREVIATURAS

AIBV - vírus da bronquite infecciosa aviária

aMPV - metapneumovírus aviário

ATP - trifosfato de adenosina

Ca^{2+} - íons cálcio

CaBP-D9K - proteína ligadora de cálcio de 9 kilodaltons (calbindina D9K)

CaBP-D28K - proteína ligadora de cálcio de 28 kilodaltons (calbindina D28K)

ECaC1 - *Epithelial Calcium Channel 1* (Canal de Cálcio Epitelial tipo 1)

ECaC2 - *Epithelial Calcium Channel 2* (Canal de Cálcio Epitelial tipo 2)

ERα - receptores de estrógenos tipo alfa

ERβ - receptores de estrógenos tipo beta

Mg^{2+} - íons magnésio

NCX1 – *sodium/calcium exchanger 1* (trocador sódio/cálcio 1)

PDZ - *post synaptic density protein; drosophila disc large tumor suppressor e zonula occludens-1 protein*

PKC - Proteína cinase C

PMCA - *plasma membrane calmodulin-dependent calcium ATPase* (ATPase de membrana plasmática dependente de calmodulina)

RNAm - RNA mensageiro

RT-PCR - reação em cadeia da polimerase via transcriptase reversa

TM - domínios transmembrana

TRPV5 - *Transient Receptor Potential Vanilloid type 5* (Receptores de Potencial Transiente da subfamília vanilóide tipo 5)

TRPV6 - *Transient Receptor Potential Vanilloid type 6* (Receptores de Potencial Transiente da subfamília vanilóide tipo 6)

VDRE - elementos responsivos a vitamina D3

vitamina D3 - 1,25 diidroxivitamina D3 – $1,25(OH)_2D_3$

RESUMO

A litíase epididimária é uma anomalia caracterizada pela presença de cálculos ricos em cálcio na região epididimária de galos. Esta disfunção causa redução na fertilidade, tendo impacto negativo na avicultura. A causa da litíase não é conhecida, mas sabe-se que a formação de cálculos acomete principalmente os dúctulos eferentes, responsáveis pela reabsorção do fluido testicular e de cálcio. O transporte transepitelial de cálcio ocorre em etapas distintas que envolvem a entrada de cálcio na célula, mediada pelos canais para cálcio epiteliais (como a TRPV6), a translocação desse íon até a membrana basolateral por proteínas ligadoras de cálcio (calbindinas) e a sua liberação para o meio extracelular mediada pelos transportadores PMCA (ATPase de membrana plasmática) e NCX1 (trocador sódio/cálcio tipo 1). Esse processo sofre influência de hormônios, como vitamina D3, andrógenos, estrógenos e seus receptores (VDR, AR e ERα e ERβ), todos estes presentes nos dúctulos eferentes. Dessa forma, considerando as funções dos dúctulos eferentes, hipotetizamos que a formação dos cálculos intraluminais poderia estar relacionada com desequilíbrio na homeostase de cálcio e/ou hormonal nos animais afetados. Corroborando esta hipótese, demonstramos que a expressão de VDR e AR encontra-se alterada na região epididimária de galos afetados pela litíase. No entanto, nada se sabe sobre a expressão de receptores de estrógenos ERα e ERβ, nem dos mediadores do transporte de cálcio TRPV6, CaBP-D28K, NCX1 e PMCA na região epididimária desses animais. Dessa forma, nossa proposta foi investigar alterações na expressão dessas proteínas, objetivando esclarecer a fisiopatologia da anomalia. Animais afetados apresentaram maior expressão de ERβ, enquanto ERα não foi alterado. Paralelamente, houve aumento local de estradiol, mas redução de vitamina D3 e testosterona. Animais com litíase apresentaram ainda aumento na expressão de TRPV6 e CaBP-D28K, mas não de PMCA e NCX1. Em adição, grânulos Alizarina-positivos foram observados nas células epiteliais de dúctulos eferentes afetados, sugerindo concentração de cálcio. Todas as alterações detectadas foram mais evidentes nos dúctulos eferentes proximais, local onde os cálculos são formados. Em conjunto, os resultados fornecem as primeiras evidências para o envolvimento de alterações hormonais e da homeostase de cálcio como um mecanismo provável para a formação de cálculos de cálcio nos dúctulos eferentes, sendo que tal desequilíbrio pode resultar na maior concentração de cálcio intraluminal e consequente formação de centros de nucleação que levam a litíase.

I Introdução e Revisão de Literatura

I- INTRODUÇÃO E REVISÃO DE LITERATURA

1. SISTEMA GENITAL MASCULINO DAS 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, mantendo-se fixos na parede 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 parcialmente 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). Diferentemente 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).

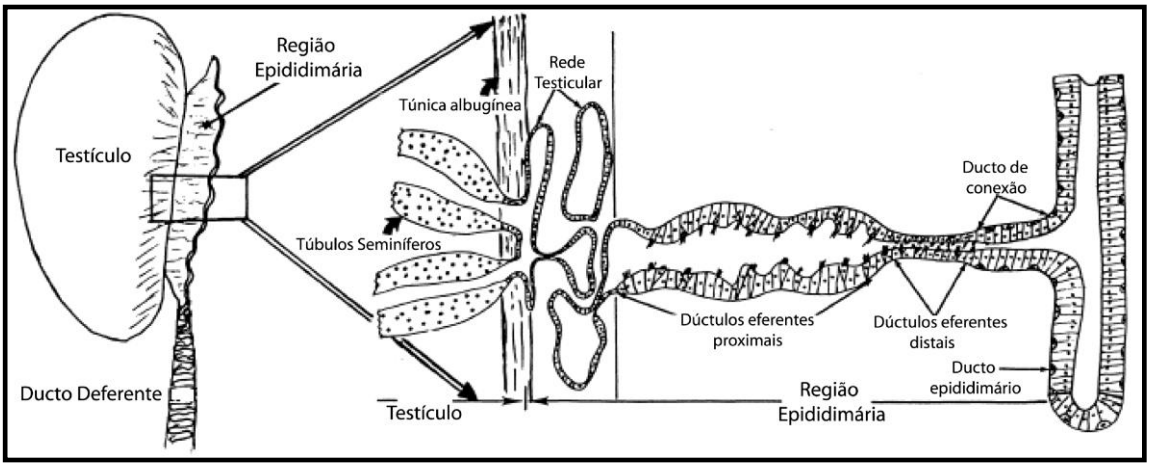

Figura 1: Desenho esquemático da região epididimária das aves. Baseado em Aire (2000).

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 porção extratesticular ocupa cerca de 10% a 13% da região epididimária dependendo da espécie estudada (Aire, 1979b; Oliveira et al, 2007) e é revestida por epitélio simples cúbico, com células apresentando curtas microvilosidades apicais. Na superfície lateral das células epiteliais 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 constituem uma série de túbulos delgados que conduzem o fluido testicular da rede testicular para o ducto epididimário. Esses dúctulos 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; Oliveira et al, 2007). 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 mais 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; Oliveira et al, 2007). Externamente, os dúctulos eferentes são envolvidos por delgada camada de musculatura lisa.

Semelhante aos de 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 coturnix japonica*), já foi demonstrado que os dúctulos eferentes são responsáveis pela reabsorção de aproximadamente 86% do fluido testicular (Clulow & Jones, 1988). Os dúctulos eferentes de aves 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). Ambos apresentam características histológicas semelhantes, sendo revestidos por epitélio pseudo-estratificado cilíndrico. Dessa forma a diferenciação morfológica desses segmentos baseia-se no fato dos ductos de conexão apresentarem menor diâmetro e epitélio mais baixo que o ducto epididimário (Aire, 1979a). 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, é que 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. 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).

De maneira geral, os constituintes da região epididimária, sobretudo os dúctulos eferentes e o ducto epididimário, relacionam-se diretamente com modificações no padrão de expressão de proteínas na superfície dos espermatozóides durante seu trânsito pelo sistema de ductos excurrentes, o que supostamente está relacionado com o processo de maturação dos mesmos (Esponda et al, 1985).

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 (Gill, 1994).

2. LITÍASE EPIDIDIMÁRIA

2.1. Características gerais

A litíase epididimária é uma disfunção reprodutiva descrita há cerca de 12 anos (Janssen et al, 2000), a qual é caracterizada pela formação de abundantes cálculos na região epididimária de galos (Fig. 2A). Acredita-se que essa disfunção reprodutiva seja restrita a essa espécie, uma vez que dentre 27 espécies de aves domésticas e selvagens investigadas, a formação dos referidos cálculos só foi encontrada em galos (Mahecha et al, 2002). Inicialmente, a litíase epididimária foi 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.

Macroscopicamente, os cálculos epididimários apresentam tamanhos e formas variadas, com diâmetros entre 9 µm a 3000 µm, sendo a superfície dos mesmos lisa ou levemente áspera, com projeções pontiagudas (Fig. 2B) (Janssen et al, 2000; Mahecha et al, 2002). A composição dos cálculos epididimários foi determinada por diversas metodologias, sendo demonstrado que o cálcio é o principal elemento encontrado nos cálculos epididimários (40%-48%). Outros átomos como oxigênio (28%) e carbono

(23,5%) também são encontrados em menores quantidades (Fig. 2C-D). Enxofre, magnésio, sódio, fósforo e material orgânico foram encontrados em quantidades muito pequenas (Janssen et al, 2000; Mahecha et al, 2002). Microscopicamente, os cálculos de cálcio são caracterizados pela presença de um centro de calcificação e pela presença de camadas alternadas de calcificação e material orgânico (Mahecha et al, 2002). A presença de espermatozóides aderidos à superfície dos cálculos e a presença de células descamadas no interior dos mesmos também são características freqüentes (Janssen et al, 2000; Mahecha et al, 2002).

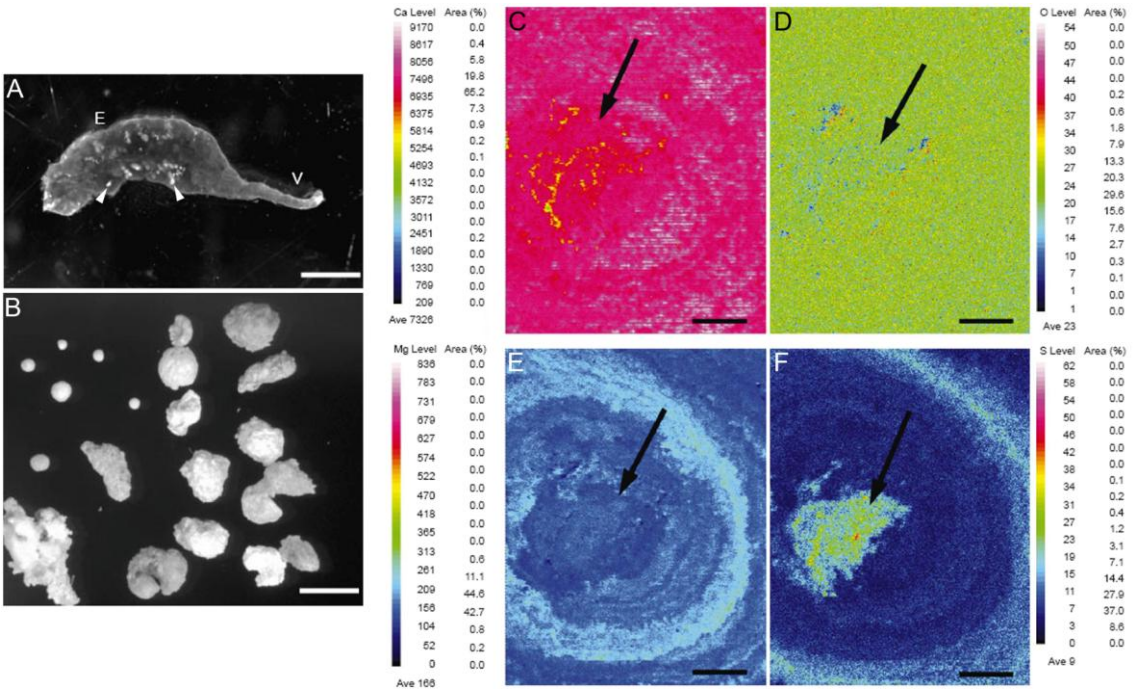

Figura 2: Aspecto geral e composição dos cálculos encontrados na litíase epididimária de galos. **(A)** Região epididimária (E) de galos afetados pela litíase com grande número de cálculos (cabeças de seta). V = ducto deferente. **(B)** Os cálculos apresentam forma e tamanho irregulares. **(C-F)** Microanálise 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 de vermelho refletem as maiores concentrações enquanto os espectros de azul, as menores concentrações. Seta = centro do cálculo. Barra em A = 0,19 mm; B = 350 µm; C-F = 50 µm. 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 inflamatórios de células mononucleares no tecido conjuntivo da região epididimária, especialmente nas adjacências dos dúctulos eferentes (Janssen et al, 2000; Mahecha et al, 2002; Oliveira et al, 2008).

Grande parte dos animais afetados apresenta as alterações supracitadas na região epididimária sem, contudo, apresentar diferenças significativas na proporção de tecido intertubular, lúmen e epitélio seminífero dos túbulos seminíferos, além da população de células de Sertoli (Oliveira et al, 2008). Corroborando esses dados, não foram observadas alterações na produção de espermatozóides por grama de testículo entre animais afetados e não-afetados pela litíase epididimária (Janssen et al, 2000). Entretanto, em alguns testículos de animais acometidos pela litíase epididimária, é possível observar áreas 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 inflamatórios de células mononucleares e perda da arquitetura testicular (Mahecha et al, 2002). A baixa prevalência de alterações testiculares significativas em galos afetados pela litíase sugere que os efeitos testiculares são secundários às alterações encontradas nas vias extratesticulares que compõem a região epididimária.

Os animais afetados pela litíase epididimária apresentam redução significativa de cerca de 65% nos níveis séricos de testosterona concomitante com um aumento na proporção de células de Leydig a qual tem sido interpretada como uma forma de

restabelecer os níveis fisiológicos desse hormônio (Janssen et al, 2000; Oliveira et al, 2008). 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 posdem ser ocasionadas por alterações na qualidade dessas células que podem refletindo alterações funcionais na região epididimária, especialmente na região dos dúctulos eferentes.

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) doença autoimune, (3) elevados níveis de cálcio e vitamina D3 (1,25 diidroxivitamina D3 – $1,25(OH)_2D_3$) presentes na dieta dos animais e (4) predisposição genética dos animais afetados.

Hipótese 1: Infecção da região epididimária

A hipótese de infecção na região epididimária como causadora da litíase epididimária foi proposta devido à presença de abundantes infiltrados inflamatórios mononucleares na região epididimária dos animais afetados (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 aviária (AIBV), uma vez que o mesmo é epiteliotrópico, possuindo afinidade especial àqueles com células ciliadas, como é o caso dos dúctulos eferentes (Jackson et al, 2006; Cavenagh et al, 2007; Shen et al, 2010). Além disso, o AIBV tem sido associado com calcificação de tecidos em outras patologias, como a urolitíase (Niznik et al, 1985; Brown et al, 1987; Fitz-Coy et al, 1988; Glahn et al, 1989). Experimentos foram realizados procurando correlacionar a infecção por AIBV com o

desenvolvimento da litíase epididimária; entretanto, o monitoramento sistemático da produção de anticorpos humorais contra esse vírus revelou que mesmo galos negativos para essa doença ainda desenvolvem a litíase epididimária (Mahecha et al, 2002). Em adição, estudos utilizando vacinação com AIBV 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 que foram utilizados como controles do experimento também desenvolveram os cálculos ricos em cálcio (Boltz et al, 2006; Jackson et al, 2006). Entretanto, esses estudos indicaram que a incidência da litíase epididimária foi maior nos animais expostos ao AIBV e que o desenvolvimento dessa disfunção reprodutiva foi acelerado nessas condições (Boltz et al, 2006; Jackson et al, 2006). Em conjunto, esses dados indicam que, mesmo que a exposição ao AIBV possa acelerar o desenvolvimento da litíase epididimária, ela não é o principal fator envolvido na sua etiologia.

Recentemente, outro vírus epiteliotrópico foi identificado no sistema genital masculino de aves. O metapneumovírus aviário (aMPV) foi identificado por RT-PCR em amostras de testículos de galos e mostrou ter alta prevalência em tecidos de animais afetados pela litíase epididimária (Villarreal et al, 2007), mas ainda não se sabe se o aMPV desempenha algum papel no desenvolvimento da litíase epididimária. Em adição, diversos agentes patológicos têm sido associados com a calcificação de tecidos (Ennever et al, 1981; Cisar et al, 2000; Kosowski et al, 2000; Colpan et al, 2003; Reyes et al, 2009). Dessa forma, a hipótese de um agente infeccioso como causa primária da litíase epididimária não pode ser descartada.

Hipótese 2: Doença autoimune

Outra explicação para a observação da inflamação crônica na região epididimária de galos com cálculos epididimários é a presença de uma doença autoimune. Em mamíferos eutérios, os dúctulos eferentes constituem os principais locais de extravasamento de antígenos presentes no lúmen e, consequentemente, os principais locais para a ocorrência de uma resposta autoimune (Suzuki and Nagano, 1978; Tung & Alexander, 1980).

Na hipótese da doença autoimune, acredita-se que a barreira dos dúctulos eferentes de galos afetados esteja, de alguma forma, alterada ou pelo menos mais permeável do que em animais não-afetados (Jackson et al, 2006). Essa permeabilização resultaria na passagem do conteúdo luminal para o tecido conjuntivo subjacente e,

consequentemente, na estimulação de células do sistema imunológico. Pouco se sabe sobre o sistema imunológico na região epididimária de galos, mas sabe-se que os dúctulos eferentes possuem a maior população de células apresentadoras de antígenos e linfócitos T $CD4^+$ e $CD8^+$ quando comparados com outros componentes da região epididimária (Yoshimura et al, 2005; Yoshimura et al, 2006). Essas células são candidatas a desempenhar papel de modulação da resposta imunológica local a antígenos luminais, resultando no recrutamento de outras células inflamatórias e na produção de anticorpos secretados no lúmen dos dúctulos eferentes. Mais estudos são necessários para determinar o papel da modulação do sistema imunológico no desenvolvimento da litíase.

Hipótese 3: Ingestão de cálcio e vitamina D3

Baseado na constituição dos cálculos epididimários, foi proposto que a origem da litíase epididimária estivesse relacionada com a ingestão de quantidades inapropriadas de cálcio e vitamina D3, sendo essa última um hormônio secosteróide que apresenta papel de destaque na manutenção da homeostase de cálcio no organismo (Mahecha et al, 2002; Bennet et al, 2006; Lips et al, 2006; de Matos, 2008). Entretanto, não foram encontradas correlações entre o número de cálculos epididimários ou peso dos testículos/região epididimária com as concentrações ingeridas de cálcio (Mahecha et al, 2002). Da mesma forma, foi observada ausência de correlação entre o consumo de vitamina D3 e a ocorrência da litíase epididimária (Jackson et al, 2006), corroborando o fato de que algumas linhagens de galos não consomem ração contendo altos níveis desse hormônio e mesmo assim apresentam os cálculos de cálcio no lúmen dos dúctulos eferentes (Mahecha et al, 2002).

Hipótese 4: Predisposição genética

Outra hipótese para explicar a origem da litíase epididimária é a predisposição genética dos animais afetados. 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 TRANSEPITELIAL DE CÁLCIO

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 consequentemente 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 intestinos, rins, glândulas mamárias e placenta, num processo mediado por complexa sequência 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 de epitélios: 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 e será detalhado nas seções subsequentes.

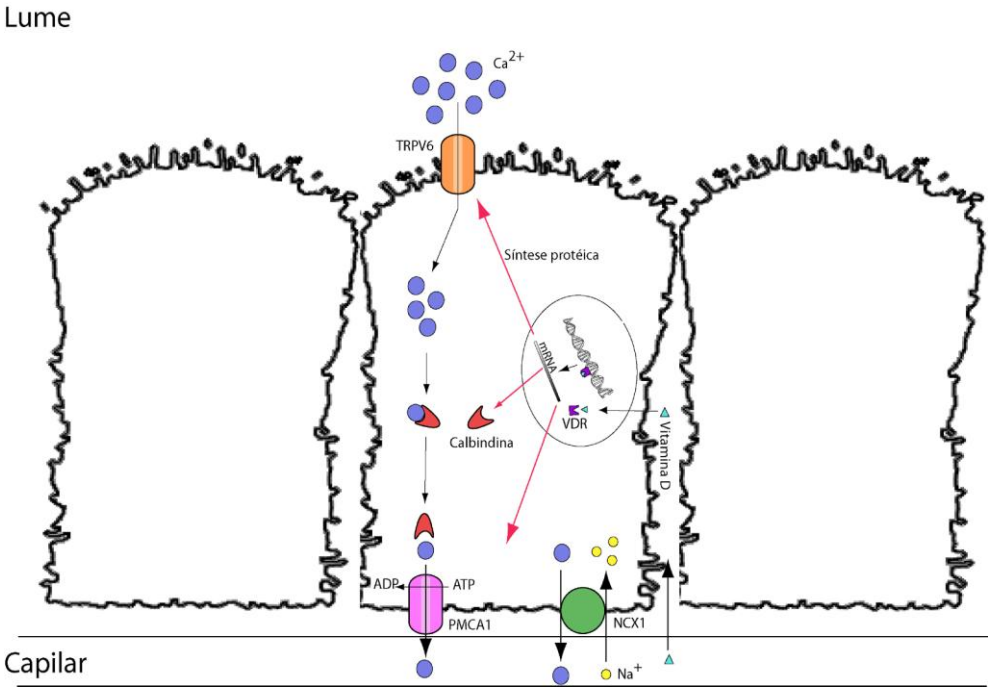

Figura 3: Desenho esquemático do transporte transepitelial de cálcio. Baseado em Hoenderop et al (2005).

3.1. Entrada de cálcio na célula: a importância dos canais para cálcio epiteliais

Acredita-se que a entrada de cálcio nas células epiteliais ocorra através de canais para cálcio localizados na membrana apical, conhecidos como TRPV5 e TRPV6 (do inglês ***T**ransient **R**eceptor **P**otential **V**anilloid",* previamente denominados *Epithelial Calcium Channel* - ECaC1 e ECaC2, respectivamente). Essas proteínas são membros de uma família de canais conhecida como "Receptores de Potencial Transiente" e, mais especificamente, são agrupadas na subfamília "Vanilóide" (Hoenderop et al, 2005), a qual possui seis componentes, sendo que desses apenas os subtipos 5 e 6 de TRPVs são capazes de transportar cálcio.

Tanto a proteína TRPV5 quanto a TRPV6 apresentam estruturas moleculares semelhantes, compartilhando 75% de similaridade na sequência de aminoácidos e uma estrutura tetramérica que forma um poro seletivo para a passagem de cálcio (Fig. 4A) (Hoenderop et al, 2005). Cada monônomero de TRPV5 ou TRPV6 é formado por aproximadamente 730 aminoácidos e contém seis domínios transmembranas (TM1-6) e extensas regiões amino- e carboxi-terminais citoplasmáticas. Entre os domínios TM5 e TM6, encontra-se uma pequena área hidrofóbica que se projeta para o interior da bicamada lipídica e que atua na formação da região do poro (Fig. 4B). Em adição, também são encontradas nesses monômeros regiões de repetição de anquirina, domínios

PDZ (***P****ost synaptic density protein;* ***D****rosophila disc large tumor suppressor e* ***Z****onula occludens-1 protein*) e sítios de fosforilação, as quais estão relacionadas com a interação dos canais com outras moléculas transportadoras, proteínas de adesão ao citoesqueleto e, ainda, proteínas reguladoras da função transportadora do canal (Fig. 4C) (Hoenderop et al, 2002; den Dekker et al, 2003; Hoenderop et al, 2005).

O movimento de cálcio para o interior das células reabsortivas ocorre de maneira dependente de gradiente eletroquímico favorável, direcionado no sentido do lúmen para o citoplasma. Quando as concentrações luminais de cálcio são pequenas, situação na qual o referido gradiente eletroquímico é pequeno, íons magnésio (Mg^{2+}) se ligam em uma região específica do poro de seletividade a cálcio, determinada por dois resíduos de aspartato nas posições 541/542, e bloqueiam o transporte de cálcio pela membrana. À medida que a concentração luminal de cálcio aumenta e o gradiente eletroquímico torna-se mais favorável ao transporte desse íon, os íons Mg^{2+} são removidos do poro do canal que passam agora a ser ocupados por íons cálcio. Nessa etapa de transição, a probabilidade de se encontrar Ca^{2+} ligado aos resíduos de aspartato 541/542 é maior devido não só a maior concentração luminal de cálcio, mas também porque a interação desse íon com a região de seletividade do poro é maior. Finalmente, quando o gradiente eletroquímico lúmen-citoplasma atinge seu limiar, os íons cálcio são removidos do poro de seletividade e direcionados ao interior do citoplasma das células epiteliais. O sítio de ligação ao cálcio é imediatamente ocupado por outro íon Ca^{2+} e o processo continua até a dissipação do gradiente eletroquímico que resulta no bloqueio dos canais TRPV5/6 por íons Mg^{2+} (den Dekker et al, 2003; Hoenderop et al, 2005).

Devido ao importante papel desempenhado tanto pela TRPV5 quanto pela TRPV6 na reabsorção de cálcio, ambas as proteínas são encontradas em epitélios envolvidos no transporte desse íon. No entanto, essas proteínas apresentam padrão de expressão distintos, uma vez que TRPV5 é encontrada principalmente nos rins, especialmente no epitélio de revestimento dos túbulos contorcidos distais e ductos de conexão, enquanto a TRPV6 apresenta distribuição mais ampla, sendo encontrada nos rins em praticamente toda a porção distal do néfron (túbulos contorcidos distais, ductos de conexão e ductos coletores), além de outros órgãos, como estômago, intestinos, fígado, pulmão, esôfago e pâncreas (den Dekker et al, 2003; Hoenderop et al, 2005). A ocorrência de canais para cálcio do tipo TRPV também já foi descrita em órgãos do sistema genital masculino, como testículos, dúctulos eferentes e próstata, sendo que

nesses órgãos apenas a proteína TRPV6 foi identificada (den Dekker et al, 2003; Jelinsky et al, 2007).

Recentemente, foi demonstrado que a TRPV6 exerce uma função essencial para a manutenção da fertilidade masculina (Weissgerber et al, 2011). Animais transgênicos que expressam a proteína TRPV6 com uma mutação no poro de seletividade ao cálcio são inférteis por apresentarem espermatozóides com baixa viabilidade e motilidade, resultado de um aumento significativo (10X) na concentração de cálcio no fluido epididimário (Weissgerber et al, 2011). Por essa razão e considerando que o órgão alvo do presente estudo são os dúctulos eferentes de galos, optamos por focar o estudo da expressão do canal TRPV6 no que se refere ao processo de reabsorção de cálcio em animais afetados pela litíase epididimária quando comparados com aqueles não-afetados.

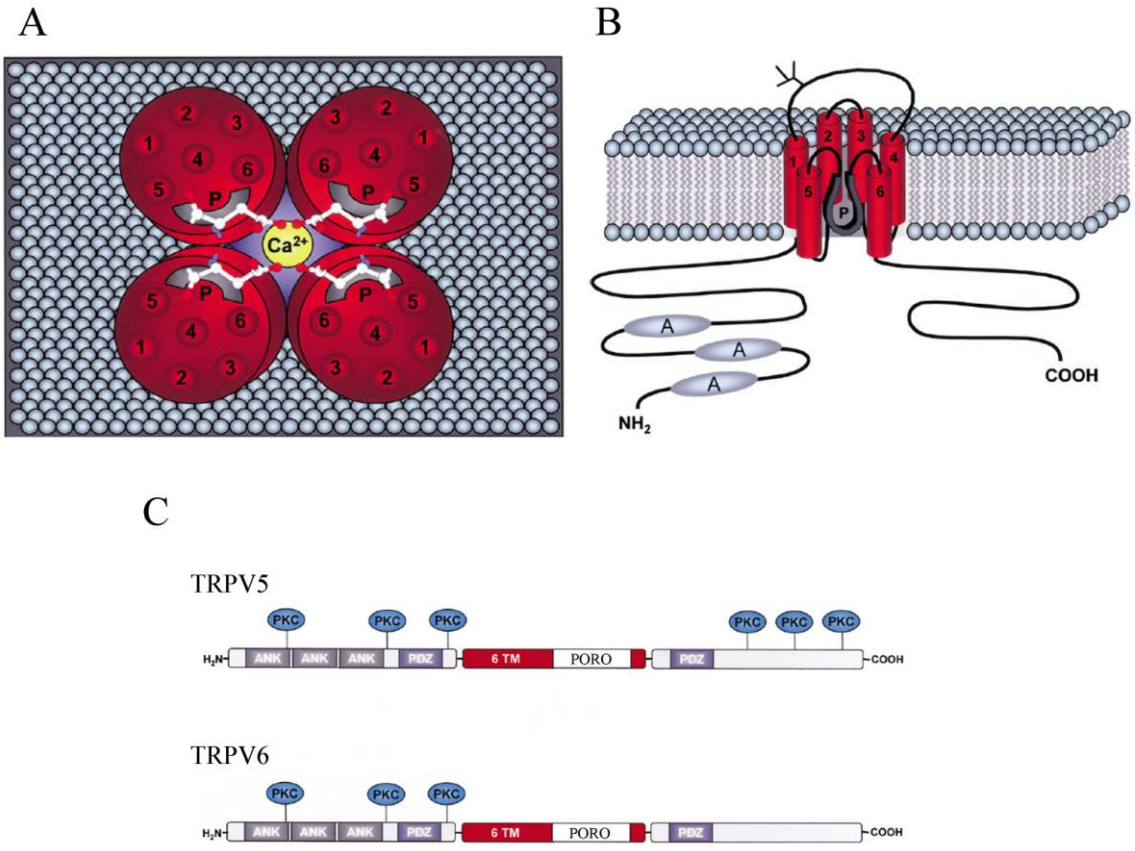

Figura 4: Estrutura molecular dos canais epiteliais para cálcio TRPV5 e TRPV6. **(A)** Visão transversal de um tetrâmero de TRPV5/6 inserido na bicamada lipídica e sua relação com íons cálcio (Ca^{2+}). Note que o poro seletivo a cálcio é formado pela região formadora do poro de cada um dos monômeros. **(B)** Estrutura terciária de um monônomero de TRPV5/6, evidenciando os 6 domínios transmembrana (numerados de 1-6), bem como a região formadora do poro (P) e as regiões de repetição de anquirina (A). **(C)** Estrutura linear de um monômero de TRPV5 e TRPV6, evidenciando sítios de fosforilação (PKC), regiões de repetição de anquirina (ANK) e domínios PDZ. A região dos 6 domínios transmembrana encontra-se representada em vermelho (6 TM) e, entre esses domínios, a região formadora do poro, representada em branco. Baseado em den Dekker et al (2003).

3.2. Difusão citoplasmática de cálcio

Uma vez no citoplasma, os íons cálcio se ligam com alta afinidade à calbindina D9K (CaBP-D9K) ou calbindina D28K (CaBP-D28K), as quais funcionam como transportadores que facilitam a difusão do cálcio entre o citoplasma apical e basolateral das células (Hoenderop et al, 2005). Devido ao fato da CaBP-D28K ser considerada a principal proteína de ligação ao cálcio em tecidos de aves (Bar et al, 2009), o presente estudo abordará as características dessa proteína apenas.

A CaBP-D28K apresenta estrutura molecular compacta, elipsóide, formada por seis domínios conhecidos como hélice E -alça ligadora de Ca^{2+}- hélice F (ou domínio-EF), e denominados de 1 a 6, sendo que os domínios 1, 3, 4 e 5 apresentam alta a moderada afinidade por cálcio enquanto os domínios 2 e 6 são considerados não-funcionais (Fig. 5) (Kojetin et al, 2006; Schwaller, 2009). Cada domínio-EF é constituído por duas α-hélices as quais são dispostas perpendicularmente e unidas por uma curta alça de aminoácidos que contêm o sítio de ligação aos íons cálcio. Para uma melhor compreensão da estrutura tridimensional desse domínio, basta posicionar a mão direita de acordo com a figura 5C. Nessa disposição, o dedo indicador representa a hélice-E, o dedo médio a alça de aminoácidos e o espaço delimitado por esse dedo a região à qual o cálcio se liga e o polegar a hélice-F (Schwaller, 2009).

A CaBP-D28K é uma proteína citoplasmática móvel a qual se difunde no citoplasma das células nas quais é expressa a uma taxa de aproximadamente $20\mu m^2/s$, o que significa que essa proteína teria a capacidade de se difundir por toda a área de citoplasma de uma célula não-ciliada dos dúctulos eferentes de aves em cerca de 10 segundos (Schmidt et al, 2005; Oliveira et al, 2007; Schwaller, 2009). Apesar de sua considerável mobilidade citoplasmática, a proteína CaBP-D28K não se encontra aleatoriamente distribuída nas células. Em células transportadoras de cálcio, essa proteína é preferencialmente localizada no citoplasma apical, sendo co-localizada com os canais para cálcio epiteliais TRPV5/6 que se encontram na membrana apical. Em menor escala, CaBP-D28K é encontrada no terço médio e basolateral do citoplasma (Lambers et al, 2006). Se considerados em conjunto, esses dados revelam que a proteína CaBP-D28K apresenta funções bastante complexas no transporte transepitelial de cálcio. O fato dessas proteínas se ligarem ao cálcio imediatamente após esse íon entrar na célula e se difundirem em direção à membrana basolateral das mesmas para a extrusão do íon, além de promover o transporte de cálcio em si, garante a manutenção do gradiente eletroquímico de cálcio e consequentemente mantém o funcionamento dos

canais TRPV5/6. Essa característica impede que a concentração de cálcio livre intracitoplasmático aumente significativamente nas células reabsortivas e resulte na ativação de vias de sinalização indesejadas ou, ainda, no desencadeamento do processo de apoptose dessas células (Hoenderop et al, 2005; Lambers et al, 2006).

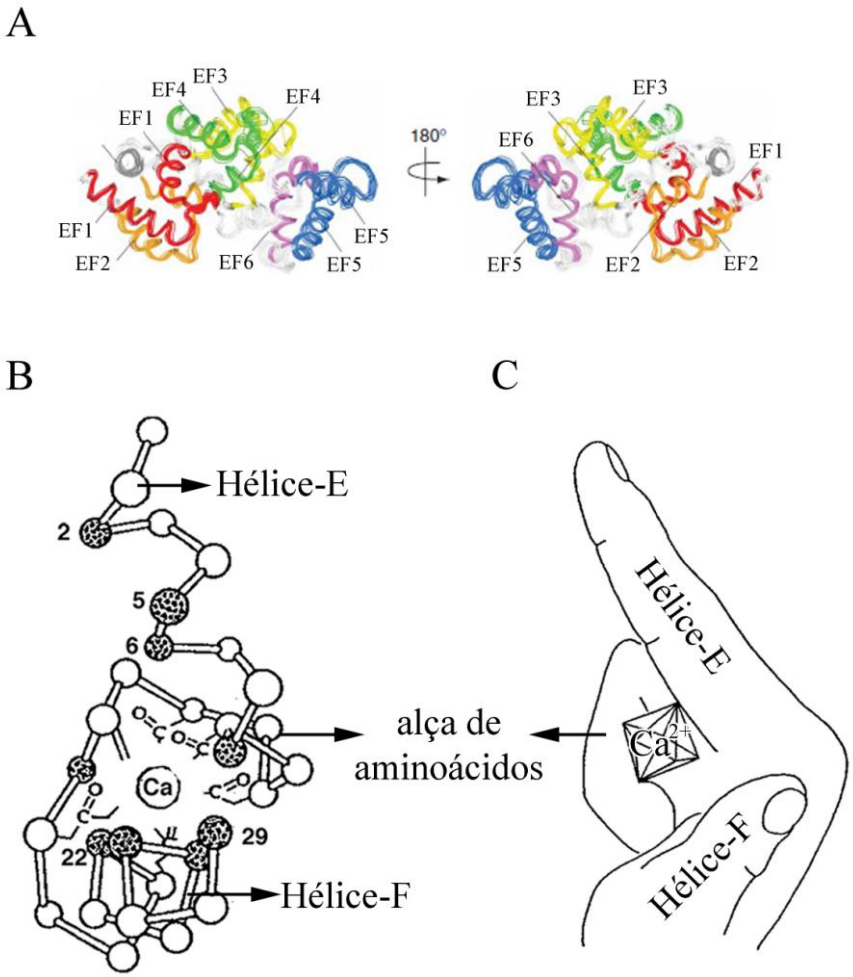

Figura 5: Estrutura molecular da proteína ligadora de cálcio Calbindina-D28K (CaBP-D28K). (A) Estrutura tridimensional da CaBP-D28K evidenciando os 6 domínios-EF, sendo as hélices vermelhas o domínio EF1; as laranjas, EF2; as amarelas EF3; as verdes, EF4; as azuis, EF5 e as roxas, EF6. (B) Estrutura molecular de um domínio-EF isolado evidenciando os seus componentes e a região ligadora de cálcio (Ca). (C) Representação didática para a visualização de um domínio-EF, no qual o dedo indicador da mão direita representa a hélice-E, os dedos médios a alça de aminoácidos que contém a região ligadora de cálcio (Ca^{2+}) e o polegar a hélice-F. Baseado em Kojetin et al (2006) e Schwaller (2009).

3.3. A extrusão do cálcio para o meio extracelular

A extrusão ou efluxo de cálcio ocorre contra um gradiente eletroquímico considerável, sendo que duas proteínas transportadoras desse íon já foram descritas nas membranas basolaterais das células reabsortivas: a PMCA (***P****lasma* ***M****embrane calmodulin-dependent* ***C****alcium* ***A****TPase*) e o trocador de sódio e cálcio NCX1 (Na^+/Ca^{2+} *exchanger*) (Bindels et al, 1991; van Baal et al, 1996; Hoenderop et al, 2005).

3.3.1. Extrusão de cálcio pela ATPase de cálcio localizada na membrana plasmática (PMCA)

As proteínas PMCA fazem parte da superfamília das P-ATPases, as quais são caracterizadas por formarem intermediários fosforilados, da subfamília IIB, sendo que até o momento foram descritas quatro isoformas, as PMCA 1 a 4. Em mamíferos eutérios, a PMCA é um produto de expressão de quatro genes distintos e é organizada na própria membrana plasmática. A estrutura molecular das PMCAs, de maneira geral, apresenta dez domínios transmembrana e quatro domínios citosólicos, sendo o primeiro a cauda N-terminal, o segundo o domínio que apresenta a região que se liga a fosfolipídeos acídicos, o terceiro o que contém os sítios catalíticos (sítios de fosforilação e, provavelmente, de ligação a íons cálcio) e ligação ao ATP e o quarto domínio, o C-terminal, que apresenta domínios de ligação à calmodulina e é responsável pela regulação da atividade da bomba de cálcio (Fig. 6) (Carafoli & Brini, 2000).

A conformação inativa das PMCAs ocorre quando os níveis de calmodulina e cálcio na célula são baixos, situação na qual o domínio C-terminal se liga às outras unidades citoplasmáticas principais e inibe o transporte de cálcio pela membrana. Dessa forma, a ativação dessas bombas requer a ligação de Ca^{2+}-calmodulina no domínio C-terminal e envolve uma alteração conformacional na proteína que culmina na interrupção da interação inibitória dessa unidade e, consequentemente, na exposição dos sítios catalíticos principais (Strehler et al, 2007). O funcionamento da ATPase de cálcio é cíclico e ocorre em etapas distintas. Antes de detalhar o processo de transporte desse íon pela membrana, é importante ressaltar que a PMCA é encontrada em duas formas: uma com sítio de ligação com alta afinidade ao cálcio voltado para o citoplasma e outra com sítio de ligação com baixa afinidade ao cálcio voltado para o meio extracelular, sendo que a proteína como um todo transita entre as duas formas. No início do ciclo,

observa-se a ligação do ATP ao seu respectivo domínio de ligação e, em seguida, a interação da PMCA com cálcio através do domínio de alta afinidade pelo íon. Após essa etapa inicial, o ATP é clivado em ADP e resulta na fosforilação de resíduos de aminoácidos específicos do sítio catalítico. Essa fosforilação resulta tanto em uma alteração conformacional na proteína, que passa a expor o sítio catalítico no lado oposto da membrana (meio extracelular), quanto na redução da afinidade de ligação ao cálcio. Dessa forma, o íon rapidamente se difunde para o meio extracelular. Finalmente, o sítio catalítico é desfosforilado e a proteína readquire sua conformação original (Carafoli & Brini, 2000).

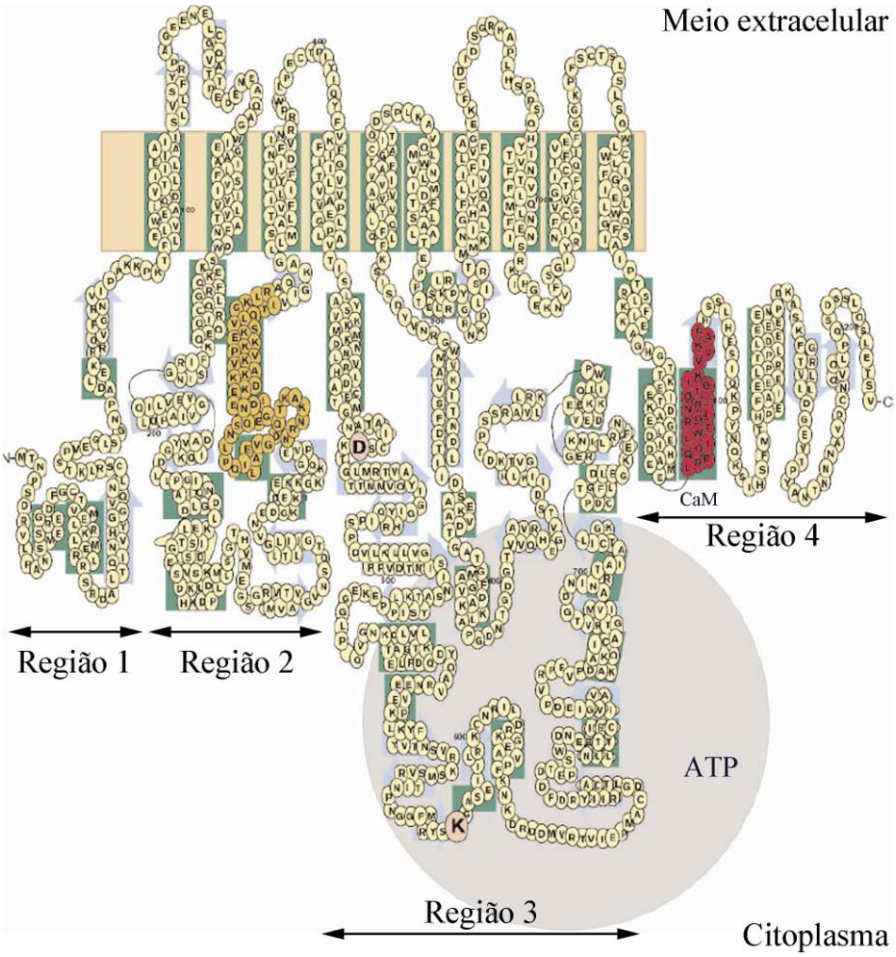

Figura 6: Estrutura molecular da PMCA. A proteína é formada por 10 domínios transmembrana e 4 regiões intracitoplasmáticas principais (evidenciadas na figura). Os resíduos de aminoácidos em laranja correspondem à região de ligação a fosfolipídeos acídicos, enquanto os resíduos corados em vermelho correspondem à região de interação com a calmodulina. Na região 3, a representação de uma molécula de ATP (em cinza) pode ser observada. Baseado em Carafoli & Brini (2000).

3.3.2. Extrusão de cálcio pelo trocador Sódio/Cálcio (NCX)

A família de proteínas NCX faz parte da superfamília de proteínas transportadoras conhecidas como CaCA (***Ca**lcium **C**ation **A**ntiporter*), cuja principal função é promover o controle do fluxo de cálcio através da membrana plasmática e/ou entre compartimentos intracelulares (Lytton, 2007). Devido ao fato da concentração de cálcio intracelular ser muito menor do que no meio extracelular – em torno de 1000 vezes – acredita-se que a principal função das proteínas NCX seja a extrusão do cálcio para o meio extracelular (Lytton, 2007, Kimura et al, 2009).

São descritas três isoformas de proteínas NCX (NCX 1 a 3). Uma característica comum a todas elas é sua estrutura molecular que, de maneira geral, apresenta nove domínios transmembrana (TM 1-9) e uma longa alça citoplasmática (Fig. 7A), localizada entre os domínios TM5 e TM6. Dessa forma, a molécula de NCX é dividida em duas subregiões: a região α1, que compreende os domínios TM 1-5 e a região α2, composta pelos domínios TM 6-9. Estudos estruturais revelaram que as duas regiões α são orientadas em direções opostas em relação à membrana, sugerindo que elas formam parte do vestíbulo que atua na ligação e transporte dos íons sódio e cálcio (Lytton, 2007, Kimura et al, 2009). Por outro lado, é na região citoplasmática da proteína que se encontram sequências de aminoácidos responsáveis pela ligação de moduladores/inibidores da função das proteínas NCX (conhecidas como sequências XIP) e um domínio formado por duas repetições de β-folhas, as quais também atuam na mediação da ligação de íons cálcio (CB1 e CB2). Finalmente, as proteínas são flanqueadas por uma região N-terminal extracelular, que contém um peptídeo sinal (clivado durante a tradução da proteína ou em modificações pós-traducionais), e uma região C-terminal, voltada para o meio intracelular (Fig. 7A) (Lytton, 2007).

A troca de íons sódio e cálcio é mediada por uma sequência de etapas envolvendo ligações iônicas nos sítios específicos, alterações conformacionais na estrutura protéica e o desligamento dos íons dos sítios de ligação (Fig. 7B). Durante essas etapas, são transportados três íons sódio para cada íon cálcio (estequiometria de 3:1). É importante ressaltar que a função transportadora das proteínas NCX é completamente reversível, ou seja, o transporte de cálcio pode ocorrer tanto para o meio extracelular, direção mais frequente, quanto para o meio intracelular, em algumas condições especiais. A direção dos movimentos iônicos depende exclusivamente do

gradiente eletroquímico e do número de íons que são ligados e transportados (Lytton, 2007).

Dentre as isoformas de NCX, o subtipo NCX1 é o que apresenta maior amplitude de distribuição, tendo sido descrito em vários órgãos como coração, cérebro, rins, enquanto NCX2 é expressa no cérebro e NCX3 na musculatura esquelética (Lytton, 2007, Kimura et al, 2009). Dessa forma, o presente trabalho focou na investigação da expressão de NCX1.

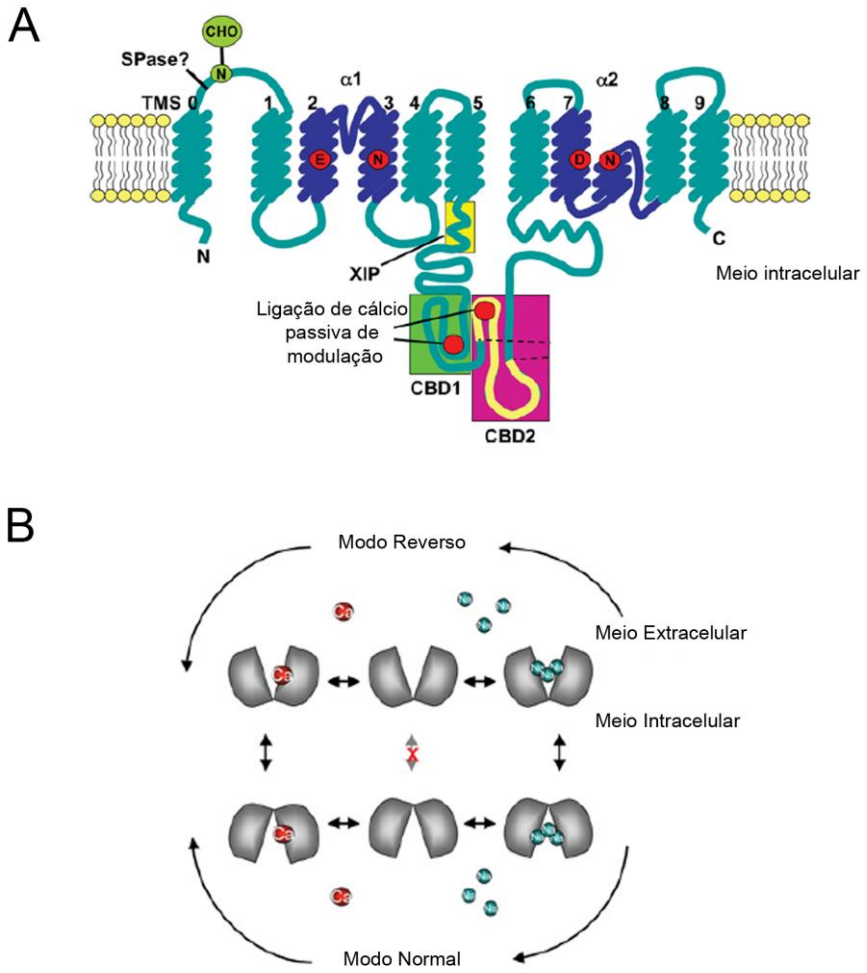

Figura 7: Estrutura molecular da NCX1. **(A)** A proteína é formada por 9 domínios transmembrana (TM 1-9) e 1 região intracitoplasmática. TM0 representa a sequência sinal da proteína e SPase o possível sítio de clivagem do peptídeo sinal. CBD1 e CBD2 representam os sítios de ligação de cálcio na região intracitoplasmática. **(B)** Esquema evidenciando a dinâmica da proteína NCX1 e o transporte de íons sódio e cálcio através da membrana plasmática. Note que a direção do transporte de íons é completamente reversível. Baseado em Lytton (2007).

3.4. Regulação hormonal do transporte transepitelial de cálcio

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 andrógenos e estrógenos (Prince, 1994; Mundy & Guise, 1999; Hoenderop et al, 2005). Como o presente estudo focará nos sistemas responsivos para vitamina D3, andrógenos e estrógenos, apenas o envolvimento desses hormônios na manutenção da homeostase de cálcio será abordado.

A vitamina D3 é descrita como sendo 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 sequência 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 e TRPV6, além de CaBP-D28K (envolvidas na entrada e difusão do cálcio na célula, respectivamente) são 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 (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).

Os efeitos de andrógenos no transporte transepitelial 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 tempo tenha sugerido a sua participação na homeostase desse íon. Recentemente, foi demonstrado que as concentrações de testosterona são inversamente proporcionais à reabsorção de cálcio, desde que a redução nos níveis desse hormônio resulta em maior expressão de TRPV5, CaBP-D28K e PMCA, enquanto efeitos exatamente opostos na expressão dessas proteínas são observados na presença de altos níveis de testosterona (Hsu et al, 2010).

Além da vitamina D3 e andrógenos, a homeostase de cálcio também é influenciada por estrógenos. Essa associação vem sendo demonstrada pelo importante papel desempenhado pelos 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 receptores de estrógenos e calcificação em condições patológicas, como a aterosclerose (Christian et al, 2006). Com relação ao transporte transepitelial de cálcio, é sabido que o hormônio 17β-estradiol induz a expressão das proteínas TRPV5, TRPV6 e CaBP-D28K, bem como aumenta a síntese e atividade de PMCA (van Abel et al, 2002; Dick et al, 2003; Oz et al, 2007). Em aves, no entanto, são poucas as informações concernentes às ações de estrógenos no transporte de cálcio através do epitélio. Em galinhas, os níveis de RNAm de canais de cálcio epiteliais TRPV5/6, calbindina-D2K e PMCA encontram-se aumentados durante a fase de atividade sexual, sendo esses efeitos mimetizados pela administração exógena de estrógenos (Bar, 2009). No entanto, dados acerca da distribuição das proteínas transportadoras de cálcio no sistema genital masculino das aves, sobretudo na região epididimária, bem como seu envolvimento na litíase epididimária, ainda são inexistentes.

4. DISTRIBUIÇÃO E IMPORTÂNCIA DE RECEPTORES DE ESTRÓGENOS ERα E ERβ NO SISTEMA GENITAL MASCULINO

As ações biológicas dos estrógenos são mediadas pelos receptores de estrógenos ERα e ERβ e são importantes para a manutenção de diversas funções biológicas, incluindo-se a reprodução masculina. Em mamíferos eutérios, já foi demonstrado que o sistema responsivo a estrógenos é essencial para a manutenção da fertilidade masculina, uma vez que a inativação de ERα e/ou ERβ, bem como da enzima aromatase (responsável pela conversão de testosterona em estradiol), resulta em machos inférteis (Hess et al, 1997a; Robertson et al, 1999; Dupont et al, 2000; Oliveira et al, 2001; Robertson et al 2001; Antal et al, 2008). A ocorrência de receptores de estrógenos já foi descrita no sistema genital masculino de diversas espécies, incluindo-se primatas humanos e não-humanos, roedores (camundongo e rato), animais domésticos (cão, gato, suíno, cabra e ovelha) bem como em espécies silvestres, como morcegos, cervo e leão marinho da Califórnia (Goyal et al, 1997; Hess et al, 1997b; Pelletier et al 2000; Sounders et al, 2001; Nie et al, 2002; Zhou et al, 2002; Ramesh et al 2007; Schon & Blottner, 2008; Colegrove et al, 2009; Oliveira et al, 2009). Em espécies de vertebrados não-mamíferos, receptores de estrógenos foram descritos no sistema genital masculino de peixes (Miura et al, 1999; Bouma et al, 2001; Vinas & Piferrer, 2008), anfíbios

(Arenas et al, 2001), lagartos (Chieffi & Varriale, 2004), tartarugas (Gist et al, 2007; Otsuka et al, 2008) e aves (Kwon et al, 1997; Nishizawa et al, 2002).

Dentre os órgãos do sistema genital masculino, chama a atenção o fato de que os dúctulos eferentes expressam níveis elevados tanto de ERα quanto de ERβ (Hess, 2003). Interessantemente, o padrão de expressão de ambos os receptores apresenta diferenças consideráveis. Nas vias extratesticulares, ERα é encontrado predominantemente nos dúctulos eferentes, enquanto a ocorrência de ERβ é mais ampla (Hess et al, 1997b; Nie et al, 2002; Zhou et al, 2002). Acredita-se que os estrógenos e seus receptores estejam associados ao controle da reabsorção do fluido testicular pelos dúctulos eferentes, uma vez que os estrógenos medeiam a regulação de diversas proteínas importantes que estão envolvidas com esse processo (Hess et al, 1997a; Lee et al, 2001; Zhou et al, 2001; Oliveira et al, 2002; Oliveira et al, 2005; Picciarelli-Lima et al, 2006).

Apesar das importantes funções dos estrógenos no sistema genital masculino de mamíferos, sobretudo nos dúctulos eferentes, há escassez de informações acerca do sistema responsivo a estrógenos na reprodução masculina de aves. Um possível papel desempenhado por estrógenos nas aves foi primeiramente proposto quando a expressão da enzima P450 aromatase foi descrita nas células germinativas dos testículos e também nos espermatozóides encontrados no lúmen das vias extratesticulares (Kwon et al, 1995). Posteriormente, foi demonstrado que a expressão testicular de ERα é alterada em galos imaturos, sexualmente maduros e senescentes, sendo mais expresso nos animais sexualmente ativos e, dessa forma, corroborando a idéia de que receptores de estrógenos podem ser importantes na regulação das funções testiculares em galos (Gonzalez-Moran et al, 2008). Com relação às vias extratesticulares das aves, as quais são alvo do presente estudo, existe apenas um estudo acerca da expressão de receptores de estrógenos. Nesse trabalho, foi demonstrado que os dúctulos eferentes apresentam altos níveis de expressão de receptores de estrógenos, semelhante aos mamíferos eutérios, mas os autores não discriminaram as duas formas de receptores, a saber, ERα e ERβ (Kwon et al, 1997). Sendo assim, é essencial que se faça uma nova abordagem em relação à distribuição de ambos os receptores de estrógenos (ERα e ERβ) na região epididimária de galos para investigar se nessa espécie há também diferenças no padrão de expressão desses receptores que poderiam refletir diferenças funcionais dos diferentes constituintes da região epididimária. Além disso, essa é uma informação também é crucial para o entendimento da litíase epididimária, tendo em vista o importante papel dos estrógenos na reabsorção de cálcio.


II
Justificativa e Objetivos

II- JUSTIFICATIVA E OBJETIVOS

1. JUSTIFICATIVA

Pouco se sabe sobre a litíase epididimária dos galos e todos os dados existentes até o momento mostram que ela tem marcantes efeitos adversos na reprodução desses animais (Janssen et al, 2000; Mahecha et al, 2002). De fato, a fertilidade de animais afetados é reduzida em 40% a 60% em relação aos animais não-afetados (Janssen et al, 2000).

Atualmente, o Brasil ocupa a primeira posição na exportação mundial de frango, sendo essa uma atividade em ampla expansão no país. De acordo com a ABEF (Associação Brasileira de Exportadores de Frango), as exportações de frango atingiram recorde desde o ano de 2007, gerando uma receita de US$ 3,53 bilhões (ABEF - www.abef.com.br), indicando que essa indústria se encontra em franca expansão no país. Dessa forma, a litíase epididimária, por afetar precocemente a fertilidade dos animais, representa um fator de impacto negativo na indústria avícola brasileira. Sendo assim, estudos que forneçam conhecimentos sobre essa anomalia tornam-se plenamente justificáveis, ainda mais levando-se em consideração a escassez de estudos relacionados à mesma.

Até o momento, praticamente todos os estudos realizados sobre a litíase epididimária focaram nas alterações morfológicas decorrentes da mesma. Só recentemente foram abordados possíveis mecanismos moleculares envolvidos na formação dos cálculos epididimários. Esses trabalhos, por nós conduzidos, revelaram alterações na expressão de VDR e AR na região epididimária de galos afetados (Oliveira et al, 2008), proteínas essas diretamente envolvidas na modulação hormonal do transporte transepitelial de íons cálcio. Dessa forma, considerando as alterações nos sistemas responsivos de vitamina D e andrógenos e devido ao fato do cálcio ser o componente principal dos cálculos epididimários formados no lúmen dos dúctulos eferentes (Janssen et al, 2000; Mahecha et al, 2002; Clulow & Jones, 2004), é possível que a litíase epididimária resulte de uma alteração da homeostase local de cálcio (Oliveira et al., 2008). Como consequência dessas alterações, a concentração de cálcio no lúmen dos dúctulos eferentes de animais afetados pela litíase seria maior e atuaria como centros de nucleação para íons cálcio (Oliveira et al, 2008).

Porém, os estrógenos e seus receptores têm surgido também como fortes candidatos a moduladores do transporte de cálcio em diversos órgãos. Sendo assim, investigar possíveis alterações nos níveis de receptores de estrógenos ERα e ERβ é uma necessidade iminente, que poderá adicionar informações essenciais que auxiliem a melhor compreensão da natureza da litíase epididimária.

Outra perspectiva importante surgida dos nossos estudos sobre a litíase epididimária é a investigação da expressão de proteínas envolvidas no transporte transepitelial de cálcio, como a TRPV6, calbindina-D28K, NCX1 e PMCA, cujas expressões são moduladas por VDR, AR, ERα, ERβ e seus respectivos ligantes. É surpreendente a escassez de informações disponíveis sobre a morfofisiologia das vias genitais masculinas de aves, e mais especificamente sobre a homeostase de fluido e eletrólitos na região epididimária. Portanto, ao fornecer dados inéditos sobre a expressão e distribuição celular e subcelular de proteínas chave na dinâmica do transporte transepitelial de cálcio, nossa investigação poderá contribuir grandemente para os conhecimentos básicos da biologia da reprodução das aves.

2. OBJETIVOS

2.1. Objetivo geral

O presente estudo tem como objetivo determinar se a desregulação hormonal e/ou da homeostase de cálcio na região epididimária de galos domésticos adultos participam na a fisiopatologia da litíase epididimária.

2.2. Objetivos específicos

O presente projeto será desenvolvido em duas etapas:

1) Analisar o possível envolvimento da desregulação hormonal como indutor da formação dos cálculos epididimários. Nessa etapa, estudaremos os níveis teciduais e plasmáticos de testosterona, estradiol e vitamina D3, além da ocorrência e distribuição subcelular de receptores de estrógenos ERα e ERβ e suas possíveis alterações na região epididimária de galos afetados, quando comparados com espécimes não-afetados.

2) Analisar possíveis alterações na homeostase de cálcio, investigando a expressão de proteínas envolvidas na dinâmica do transporte transepitelial de cálcio, tais como

TRPV6, CaBP-D28K, NCX1 e PMCA, bem como a concentração tecidual de cálcio em animais afetados e não-afetados pela litíase epididimária.

Resultados

ARTIGO 1

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Occurrence and cellular distribution of estrogen receptors ERα and ERβ in the testis and epididymal region of roosters

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ABSTRACT

Estrogen signaling is required for the maintenance of male reproductive function and is mediated by the estrogen receptors ERα and ERβ. These receptors are widely distributed in mammalian reproductive tissues, but information is limited in non-mammalian species including birds. The aim of this study was to investigate the occurrence and cellular distribution of ERα and ERβ in the testis and epididymal region of roosters. The results showed for the first time that ERβ was the predominant receptor detected in the testis, being expressed in the somatic and some germ cells. Within the epididymal region, ERβ was strongly expressed in all segments, whereas the most intense reaction for ERα was found in the distal efferent ductules. The differential expression of ERα and ERβ within the rooster testis and epididymal region suggests that these organs may be a target for different actions of estrogen.

1. Introduction

Estrogen signaling mediated by the estrogen receptors ERα and ERβ is important for the maintenance and regulation of several biological functions, including male reproduction. In mammals, it has been demonstrated that the estrogen responsive system is crucial for the maintenance of male fertility since the inactivation of ERα and/or ERβ, as well as the enzyme P450 aromatase, results in infertile males [7,27,36,52,59,60]. Estrogen receptor localization have been described in the male genital system of several mammalian species, including primates (humans and non-humans), rodents (mice and rats), domestic animals (dogs, cats, porcine, sheep and goat) as well as wild species, such as bats, roe deer and californian sea lion [21,33,37,47,54,56,58,62,63,72]. In non-mammalian vertebrates, ERs have been found in the reproductive systems of fish [12,45,67], amphibians [8], lizards [18], turtles [31,55], and birds [41,49].

Although roosters represent one of the avian species most extensively used for commercial and experimental purposes, the occurrence and functional role of the estrogen responsive system in their genital organs are still poorly determined. A possible role for estrogen receptor signaling in this species was first proposed when high levels of the enzyme P450 aromatase were found in the germinal cells in the testis, as well as in the sperm traversing the male tract [42]. More recently, it was shown that testicular ERα levels change in immature, mature and aged roosters, being more highly expressed in sexually active animals, thus confirming that ER may be important for modulating rooster testicular functions [32]. There is only one report on ER expression in avian male reproductive tracts; however, the authors did not differentiate ERα and ERβ [41]. To address this issue is highly important, since, although ERα and ERβ shares structural similarity, they also present important functional differences [22,34,40].

A remarkable characteristic of the rooster male tract is the prominence of the efferent ductules, which compose up to 50% of the epididymal region, contrasting with a short and non-differentiated epididymal duct [3,50]. The avian efferent ductules are responsible for reabsorption of more than 90% of the fluid coming from the testis, with a rate of reabsorption that is greater than in mammals [20]. Regulation of the fluid reabsorption is a known function attributed to estrogens in the mammalian efferent ductules [35,36]. However, the molecular mechanism regulating this important function is still not determined for birds. It is consensual in the literature that, among the male genital tract of mammals, the efferent ductules are the most sensitive to estrogens, presenting higher labeling for ERα [29,37,47,48,62]. Higher ERα expression is also observed in turtle efferent ductules [31,55], but it is still uncertain for roosters. Further investigation detailing the distribution of both estrogen receptors in the rooster epididymal region would be helpful to clarify whether or not there is different sensitivity to estrogens that would predict functional differences within the reproductive tracts. Therefore, to contribute with information about occurrence and precise cellular distribution of ERα and ERβ in the testis and each segment composing the epididymal region of roosters is the aim of the present study.

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2. Materials and methods

2.1. Animals

The study was performed on the epididymal region of adult roosters (*Gallus gallus*) ($n = 10$) in reproductive activity obtained from commercial sources and housed at the Federal University of Minas Gerais facilities. Since it is difficult to determine the precise age of the animals, parameters as the body weight as well as absolute and relative testis weights were taken to emphasize the homogeneity of the group (Table 1). The animals received water and commercial chow (Socil Guyomarc'h, Brazil) *ad libitum*. Principles of research involving animals followed those published by the local ethical committee (http://www.ufmg.br/bioetica/coep).

2.2. Tissue preparation

Roosters were weighed, anesthetized (i.p. sodium pentobarbital 50 mg/kg of body weight) and perfused intracardially with saline solution followed by 10% neutral buffered formalin (NBF) for immunohistochemistry studies. For Western Blotting analysis, animals were perfused with saline solution only, and after the dissection of the testis and epididymal region, fragments of tissues were frozen in liquid nitrogen.

2.3. Western blotting

Western blotting analyses were performed in order to investigate the expression of ERα and ERβ in the testis and epididymal region of roosters, as well as to test the specificity of the antibodies used. Fragments of tissue frozen in liquid nitrogen ($n = 6$) were macerated in dry ice and submitted to total protein extraction. After this step, the samples were subjected to electrophoresis using 10% SDS–PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) and transferred to nitrocellulose membranes. The membranes were blocked with 10% normal goat serum (NGS) and incubated with rabbit anti-ERα (clone 60C, Millipore, USA) or mouse anti-ERβ (NCL-ERbeta, Novocastra, USA) antibodies diluted at 1:300 or 1:150, respectively. After several washes in phosphate buffer saline (PBS) – tween 0.05%, the membranes were incubated with the biotinilated goat anti-rabbit (for ERα) or goat anti-mouse (for ERβ) secondary antibodies (Dako, USA) diluted at 1:1000. The membranes were then incubated with the avidin–biotin complex (Vectastain Standard ABC kit – Vector Laboratories, USA) for 30 min and the immunolabeling was visualized with a solution of 0.1% 3,3′diaminobenzidine in PBS containing 0.05% chloronaphtol, 16.6% methanol and 0.04% H_2O_2. Both antibodies used in this technique were raised against a synthetic peptide of human ERα or ERβ, which share, respectively, 98% and 96% of similarity with chicken sequences according to BLASTp analysis [5,6].

2.4. Immunohistochemistry

Aiming to determine the cellular and regional distribution of ERα and ERβ proteins, fragments of the testis and epididymal regions fixed in NBF ($n = 4$) were embedded in paraffin, sectioned (5 μm) and used for immunohistochemistry. Sections were blocked for endogenous peroxidase activity with 0.6% H_2O_2 in methanol and subjected to a standard protocol for antigen retrieval in citrate buffer and microwave. Then, non-specific activity was blocked by the incubation of slides in 10% NGS prior to the incubation with the anti-ERα antibody (clone 60C, Millipore, USA) diluted at 1:50 for 12 h or with the anti-ERβ antibody (NCL-ERbeta, Novocastra, USA) diluted at 1:25 for 48 h. For negative control, sections were incubated with PBS instead of the primary antibody. Following the incubation of sections with the biotinilated goat anti-rabbit (for ERα) or goat anti-mouse (for ERβ) secondary antibodies (Dako, USA) diluted at 1:50, sections were exposed to the avidin–biotin complex (Vectastain ABC kit – Vector Laboratories, USA). After this step, the immunoreactions were visualized using diaminobenzidine containing 0.01% H_2O_2 in 0.05 M Tris–HCl buffer, pH 7.6. Sections were slightly counterstained with Meyer's hematoxilin.

Table 1
Body and testis/epididymal region weights of roosters.

Body weight (kg)	Testis/epididymal region absolute weight (g)		Testicular/epididymal region relative weight (g/100 g BW)	
	Fixed[a]	Frozen[b]	Fixed[a]	Frozen[b]
2.8 ± 0.2	25 ± 4	24 ± 3	0.9 ± 0.2	0.9 ± 0.1

Data are presented as mean ± SEM.
[a] Tissues fixed in NBF for immunohistochemistry ($n = 4$).
[b] Tissues frozen in liquid nitrogen for Western blotting ($n = 6$). BW = Body weight.

2.5. Morphometry

The differences in ERα and ERβ immunostaining intensity among the segments composing the epididymal region were quantified by using computer-assisted image analysis [26,66]. Digital pictures of 5 random sections of each segment analyzed were taken, converted to grayscale mode and then inverted. The images were exported to Image-Tool software (Version 3.00; University of Texas Health Sciences Center, USA), for quantitative analysis. The stained nucleus were traced and 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 and subtracted from values detected in the labeled regions. ERα and ERβ staining were quantified in 50 cells of each segment studied per animal ($n = 4$).

2.6. Statistical analysis

Differences in the immunostaining intensity of ERα and ERβ in the rooster epididymal region were assessed by Student's *t*-test (for ERα) or analysis of multiple variance (ANOVA) followed by the Newman-Keuls *post hoc* test (for ERβ). Differences were considered statistically significant when $P \leqslant 0.05$.

3. Results

3.1. Western blotting

The ERα and ERβ antibodies recognized a single protein band of about 67 kDa and 54 kDa, respectively, in the total protein extracts of both testis and epididymal region (Fig. 1). These molecular weights are in agreement with those previously described for both proteins in different species [31,32,47,54,72]. However, since

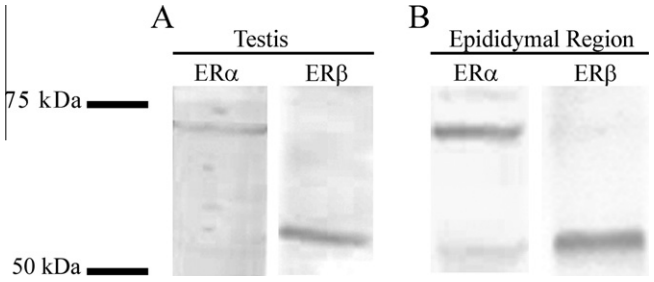

Fig. 1. Western blotting analysis of ERα and ERβ expression in (A) testis and (B) epdidymal region of roosters; $n = 6$.

Table 2
Immunostaining intensity for estrogen receptors ERα and ERβ in the ducts composing the epididymal region of roosters.

	ERα	ERβ
Testis		
Interstitial compartment		
Leydig cells	–	+++
Endothelium	–	+++
Blood vessels smooth muscle cells	–	+++
Tubular compartment		
Sertoli cells	–	++/+
Spermatogonia	–	++
Spermatocytes	–	+/–
Peritubular myoid cells	–	+++/++
Epididymal region		
Rete testis		
Epithelial cells	–	+++
Proximal efferent ductules		
Epithelial non-ciliated cells	+/–	+++
Epithelial ciliated cells	–	++
Distal efferent ductules		
Epithelial non-ciliated cells	+++	+++
Epithelial ciliated cells	–	++
Epididymal duct		
Epithelial principal cells	++	+++
Epithelial basal cells	++	+++
Connective tissue cells	–	+++

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

rooster ERα and ERβ proteins are not commercially available, it is difficult to fully determine the antibodies specificity by direct means.

3.2. Immunoreactivity for ERα

ERα protein was found on the nuclei of epithelial cells in the proximal efferent ductules, distal efferent ductules and epididymal duct; however, with remarkable differences in the intensity and pattern of the immunoreaction (Table 2 and Fig. 2A–D). In the efferent ductules, only the non-ciliated cells lining the epithelia were positive for ERα, whereas the ciliated cells were not reactive for this protein. Regarding the proximal efferent ductules, a weak and intermittent staining for the receptor was observed, as only a few non-ciliated cells, scattered in the epithelia, were positive. On the other hand, the frequency of positive non-ciliated cells in the distal efferent ductules was higher and the cells presented the strongest staining for ERα among all segments analyzed (Fig. 3A). Finally, the principal and basal cells of the epididymal duct showed a moderate immunostaining for ERα protein. The testis, rete testis epithelial cells as well as connective tissue cells in the epididymal region were not immunoreactive for ERα (Table 2 and Fig. 2). Since ERα reaction was negative in the rete testis and intermittent in the proximal efferent ductules, these segments were not included in the morphometrical analysis.

3.3. Immunoreactivity for ERβ

ERβ immunostaining was found in the nuclei of Leydig cells, as well as endothelial and smooth muscle cells of blood vessels, in the testis interstitium (Table 2 and Fig. 4A and B). Within the tubular compartment, ERβ was found in Sertoli cells, peritubular myoid cells and some germ cells, including spermatogonias and early spermatocytes. The staining intensity in the spermatogonia did not differ among different sections of the seminiferous tubules; however, the Sertoli cells and spermatocytes immunoreactivity varied considerably. Round and elongated spermatids were negative for ERβ.

Labeling for ERβ protein was also observed in the nuclei of cells lining the rete testis, proximal and distal efferent ductules as well as in the epididymal duct with no detectable regional differences between segments (Table 2, Fig. 3B, Fig. 4C–F). In the rete testis, ERβ staining was observed in the nuclei of cuboidal epithelial cells and in luminal cells morphologically similar to macrophages. In the efferent ductules, positivity was observed in the non-ciliated and

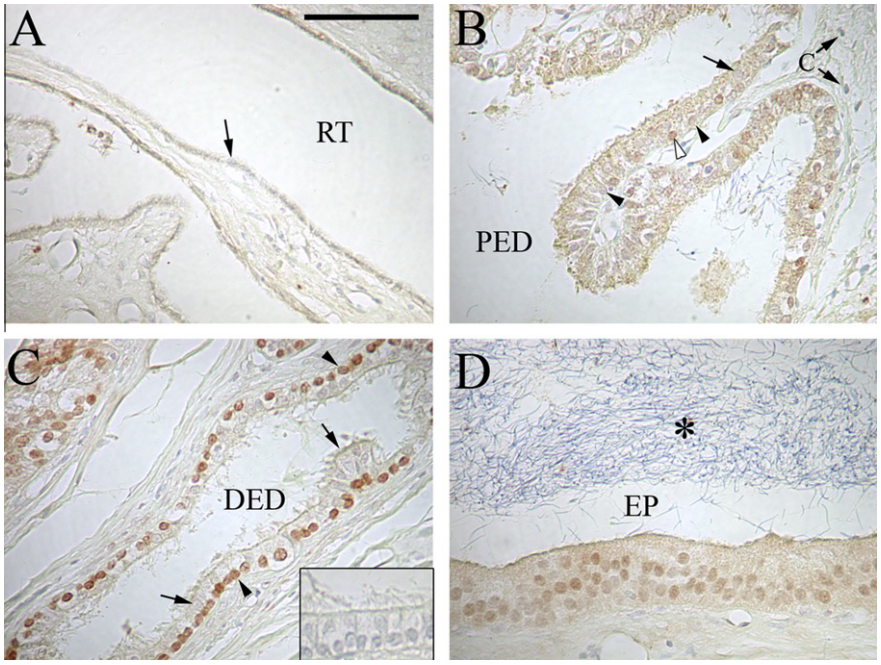

Fig. 2. Expression of ERα in the epididymal region. (A) Rete testis (RT) epithelial cells were negative for ERα immunostaining (arrow). (B) Proximal efferent ductules (PED) epithelia showed non-ciliated cells negative (black arrowheads) or weakly positive for ERα (white arrowhead), whereas ciliated cells (arrow) and connective tissue cells (C) were negative. (C) Distal efferent ductules (DED) non-ciliated cells (arrowheads) showed the strongest ERα staining. Ciliated cells were also negative for this protein (arrow). (D) Epididymal duct (EP) presented moderate ERα positivity in the principal and basal cells. ∗ = spermatozoa. Insert in C = negative control. Bar = 50 μm.

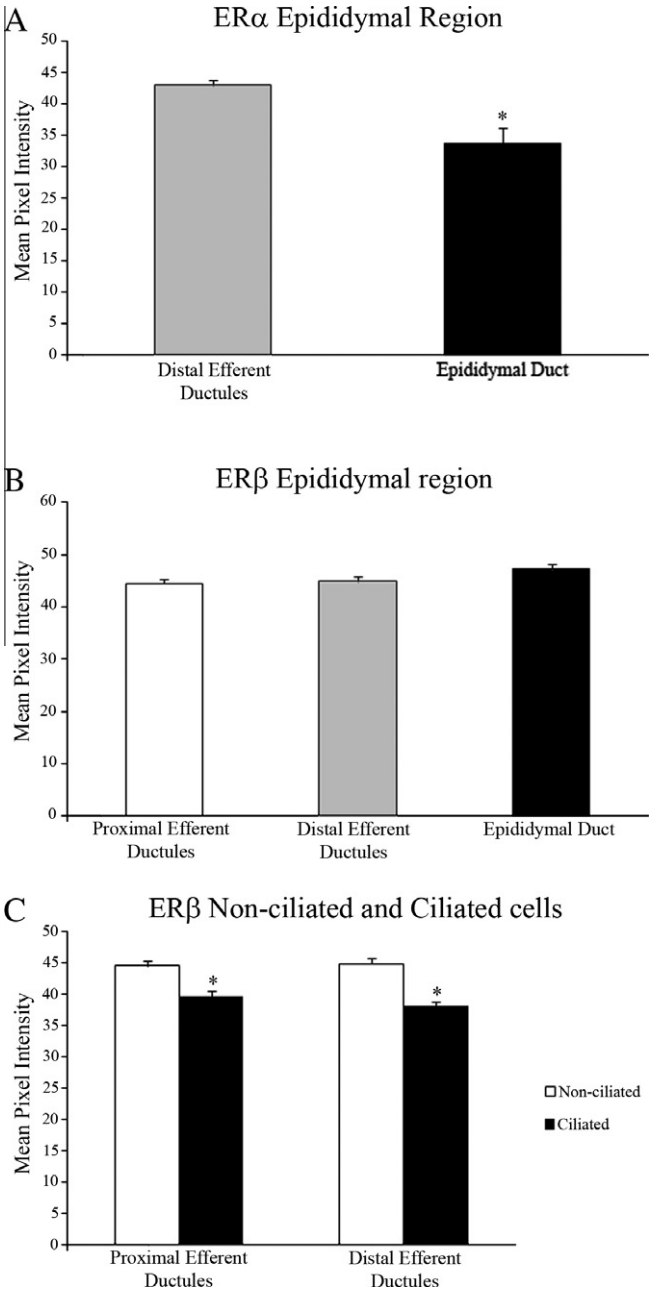

Fig. 3. Quantification of the immunoreactions for ERα and ERβ within the epididymal region. (A) ERα analysis in the segments that showed positive immunostaining. (B) ERβ quantification in the segments positive for this receptor. (C) ERβ comparative analysis between non-ciliated and ciliated cells of the distal efferent ductules. $* = P \leqslant 0.05$. $n = 4$.

ciliated cells in both proximal and distal segments. When comparing the cell types lining the epithelia, it was evidenced that ERβ expression was higher in the non-ciliated cells than in the ciliated cells in both segments (Fig. 3C). Regarding the epidydimal duct, ERβ immunostaining was found on the nuclei of basal and principal cells with no evident differences in the staining intensity between both cell types. Some cells of the connective tissue were also positive for this receptor.

4. Discussion

The present study showed for the first time that estrogen receptor ERα and ERβ present differential cellular and regional distribution in the testis and epididymal region of roosters. ERβ was the predominant receptor detected in the testis, being expressed in the somatic and some germ cells. In the epididymal region, the most intense reaction for ERα was found in the distal efferent ductules, whereas ERβ was strongly expressed in all segments. Together, these results indicate that differential estrogen signaling via ERs may occur in testis and male tract of roosters.

The occurrence of ERα and ERβ in the rooster extratesticular excurrent ducts with highest ERα immunostaining observed in the efferent ductules is in agreement with other findings in mammalian and non-mammalian species [31,37,47,48,55,63]. It has been proven that estrogens rather than androgens control the bulk of testicular fluid reabsorption which occurs in the mammal efferent ductules, as estrogens mediate the regulation of several key proteins involved in this process [36,43,51,53,57,71]. Similar to mammals, the avian efferent ductules are responsible for the reabsorption of more than 90% of the testicular fluid [2,20,50], in a process involving Na^+/K^+ ATPase, carbonic anhydrase, Na^+/H^+ exchanger (NHE3) and aquaporins [9,70]. Most of these proteins have been shown to be modulated by estrogen in mammals [43,51,53,57,61,71]. Therefore, it is plausible to infer that the estrogen responsive system may also have a role in regulating avian efferent ductules reabsorption. Corroborating this point of view, there is evidence that estrogenic/antiestrogenic compounds affects the structure of the avian epididymal region, as well as testis, including formation of blisters filled with fluid or seminiferous tubules dilation and reduced semen formation [11,13,30,50,69]. These testicular alterations are suggestive of disruption of efferent ductule reabsortive function in roosters with consequent back up of fluid in the testis, similar to that found in mammals with disruption of estrogen action [36,52,53].

Higher ERα labeling was found in the distal efferent ductules whereas the proximal segment presented a slight and intermittent immunopositivity. Differential expression of ERα does not appear to be restricted to roosters, as higher immunoreactivity for this receptor in the distal efferent ductules has also been observed in turtles [55]. Differences in distribution of other important receptors, such as androgen and vitamin D3 receptors, has already been described between proximal and distal efferent ductules of roosters [25,26]. One possible explanation for these differences can be the distinct embryological origin of both segments, as the proximal efferent ductules develop from the capsule of mesonephric corpuscles, whereas the distal efferent ductules originate from the mesonephric tubules [14,15]. Therefore, despite the common name, these ductules present considerable morphological differences and thus may play different functional and physiological roles in avian reproduction as already suggested [1,2].

Principal and basal cells of the epididymal duct were positive for ERα and ERβ, even though the ERα labeling was just slight in both cell types. Indeed, the occurrence of ERα protein within the epididymal duct has been controversial, as it was reported in some species [4,31,35,47,55,72] but appears absent in others [33,35,47,48,56,63]. The functions of estrogen receptors in the epididymal duct are still a matter of debate, but there is evidence that ERα may play a role in the maintenance of normal luminal *milieu*, by regulating the fluid osmolality and acidification, which is essential for sperm maturation and function [38,39]. The avian epididymal region is also thought to play an important role in the composition of luminal fluid to provide sperm maturation, since sperm undergoes changes in the pattern of surface protein expression that are correlated with changes in fluid composition [28]. The changes in avian epididymal fluid include creation and maintenance of a hyper-osmotic environment [19], a function compatible with the expression of specific water and ion transporters [9,70], known to be regulated by estrogen. Therefore, a speculative role for estrogen in the avian epididymal duct may be related to regulation of

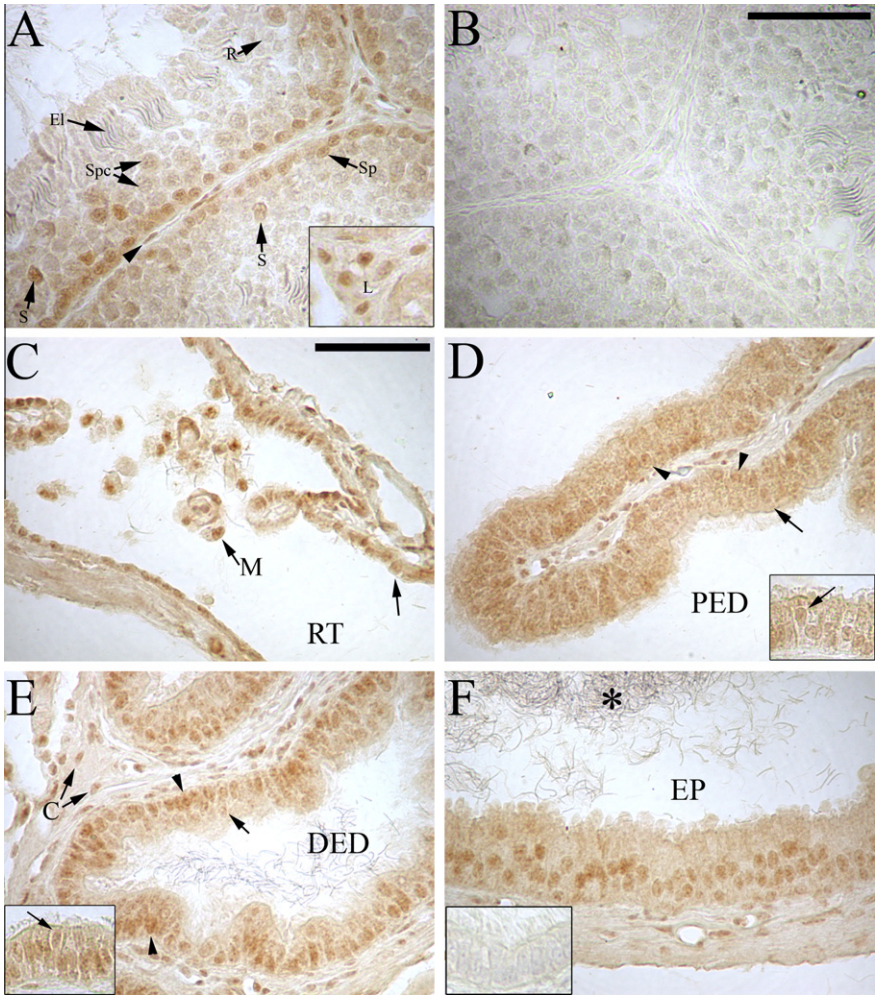

Fig. 4. Expression of ERβ in the testis (A, B) and epididymal region (C–F). (A) ERβ positivity was observed in Sertoli cells (S), spermatogonia (Sp), spermatocytes (Spc) and peritubular myoid cells (arrowhead) within the seminiferous tubules. In the testis interstitium, Leydig cells (L in the insert), as well as endothelial and smooth muscle cells of blood vessels were also positive. (B) Negative control of the immunostaining in the testis. (C) Rete testis epithelial cells (arrow) were positive for ERβ as well as luminal macrophages (M). (D, E) Proximal (D) and distal (E) efferent ductules had strong ERβ staining in the non-ciliated cells (arrowheads), whereas the ciliated cells (arrows) presented a moderate immunoreaction. Connective tissue cells (C) were also positive. Inserts in D and E highlight positivity in ciliated cells. (F) Basal and principal epithelial cells of the epididymal duct were labeled for ERβ. * = spermatozoa. RT = rete testis; PED = proximal efferent ductules; DED = distal efferent ductules; EP = epididymal duct. Insert in F = Negative control of the immunostaining in the epididymal region. Bar in B = A: 50 μm; Bar in C = D = E = F: 50 μm.

fluid/ion transporters involved in the maintenance of the proper luminal environment.

Within the rooster testis, ERβ was detected in most somatic cells and some germ cells, which is consistent with the findings for other species [8,10,16,18,47,54,62,65,72]. Contrasting with ERβ, no labeling above background was observed for ERα in the rooster testis (even after using several different antibodies, data not shown), even thought the protein was detected by Western blotting assays. This result may be explained by differences in assay sensitivity, as Western blotting is known to be more sensitive for detection of small amounts of proteins than immunohistochemistry. In addition, ERα mRNA has been reported in the testis of roosters [68], being consistent with the protein expression found in this study by Western blotting. In agreement with our results, weak signal in ERα immunohistochemistry or negativity at all has been described for other species, including goat, roe deer, bat, human and non-human primates [10,33,54,62,63]. Estrogen action has been associated with spermatogenic cell proliferation, development and survival in the testis of different vertebrate species [17,23,44,45,64]. These functions appear to be mediated by factors produced by Sertoli cells [46], by a direct action of estrogen on germ cells or both [24]. Considering the ubiquitous cell distribution in the testis, it is believed that ERβ is the estrogen receptor subtype directly involved in this testicular estrogen signaling.

In conclusion, ERα and ERβ have distinct pattern of expression in the testis and epididymal region of roosters, as ERα expression was more pronounced in the distal efferent ductules whereas ERβ was more widely distributed along the rooster male genital organs. These data confirm the assumption that all components of the epididymal region may be a target for estrogen action, but cellular- and regional-specific functions for estrogens acting through ERα and ERβ are predicted.

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REPRODUCTION

RESEARCH

Roosters affected by epididymal lithiasis present local alteration in vitamin D3, testosterone and estradiol levels as well as estrogen receptor 2 (β) expression

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Abstract

Epididymal lithiasis is a reproductive dysfunction of roosters that is associated with loss of fertility and is characterized by the formation of calcium stones in the lumen of the efferent ductules of the epididymal region. The efferent ductules of birds are responsible for the reabsorption of the fluid coming from the testis as well as luminal calcium. It has been hypothesized that the epididymal stone formation may be related to the impairment of local fluid or calcium homeostasis, which depends on hormones such as estradiol (E_2). Therefore, this study aimed to investigate possible alterations in the expression of ERα (ESR1) and ERβ (ESR2) in the epididymal region of roosters affected by epididymal lithiasis. The study was performed by immunohistochemistry and western blotting assays. In addition, the concentrations of E_2, vitamin D3, and testosterone, which are also key hormones in maintenance of calcium homeostasis, were determined in the plasma and epididymal region, by ELISA. It was observed that ESR2 expression is increased in all segments of the epididymal region of affected roosters, whereas ESR1 levels are not altered. Moreover, the hormone concentration profiles were changed, as in the epididymal region of roosters with lithiasis the E_2 levels were increased and vitamin D3 as well as testosterone concentrations were significantly decreased. These results suggest that a hormonal imbalance may be involved with the origin and progression of the epididymal lithiasis, possibly by affecting the local fluid or calcium homeostasis.

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Introduction

Epididymal lithiasis is a reproductive dysfunction characterized by the formation of luminal stones rich in calcium in the rooster's epididymal region (Janssen *et al*. 2000, Mahecha *et al*. 2002, Oliveira & Oliveira 2011). Animals affected by this anomaly present severe testicular and epididymal alterations. Testicular damage includes dilation of seminiferous tubules, sloughing of the seminiferous epithelium, and increase in Leydig cell frequency in the interstitial tissue (Janssen *et al*. 2000, Mahecha *et al*. 2002, Oliveira *et al*. 2008). The testicular alterations result in reduced fertility in affected animals (Janssen *et al*. 2000). Interestingly, the reduction in fertility was observed after natural and even artificial insemination with equivalent numbers of sperm obtained from the semen of affected animals (Janssen *et al*. 2000). This result suggested that adverse effects of epididymal lithiasis on fertility could be attributed to alterations in the production of sperm at the testicular level, but also possibly as a consequence of impaired maturation as they traverse the epididymal region. In birds, the epididymal region is composed of the rete testis, proximal and distal efferent ductules and epididymal duct (Aire 1979*a*, Aire & Soley 2000, Oliveira *et al*. 2007), which are also affected by the presence of the stones (Janssen *et al*. 2000, Mahecha *et al*. 2002, Oliveira *et al*. 2008).

The efferent ductules are the most affected segment of the rooster's genital tract in animals with epididymal lithiasis (Janssen *et al*. 2000, Mahecha *et al*. 2002). Besides the fact that the formation of the calcium stones is restricted to the efferent ductules, epithelial injury as well as the occurrence of frequent mononuclear cell infiltrates adjacent to affected ductules are common features in affected animals (Janssen *et al*. 2000, Mahecha *et al*. 2002, Boltz *et al*. 2004, 2006, Jackson *et al*. 2006, Oliveira *et al*. 2008). In birds, the efferent ductules compose up to 60% of the epididymal region (Aire 1979*b*, Oliveira *et al*. 2007). It has been shown that these ductules are responsible for testicular fluid and calcium reabsorption, an essential function involved in

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sperm concentration and maturation (Clulow & Jones 1988, 2004).

It is well known that the fluid reabsorptive function of the mammalian efferent ductules are under the influence of estrogen and its receptors ESR1 and ESR2, that are highly expressed within the epithelial cells of these ductules (Goyal *et al*. 1997, Hess *et al*. 1997, Nielsen *et al*. 2001, Zhou *et al*. 2001, Nie *et al*. 2002, Oliveira *et al*. 2002, 2005, Picciarelli-Lima *et al*. 2006, Joseph *et al*. 2011). In addition to the regulation of fluid transport, estrogens also participate in the maintenance of calcium homeostasis in several tissues, including in birds, by regulating the expression and activity of proteins involved in the calcium homeostasis (Hoenderop *et al*. 2005, de Matos 2008). In roosters, the relevance of estrogen action in the epididymal region has not been determined. Previous studies have shown that P450 aromatase, the enzyme responsible for estrogen production (Kwon *et al*. 1995), as well as estrogen receptor (ER), are present in the rooster epididymal region (Kwon *et al*. 1997). However, a differential expression of ESR1 and ESR2 has recently been described for this species (Oliveira *et al*. 2011). It was shown that ESR2 is widely expressed within the epididymal region, whereas ESR1 was mostly found in the distal efferent ductules, suggesting that, as in mammals, these ductules may be important targets for estrogen action in roosters (Oliveira *et al*. 2011).

It has been hypothesized that the epididymal stone formation may be related to impairment of fluid or calcium transepithelial transport in the efferent ductules (Oliveira *et al*. 2008, Oliveira & Oliveira 2011). Therefore, based on the dual role of estrogens and their receptors in the transepithelial transport of fluid and calcium, this study aimed to investigate the expression of ESR1 and ESR2 in the epididymal region of roosters affected by epididymal lithiasis as well as the concentrations of estrogens in the plasma and epididymal region. In addition, we also addressed the levels of vitamin D3 and testosterone, considering that previous studies have shown alterations in both vitamin D3 receptor (VDR) and androgen receptor in the epididymal region of roosters affected by lithiasis (Oliveira *et al*. 2008).

Results

Epididymal region

The epididymal region of roosters lies closely attached to the testis and is formed by the extratesticular rete testis, proximal, and distal efferent ductules as well as connecting and epididymal ducts (Fig. 1). In males affected by the epididymal lithiasis, stones were visible macro- and microscopically within the lumen of efferent ductules, especially in the proximal efferent ductules that also presented loss of epithelial folding (Fig. 1).

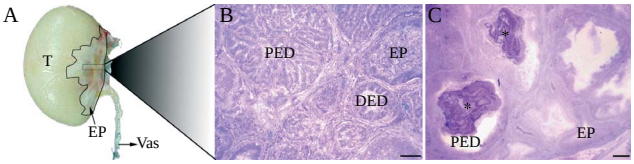

Figure 1 The epididymal region of roosters. (A) Macroscopical view of the testis and epididymal region (highlighted area). (B) The epididymal region of non-affected animals showing proximal efferent ductules with highly folded epithelium (PED), distal efferent ductules (DED), and epididymal duct (EP). (C) Epididymal region of roosters affected by epididymal lithiasis showing luminal stones (*) and loss of epithelial folding in proximal efferent ductules (PED). Epididymal duct (EP) shows no evident alterations. Bar in B and C = 100 μm. T, testis; EP, epididymal region; Vas, deferent duct.

The efferent ductules compose the majority of the volume (23%) of the ducts in the epididymal region of the roosters, followed by the rete testis (16%) and connecting ducts/epididymal duct (10%). A great proportion of the region is formed by connective tissue (51%). No alterations in the proportion of the epididymal region components were found between animals non-affected and affected males.

ESR1 and ESR2 detection

Western blotting assays for ESR1 and ESR2 detected specific positive protein bands of 67 and 54 kDa respectively (Fig. 2). These results are in agreement with previous published data for these receptors in birds (Gonzalez-Moran *et al*. 2008, Oliveira *et al*. 2011). A significant increase (26%) in ESR2 expression was observed in the epididymal region of roosters affected by epididymal lithiasis compared with non-affected animals (Fig. 2B). On the other hand, ESR1 expression was not statistically significant between the groups (Fig. 2A).

The highest ESR1 labeling was found in the nuclei of non-ciliated cells of the distal efferent ductules, followed by principal and basal cells in the epididymal duct (Fig. 3A–C). On the other hand, the non-ciliated cells of the proximal efferent ductules were intermittently positive for ESR1. Cells of the connective tissue and ciliated cells of the efferent ductules were not immunolabeled for this protein. Compared with non-affected animals, roosters affected by epididymal lithiasis did not show evident alterations in ESR1 (Fig. 3D–F).

ESR2 was widely expressed in the epididymal region of roosters, as all epithelial cell nuclei as well as connective tissue cells were positive for this protein (Fig. 4A–C). In the efferent ductules, ESR2 immunoreaction was observed in both non-ciliated and ciliated cells lining the epithelium, as well as in principal and basal cells of the epididymal duct. There were no significant differences in ESR2 immunostaining between the segments of the epididymal region in non-affected animals. Compared with non-affected roosters, animals affected by lithiasis presented increased ESR2 labeling in

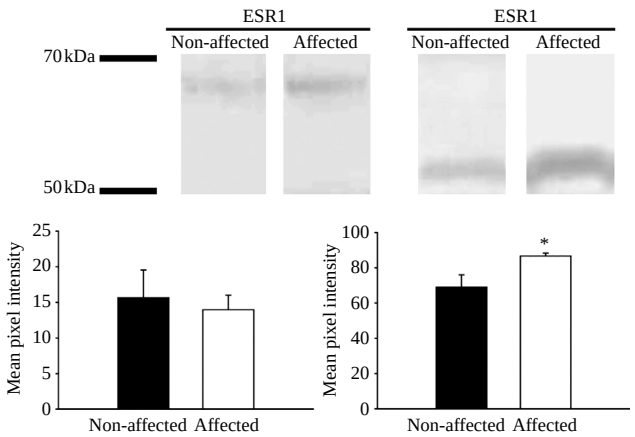

Figure 2 Western blotting analysis of ESR1 and ESR2 in the epididymal region of roosters affected and non-affected by epididymal lithiasis (A). Graphical representation of the image analysis of the ESR1 (B) and ESR2 (C) western blots. $n=5$; $^{*}P \leq 0.05$.

the epithelium of all segments analyzed. On the other hand, the immunoreaction for this receptor in the connective tissue cells was similar in both groups (Fig. 4D–F). In the proximal efferent ductules, ESR2 levels were higher in the non-ciliated cells (38%) and in ciliated cells (44%); whereas in the distal efferent ductules the increase in staining was in the magnitude of 44 and 45% respectively (Fig. 4G and H). In addition, principal cells lining the epididymal duct showed an increase of 41% in the immunostaining for this receptor when affected and non-affected animals were compared (Fig. 4I). It is important to highlight that ESR2 was not detected in the mononuclear cells present in the infiltrates adjacent to affected efferent ductules.

Hormone levels

Estradiol (E_2) levels were increased 95% in the epididymal region of roosters affected by epididymal lithiasis compared with those not affected, whereas plasma levels were decreased 30% in these animals (Fig. 5A and B). Conversely, vitamin D3 concentrations presented a decrease of 86% in the epididymal region and a remarkable increase of about 11-fold in plasma of affected animals (Fig. 5C and D). Regarding testosterone levels, it was observed a drastic reduction in the concentration of this hormone within the epididymal region and plasma of roosters affected by epididymal lithiasis compared with non-affected animals (84 and 60%, respectively; Fig. 5E and F).

Discussion

This study demonstrated that roosters affected by epididymal lithiasis had altered expression patterns of estrogen receptors ESR1 and ESR2 compared with control roosters. Paralleling these changes, E_2 levels were increased in the epididymal region, suggesting that alterations in the estrogen signaling may be associated with epididymal lithiasis. In addition, vitamin D3 and testosterone levels were decreased in the epididymal region of affected roosters. These results add to the limited data about epididymal lithiasis, pointing out that a local hormonal imbalance may be involved with the origin and progression of this anomaly, possibly by affecting the transepithelial calcium transport and/or fluid reabsorption in the efferent ductules.

Roosters affected by the epididymal lithiasis presented decreased E_2 and testosterone, but increased vitamin D3 plasma levels. It is difficult to determine the consequence of such alterations in calcium stone formation, but, corroborating our findings, altered hormonal profiles have been also associated with the development of calcium stones in the kidney of rodent and human (Iguchi *et al.* 1999, Yoshioka *et al.* 2010). In most cases, high levels of circulating estrogen prevents calcium crystal growth and aggregation (Iguchi *et al.* 1999, Yoshioka *et al.* 2010), whereas increased circulating vitamin D3 parallel a higher incidence of kidney stones (Worcester & Coe 2008, Shakhssalim *et al.* 2011). Because the circulating levels of hormones may not reflect exactly the physiology and function of specific organs, tissue concentrations of E_2, vitamin D3, and testosterone, were also investigated.

A remarkable characteristic of the avian efferent ductules is their participation in the reabsorption of a

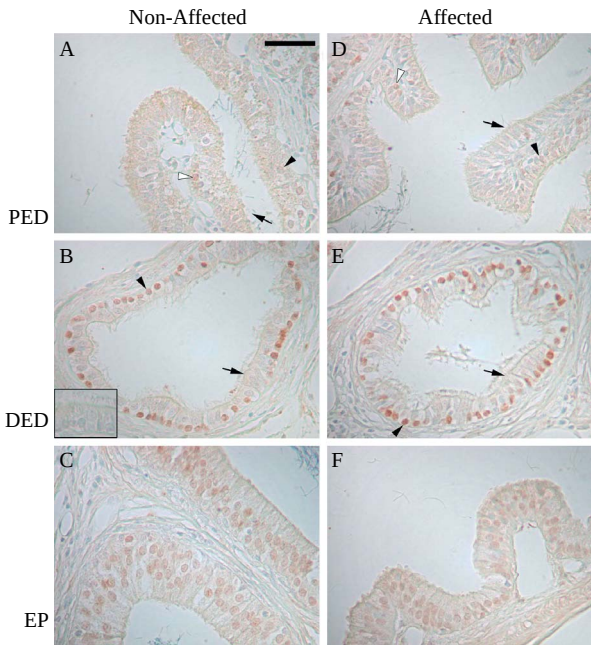

Figure 3 Immunodetection of ESR1 in the epididymal region of roosters not affected (A–C) and affected (D–F) by epididymal lithiasis. PED, proximal efferent ductule; DED, distal efferent ductule; EP, epididymal duct; white arrowheads, ESR1 positive non-ciliated cells; black arrowheads, ESR1 negative non-ciliated cells; arrows, ciliated cells; bar in A, 30 μm; inset in B, negative control.

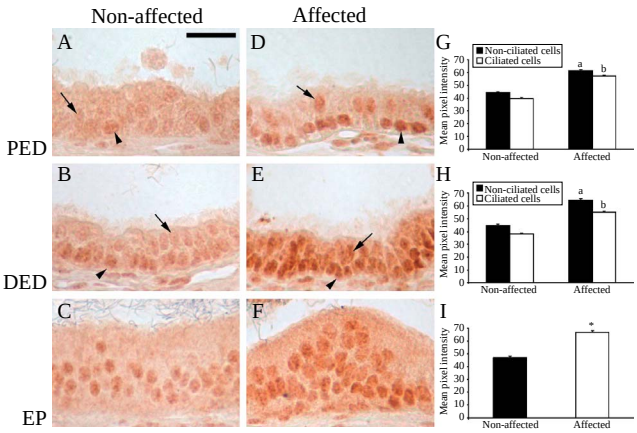

Figure 4 Immunodetection of ESR2 in the epididymal region of roosters non-affected (A–C) and affected (D–F) by epididymal lithiasis. (G–I) Graphical representation of the immunohistochemistry image analysis. PED, proximal efferent ductule; DED, distal efferent ductule; EP, epididymal duct; arrowheads: non-ciliated cells; arrows, ciliated cells. $n=4$; $a=P\leq0.05$ between non-ciliated cells and $b=P\leq0.05$ between ciliated cells; $*P\leq0.05$.

great amount of calcium, besides fluid reabsorption (Clulow & Jones 2004). Vitamin D3 is recognized as a key hormone in tissue calcium homeostasis (Cai *et al.* 1993, Hoenderop *et al.* 2001, 2005, Dick *et al.* 2003, Meyer *et al.* 2007). VDR is expressed within the mammalian male genital system (Stumpf *et al.* 1987, Schleicher *et al.* 1989, Johnson *et al.* 1996, Blomberg Jensen *et al.* 2010), playing an important role in sperm maturation (Blomberg Jensen *et al.* 2010). In birds, the efferent ductules exhibit greater amount of this receptor among the components of the epididymal region (Dornas *et al.* 2007). Because VDR was increased in the efferent ductules of roosters affected by epididymal lithiasis (Oliveira *et al.* 2008), the reduction in local vitamin D3 levels presently found suggests that the VDR upregulation was possibly a compensatory mechanism to reestablish local vitamin D3 action.

E_2 level was higher within the epididymal region of roosters with epididymal lithiasis than in non-affected animals. It is known that the biological function of E_2 is mediated by estrogen receptors ESR1 and ESR2 (Carreau & Hess 2010), which shares structural similarities but also present functional differences (Kuiper *et al.* 1996, Heldring *et al.* 2007). Coincident with the E_2 levels, ESR2 was also overexpressed in all segments within the epididymal region, whereas ESR1 levels were not affected. Involvement of ESR2 in other pathological conditions with soft-tissue calcification has been already demonstrated in other species, whereas ESR1 is commonly associated with a protective effect against calcification (Hodgin *et al.* 2001, Christian *et al.* 2006). It is known that estrogens are involved in regulation of the male tract luminal osmolarity and acidification (Joseph *et al.* 2010*a*, 2010*b*). Interestingly, acidification of luminal fluid inhibits calcium transcellular reabsorption in the kidney of mammals (Bindels *et al.* 1994, Hoenderop *et al.* 2005, Topala *et al.* 2007). Therefore, it is possible that the impairment in estrogen responsive system within the epididymal region of roosters affected by epididymal lithiasis may also result in alterations in the luminal fluid pH with consequent negative effects in the local calcium homeostasis.

Vitamin D3, E_2, and testosterone play important roles in the maintenance of local calcium homeostasis as these hormones modulate the expression of key proteins involved in the transepithelial calcium transport. High levels of vitamin D3 and E_2 are associated with increased calcium reabsorption, as they promote the expression of the transient receptor potential vanilloid channels (TRPV5), calbindin-D28k (CaBP-D28K), and plasma membrane calcium ATPase (PMCA), including in birds (Cai *et al.* 1993, Hoenderop *et al.* 2001, 2005, Van Abel *et al.* 2002, Dick *et al.* 2003, Kip & Strehler 2004, Oz *et al.* 2007). These proteins participate on the entry of calcium within the cell, its diffusion throughout the cell cytoplasm to the basolateral membranes and calcium extrusion to the extracellular environment respectively (Hoenderop *et al.* 2005). Moreover, low levels of testosterone increase the expression of the TRPV5 and CaBP-D28k (Hsu *et al.* 2010). Furthermore, besides the transcellular pathway, vitamin D3, E_2, and testosterone may also influence the paracellular calcium transport (Wada-Hiraike *et al.* 2006, Fujita *et al.* 2008, Kong *et al.* 2008, Braniste *et al.* 2009, Park *et al.* 2011). Therefore, even though E_2 and testosterone are not considered as calciotropic factors, they may also affect the epithelial calcium transport both through the transcellular and paracellular pathways. Based on this information, and considering that the luminal stones consist primarily by calcium (Janssen *et al.* 2000, Mahecha *et al.* 2002), we hypothesize that the imbalance in vitaminD3, estrogen, and androgen responsive systems may result in calcium transport alterations through the transcellular and/or paracellular pathways (Fig. 6). Future detailed studies on the expression of key proteins involved in epithelial calcium transport should better clarify the mechanism of calcium handling in the epididymal region of roosters.

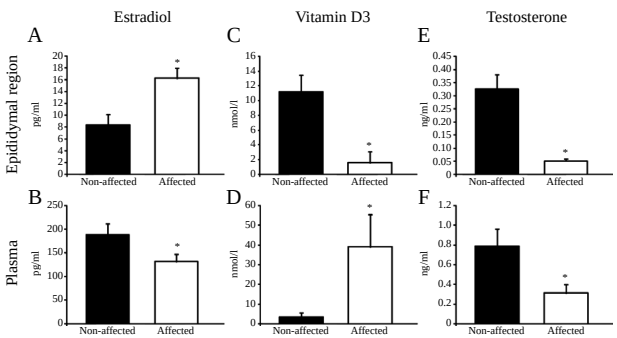

Figure 5 Hormone levels of (A and B) E_2, (C and D) vitamin D3, and (E and F) testosterone in the epididymal region and plasma of roosters non-affected and affected by epididymal lithiasis. $n=5$; $*P\leq0.05$.

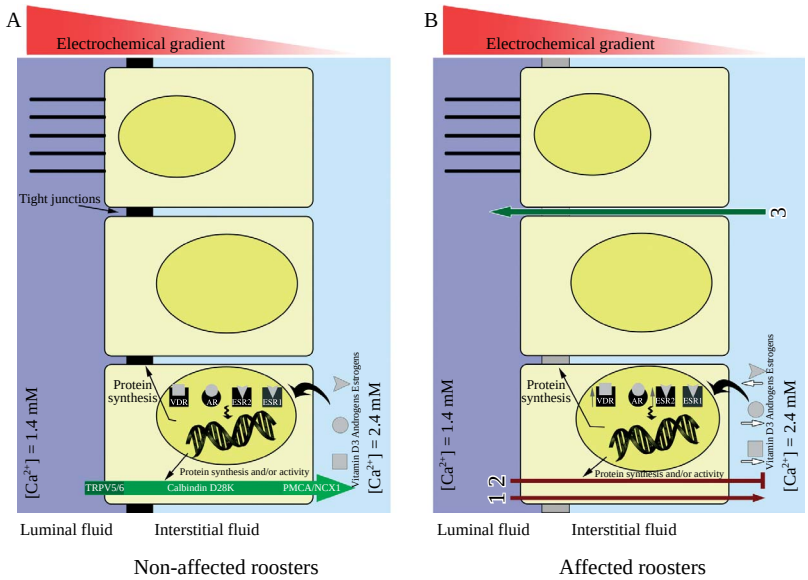

Figure 6 Proposed mechanism for luminal calcium stone formation in the efferent ductules of roosters. (A) In roosters non-affected by epididymal lithiasis, calcium is reabsorbed against an electrochemical gradient (Clulow & Jones 2004) possibly by the transcellular pathway. This process is mediated by different key proteins (TRPV5 or TRPV6, calbindin-D28K as well as PMCA and NCX1) and regulated by vitamin D3, estrogens, androgens, and their receptors VDR, ESR1/ESR2, and AR (Hoenderop *et al.* 2005, Hsu *et al.* 2010). (B) In affected roosters, it is possible that the imbalance of VDR (Oliveira *et al.* 2008) and ESR2 levels as well as the local concentrations of vitamin D3, E_2, and testosterone culminate in reduced (1) or impairment (2) in the transepithelial calcium transport. Also, the hormonal imbalance could interfere with paracellular calcium transport, resulting in the transport of this ion toward the lumen (3). As a consequence of events 1–3, individually or synergistically, calcium concentration within the lumen of efferent ductules would be increased creating a favorable microenvironment for calcium aggregation.

In conclusion, vitamin D3, E_2, and testosterone levels as well as ESR2, but not ESR1, expression were altered in the epididymal region of roosters affected by epididymal lithiasis. These findings point out that alteration in these hormone responsive systems may result in disruption of local calcium homeostasis, which would culminate in the formation of a favorable microenvironment for the aggregation and development of the luminal calcium stones.

Materials and Methods

Animals and tissue preparation

The investigation was performed on the epididymal region of 18 adult cross breed roosters (*Gallus domesticus*) ~1–2 years old obtained from commercial sources and housed at the Universidade Federal de Minas Gerais facilities. The animals were kept under natural conditions of light, humidity, and temperature and were allowed free access to water and food (Socil III Guyomarc, Belo Horizonte, Brazil). The principles of research involving animals followed those advocated by the local Ethics Committee published by the CETEA/UFMG (http://www.ufmg.br/bioetica/coep).

After weighting, the roosters were anesthetized (i.p. lethal dose of sodium pentobarbital 50 mg/kg body weight) and blood samples were collected by cardiac puncture. The plasma was separated by centrifugation and stored at −20 °C for subsequent hormone measurements. Animals were then perfused intracardially with saline and 10% neutral buffered formalin (NBF) for immunohistochemical studies. After fixation, the epididymal regions were dissected out from the testis. Alternatively, animals were perfused with saline solution only and after dissection; tissues were frozen in liquid nitrogen for western blotting and ELISA analysis.

Diagnostics of epididymal lithiasis

The macroscopic diagnostic of epididymal lithiasis was performed by the tissue clearing methodology as previously described and validated (Mahecha *et al.* 2002, Oliveira *et al.* 2008). Briefly, tissues were rinsed in PBS, transferred to 0.5% (w/v) potassium hydroxide and 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 in the epididymal region. Macroscopical findings were validated by histological evaluations of fixed epididymal fragments that were stained with hematoxylin and eosin (H&E).

Histology and morphometry

NBF fixed tissues were processed routinely for paraffin embedding and sectioned at 5 µm. Then, sections were stained with H&E and used for histological studies and morphometrical analysis by classical methodology (Weibel 1969, Oliveira *et al.* 2007). The volumetric density (Vv%) of the rete testis, proximal and distal efferent ductules, connecting ducts and epididymal duct as well as the connective tissue in the epididymal region was analyzed using a grid of 400 points. All the points of the grid incident in the ducts of the region were

scored and the result for each duct was divided by the sum of all points scored to obtain the Vv% of the parameters analyzed. Because differentiation between the connecting and epididymal ducts is difficult (Aire & Soley 2000, Oliveira *et al.* 2007), they were considered together.

Western blotting

For the western blotting assays, epididymal regions of non-affected and affected roosters ($n=5$ per group) frozen in liquid nitrogen were used. Following total protein extraction, samples were subjected to continuous electrophoresis using 10% SDS–PAGE. The separated proteins were transferred to nitrocellulose membranes and blocked with 10% normal goat serum for 1 h at room temperature. After incubation for 1 h with rabbit anti-ESR1 (Clone 60C – Millipore, Temecula, CA, USA) or mouse anti-ESR2 (NCL-ERβ – Novocastra Laboratories, New Castle, UK) antibodies, used at 1:300 and 1:150 dilution, respectively, the blots were washed in PBS Tween 0.05% and then incubated in a biotinylated secondary antibody (Dako, Glostrup, Denmark) goat anti-rabbit (for ESR1) or goat anti-mouse (for ESR2) at 1:1000. The membranes were then incubated with the avidin–biotin complex (Vectastain Elite ABC Kit – Vector Laboratories, Burlingame, CA, USA) for 30 min and the immunolabeling was visualized with a solution of 0.1% (w/v) 3,3′-diaminobenzidine in PBS containing 0.05 chloronaphthol (w/v), 16.6 methanol (v/v), and 0.04% (v/v) H_2O_2. The quantification of ESR1 and ESR2 positive bands were estimated as described previously (Oliveira *et al.* 2008).

Immunohistochemistry

NBF fixed, paraffin embedded fragments of the epididymal region of non-affected and affected animals ($n=4$ per group) were used for immunohistochemical studies. To allow comparison between animals, the sections were run in parallel, and the staining was performed in two different sets to confirm the results. Sections were deparaffinized, rehydrated, blocked for endogenous peroxidase and submitted to antigen retrieval by a standard microwave method. The avidin–biotin non-specific binding was blocked using the Vector blocking kit (Vector Laboratories). Sections were then incubated in 10% normal goat serum to block non-specific binding and then with the primary rabbit anti-ESR1 (Clone 60C – Millipore) or mouse anti-ESR2 (NCL-ERβ – Novocastra Laboratories) antibodies at the dilution of 1:50 and 1:25 respectively. The use of these antibodies has already being validated for rooster tissues (Oliveira *et al.* 2011). After several washes in PBS, the sections were exposed to a biotinylated goat anti-rabbit (for ESR1) or goat anti-mouse (for ESR2) secondary antibody (Dako) used at 1:50 dilution. The negative controls were obtained by the omission of the primary antibodies followed by the incubation with goat anti-rabbit IgG and goat anti-mouse IgG diluted 1:50 for ESR1 and ESR2 respectively. The sections were then incubated in the avidin–biotin complex (Vectastain Elite ABC Kit – Vector Laboratories). Finally, the immunoreaction was developed in 0.05% 3,3′-diaminobenzidine containing 0.01% (v/v) H_2O_2 in 0.05 M Tris–HCl buffer, pH 7.6 and stopped in distilled water. Sections were counterstained with Mayer's hematoxylin, dehydrated in ethanol, cleared in xylene, and mounted.

Semi-quantitative immunohistochemical studies

The intensity of ESR1 and ESR2 immunostaining was estimated by computer-assisted analysis, based on previously reported protocols (Oliveira *et al.* 2008). Digital images from five different areas of the proximal and distal efferent ductules as well as the epididymal duct of each animal were taken by a Nikon Eclipse E600 microscope (Nikon Co., Melville, NY, USA). The images were processed with Adobe Photoshop (Adobe Systems), converted to the grayscale mode and inverted. The images were then exported to Image-Tool Software (version 3.00, University of Texas Health Sciences Center, USA), for quantitative analysis. For this purpose, 25 nuclei of each cell type positive for the proteins investigated in the efferent ductules and epididymal duct 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.

Hormone measurements

Vitamin D3, E_2, and testosterone levels were dosed in the epididymal region and plasma by ELISA assays (Akhlaghi & Zamiri 2007, Ruwaan *et al.* 2010). For tissue analysis, epididymal region of affected and non-affected animals ($n=5$) were frozen in liquid nitrogen and macerated in dry ice. Then, 100 mg tissue were suspended in 250 µl of PBS (pH 7.4), homogenized and sonicated for 1 min. To optimize the results for E_2 and testosterone, lipid extraction enrichment with diethyl ether was performed according to a previous study (Hany *et al.* 1999). The enrichment of vitamin D3 in samples was performed by following manufacturer's instructions. The plasma used in the experiments was obtained after centrifugation of total blood (1800 ***g*** for 10 min) in heparin-coated tubes. ELISA measurements were performed according to the protocols provided by the manufacturers of the kits (E_2 and vitamin D3 – DRG Instruments GmbH, Marburg, Germany and testosterone Interkit – Bio Check, Foster City, CA, USA). It was used the same volume of samples per well of the ELISA plates. All samples were measured in duplicate within each assay and all the experiments were repeated in two independent assays. The intra- and inter-assay coefficients of variation (CVs) were 10 and 12% for vitamin D3, 4.6 and 7.8% for E_2, and 7.4 and 5.2% for testosterone respectively. The sensitivity of the assays was 5.6 nmol/l, 9.7 pg/ml, and 0.083 ng/ml for vitamin D3, E_2, and testosterone ELISA Kits respectively.

Statistical analysis

Differences in hormone concentrations and ESR1 and ESR2 expression in the epididymal region of roosters non-affected and affected by epididymal lithiasis were statistically analyzed by the Student's *t*-test. The analysis of the morphometrical data (Vv%) was performed by using ANOVA and Newman–Keuls as *post hoc* for pairwise comparisons. Differences were considered significant at $P \leq 0.05$.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Preface

International symposium on cell signaling

The first and pioneering "International Symposium on Cell Signaling" was held in the beautiful city of Florianópolis in Santa Catarina State, Brazil, from October 28 to 31, 2010 at IL Campanario Villaggio Resort on Jurerê International Beach. This special issue of *Life Sciences* focuses on the invited speakers' contributions to the discussion of molecular and structural biology, molecular toxicology and signaling transduction.

The field of structural molecular biology has grown steadily in the last 10 years due to enormous advances in protein structure determination techniques, such as X-ray diffraction, nuclear magnetic resonance and biophysical analysis of protein–ligand interaction (using circular dichroism, fluorescence and surface plasmon resonance). Several Nobel prizes in the past decade were awarded in protein structure-related subjects, emphasizing the impact of structural biology on general science. We are now awaiting the discovery of new drugs, therapies and metabolic and signal transduction pathways, based on our increasing knowledge and expansion of the protein folding universe.

Living organisms might be exposed to a myriad of potentially toxic substances (e.g., environmental pollutants, medicines, abused drugs), whose effects are diverse and may range from no evident outcomes to significant toxicity, culminating in the occurrence of disease and, eventually, death.

Molecular toxicology represents a specific field that delves into the essential molecular mechanisms involved in the cascade of the effects of these substances and the factors of individual susceptibility modulating such effects. During the last decade, knowledge in this field has increased enormously; this has made possible the achievement of (i) fast and accurate diagnoses of toxicities, (ii) specific acceptable limits of exposure to toxic substances and (iii) potential antidotal strategies to treat poisoned humans/animals. Molecular toxicology plays a crucial role in the maintenance of life because it allows comprehension of the relationship between the toxic substances and the exposed organisms from a molecular point of view.

The study of signal transduction mechanisms of endogenous and exogenous biologically active molecules has been challenging in the last 30 years. The discovery of new substances and new intracellular signaling pathways has enriched current therapy options and become objects of continued interest in both academia and industry. Particularly, the development of a wide array of analytical techniques (including nanotechnology, patch clamp, purified cell lines, *in silico* procedures, protein transfection and siRNAs) as gold-standard assays has dramatically increased biopharmaceutical knowledge and enabled further appreciation of signal transduction cascades induced by natural and synthetic compounds. Given the growth of this field, we hope that new drugs will be developed to specifically act efficiently against infertility, immune and neurological disorders, respiratory infection, cancer, mineral diseases, multiple sclerosis and Chagas disease.

The "International Symposium on Cell Signaling" was of major interest to biochemists, pharmacologists, molecular biologists, cell biologists, physiologists and other scientists. The speakers presented their exciting new findings in plenary sessions and a wide array of symposia. The participants had the opportunity to exchange knowledge, experiences and science and to visit Florianópolis' natural tourist attractions.

The main objective of the "International Symposium on Cell Signaling" was to promote an integrative approach to research on cell signaling. We scheduled a large national and international program and invited outstanding scientists to contribute presentations in the areas of molecular signaling at the plasma membrane, "steroid–vitamin membrane interactions", "genomic/non-genomic translational control" and "ion channels and signal transduction". These scientific talks promoted fruitful discussions and the possibility to initiate new friendships, research collaborations and joint projects. During the symposia, all members of the audience were welcome ask any questions and make comments.

PhD students and post-doctoral trainees were encouraged to apply for the "Young Scientist Forum," which was an outstanding opportunity for young scientists to participate in a meeting promoting their scientific future. The organizing committee selected eight applicants to present their results orally, and a financial award was given to the best oral presentation. André Gustavo de Oliveira (UFMG) and Flávia Carla Meotti (UFSC) were jointly awarded first prize. An honorable mention was given to the other young investigators who submitted and presented meritorious work to the forum. Additionally, an award was given for the best poster presentation to Christiane M. Freitas (UFMG). The forum provided the opportunity for young investigators to exchange scientific experiences, present their work and interact with international senior scientists.

The publication of this special issue of "Life Science" represents the official report of the mini-reviews written by the speakers invited to present lectures in "International Symposium on Cell Signaling". Additionally, the young scientists who received awards were invited to publish a mini-review in this special issue.

The idea of hosting this first meeting in Florianópolis was conceptualized by researchers from the recently created Postgraduate Program of Biochemistry (PPG-BQA) at Federal University of Santa Catarina (UFSC). The main goal of the meeting was to optimize international cooperative work and national collaborations between PPG-BQA and several research groups around the world by promoting researcher topic integration through a wide discussion during the symposium.

The organizers acknowledge the financial support of the many sponsors of the "International Symposium on Cell Signaling". Without generous multi-corporation financial support, it would have been

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impossible to have a successful symposium with international participants from throughout the world. A tabulation of these sponsors appears below. We extend special thanks to CAPES (Coordenação de pessoal de nível superior), CNPq (Conselho nacional de desenvolvimento científico e tecnológico), FINEP (Financiadora de estudos e projetos), FAPESC (Fundação de amparo a pesquisa do estado de Santa Catarina) and UFSC for their financial support. The organizing and scientific committee would like to particularly acknowledge "CAPES–COFECUB (Brazil–France)", "CNPq–FNRS, Unité PMNT — Université du Louvain/ Bruxelas (Brazil–Belgium)", "CAPES–SPU (Brazil–Argentine)", "CAPES–DGU Universidad Autonoma de Madri (Brazil–Spain)", "UFSC, University of Plymouth" and "CAPES–PRODOC (UFSC–Brazil)", for their important contributions.

We would also like to give special thanks to all members of the organizing committee, our dedicated students, who worked diligently and helped to make this symposium a reality. We hope that the Symposium has represented a great scientific success and an enjoyable experience for all 185 participants.

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Minireview

Epididymal lithiasis in roosters: In the middle of the way there was a stone

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ABSTRACT

The epididymal region plays an important role in the reproduction of roosters, as it is the site of functions important in the maintenance of fertility, including fluid and calcium reabsorption and sperm surface modifications. About 10 years ago, a reproductive dysfunction characterized by the formation of luminal calcium stones in the epididymal region of roosters was described. This anomaly, known as epididymal lithiasis, is associated with a significant decrease in the fertility of affected roosters. This reproductive anomaly has been observed in multiple countries and is thought to negatively impact the poultry industry; however, the cause of epididymal lithiasis has not been fully determined. Several hypotheses have been proposed to explain the origin of epididymal lithiasis, including the presence of an infectious agent within the epididymal region, an autoimmune response, increased dietary calcium and vitamin D3 intake and the presence of genetic susceptibility factors; however, none of these has been proven to be the primary cause of the calcium stone formation. Nonetheless, considerable evidence suggests that regardless of the primary cause of epididymal lithiasis, this anomaly could result from a hormonal imbalance or a local impairment of calcium homeostasis in the epididymal region. The objectives of this mini-review are to 1) summarize the reproductive alterations observed in animals affected by epididymal lithiasis, 2) discuss the hypotheses proposed to explain the cause of luminal stone formation and 3) provide perspectives for future studies of this reproductive disorder.

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Introduction

Consumption of chicken, which serves as a major source of dietary intake for humans, has climbed worldwide, increasing about 20% in the last ten years (MAPA, 2007; USDA, 2010). As a consequence, an increase in chicken production has been necessary to meet increased demand. Despite this increased demand, chicken production is limited by fertility constraints. Even when kept under controlled conditions, the fertility rate of domestic roosters peaks at 96% at 37 weeks of age. This period of peak fertility is followed by a drastic and rapid decrease in reproductive success, with fertility levels reaching 5% at 110 weeks (Rosenstrauch et al., 1994; Muncher et al., 1995; Weil et al., 1999).

Furthermore, a recent study described a reproductive dysfunction characterized by the presence of calcium stones within the rooster epididymal region that is associated with early loss of fertility in affected

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animals (Janssen et al., 2000). This anomaly, termed epididymal lithiasis, was first identified in the United States and Japan, where its prevalence was about 75%. Epididymal lithiasis was later described in other countries, including Brazil, where lithiasis affects up to 90% of roosters (Mahecha et al., 2002), and Colombia (Personal communication), indicating that the occurrence and distribution of this anomaly may be wider than originally thought.

The avian genital system

In birds, the genital system is composed of the testes, epididymal region, deferent duct and phallus (Fig. 1) (Lake, 1981). The epididymal region is positioned at the dorso-medial surface of the testes and consists of the rete testis, efferent ductules, connecting ducts and a short and non-differentiated epididymal duct (Fig. 1) (King, 1975; Aire, 1979a, 2000). All of these components are surrounded by abundant connective tissue.

Among the components of the epididymal region, the efferent ductules are the most prominent, comprising up to 40%–60% of the epididymal region depending on the species considered (Aire, 1979b; Clulow and Jones, 1988; Oliveira et al., 2007). These ductules can be divided into two continuous segments with distinct morphological characteristics: the proximal and distal efferent ductules. The proximal efferent ductules are characterized by wide lumen and highly folded epithelium, whereas the distal ductules have narrower lumen and fewer epithelial folds (Fig. 1C–D) (Aire, 1979a; Lake, 1981). Both regions are lined by a columnar epithelium formed by non-ciliated and ciliated cells, the latter being more numerous in the distal segments (Aire, 1979a). As in mammals, the efferent ductules of birds are involved in the reabsorption of about 86% of the testicular fluid (Clulow and Jones, 1988); however, they also play an important role in the reabsorption of calcium (Clulow and Jones, 2004). Therefore, the efferent ductules of birds are considered to have an essential function in the concentration and maturation of sperm through their involvement in the maintenance of fluid and calcium homeostasis throughout the avian excurrent ducts.

Epididymal lithiasis

Epididymal lithiasis is a reproductive dysfunction described about 10 years ago (Janssen et al., 2000) that is characterized by the formation of stones within the epididymal region of roosters (Fig. 2A,

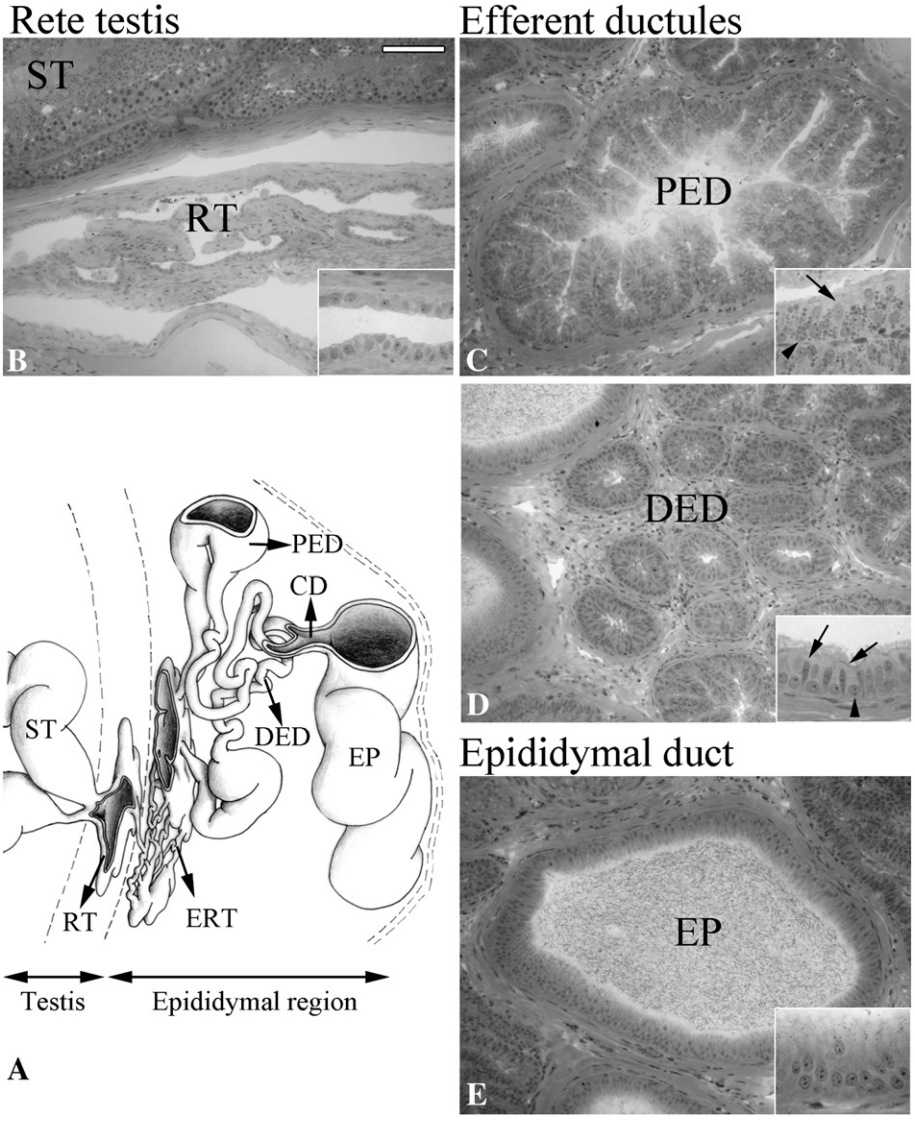

Fig. 1. The avian epididymal region. (A) Schematic representation of the avian epididymal region. (B–E) Histology of the major components of the epididymal region. The inserts highlight the epithelium lining each segment. ST = seminiferous tubules; RT = rete testis; ERT = extratesticular rete testis; PED = proximal efferent ductule; DED = distal efferent ductule; CD = connecting duct; EP = epididymal duct. Arrows in C–D = ciliated cells; arrowheads in C–D = non-ciliated cells. Bar in B–E = 100 μm.

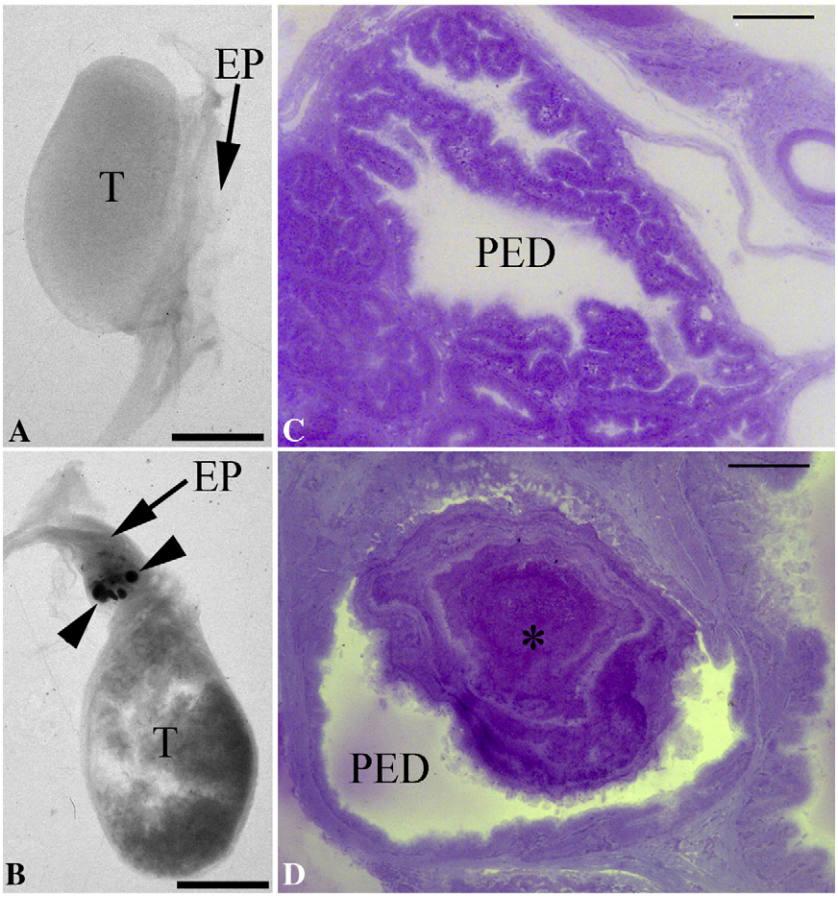

Fig. 2. Epididymal lithiasis. (A–B) Fragments of the epididymal region after clearing in potassium hydroxide and glycerin showing the epididymal region (EP) of non-affected roosters is completely transparent (A), whereas in animals affected by epididymal lithiasis stones (arrowheads) are visible within this region (B). (C) Proximal efferent ductule (PED) of non-affected animal showing a columnar and highly folded epithelium. (D) Proximal efferent ductule (PED) presenting a luminal calcium stone (*). Note the loss of epithelial folding. T = testis. Bar in A and B = 0.5 cm; Bar in C and D = 200 μm.

B). This dysfunction is thought to be restricted to this species, as among the 27 species of domestic and wild birds investigated, stone formation was only found in roosters (Mahecha et al., 2002).

The first signs of epididymal lithiasis are observed by 18 weeks of age, when luminal inclusions become evident within the efferent ductules (Janssen et al., 2000). Between 18 and 26 weeks of age, these inclusions become filled by an opaque fluid, and by 26 weeks, most of these cysts become solid and form stones within the epididymal region. The number and size of stones are thought to increase with age (Janssen et al., 2000). A noteworthy characteristic of affected animals is chronic inflammation characterized by the presence of abundant mononuclear cell infiltrates beginning at 11 weeks of age, several weeks prior to the onset of the appearance of epididymal stones (Janssen et al., 2000). The inflammatory process persists throughout adulthood (Janssen et al., 2000).

Macroscopically, the epididymal stones are irregular in size and shape, with diameters varying from 9 μm to 3000 μm and ranging from smooth or slightly rough to having sharp-pointed surfaces (Janssen et al., 2000; Mahecha et al., 2002). The epididymal stones are composed mainly of calcium (40%–48%), but other substances, including oxygen (28%), carbon (23.5%), sulfur, magnesium, sodium, phosphorus and organic matter are also present (Janssen et al., 2000; Mahecha et al., 2002). Microscopically, the calcium stones are characterized by the presence of a calcified nidus at the core surrounded by alternating layers of calcified and organic matter (Mahecha et al., 2002). It is also common to observe the presence of sperm attached to the surface of the stones and sloughed cells within the stones (Janssen et al., 2000; Mahecha et al., 2002).

Reproductive alterations

All studies to date have shown the efferent ductules to be the segment most affected by epididymal lithiasis (Janssen et al., 2000; Mahecha et al., 2002; Boltz et al., 2004, 2006; Jackson et al., 2006; Oliveira et al., 2008). This observation is based not only on the fact that the calcium stones are formed and retained within the lumen of the ductules, but also on the drastic morphological alterations observed in these ductules (Fig. 2C, D) (Janssen et al., 2000; Mahecha et al., 2002; Boltz et al., 2004, 2006; Jackson et al., 2006; Oliveira et al., 2008). Some efferent ductules present reductions in epithelial folding and height, decreased luminal area and discrete cytoplasmic vacuolization in non-ciliated cells, whereas in other ductules, these vacuoles occupy most of the cytoplasm (Janssen et al., 2000; Mahecha et al., 2002). In addition, numerous mononuclear cell infiltrates are observed adjacent to the efferent ductules (Janssen et al., 2000; Mahecha et al., 2002; Boltz et al., 2004, 2006; Jackson et al., 2006; Oliveira et al., 2008). In some areas, recanalization of these ductules occurs, possibly as an attempt to isolate the luminal stones and allow free transit of testicular secretions and spermatozoa; however, in most affected areas, efferent ductules atrophy is observed (Mahecha et al., 2002). Although the presence of epididymal stones in other segments of the epididymal region is not a frequent finding, stones can occasionally be found in the lumen of the epididymal duct surrounded by mucous substances that may facilitate their passage to the cloaca and excreta (Mahecha et al., 2002).

In contrast to the observation of the aforementioned alterations in the efferent ductules of most affected animals, testicular alterations are generally mild, as no significant changes are found in the volumetric

proportion of the interstitial tissue, seminiferous tubule lumen and epithelium or in the of Sertoli cell population (Oliveira et al., 2008). Furthermore, no differences in sperm production per gram of testis are observed between affected and non-affected roosters (Janssen et al., 2000). In the testes of some affected roosters, however, it is possible to observe areas with moderate sloughing of seminiferous tubules. In some cases, the seminiferous tubules are characterized by a reduced diameter and intense cellular sloughing. In these tubules, the seminiferous epithelium is formed mostly by Sertoli cells and a few spermatogonia, and mononuclear cell infiltrates may also be present (Mahecha et al., 2002). The rarity of major morphological alterations within the testis of roosters affected by epididymal lithiasis reinforces the proposition that the testicular effects of this disorder may be secondary to alterations found in the ducts of the epididymal region.

Affected roosters present a decrease of approximately 65% in circulating levels of testosterone. This decrease in testosterone is paralleled by an increase in the frequency of Leydig cells, which has been interpreted as an attempt to re-establish normal testosterone levels (Janssen et al., 2000; Oliveira et al., 2008). Experiments using natural mating show that together, all of the alterations in the male genital system caused by epididymal lithiasis result in significantly reduced fertility in affected animals, as the production of fertilized eggs is dramatically decreased (62%) in affected animals. Surprisingly, the artificial insemination of chickens with equivalent numbers of spermatozoa obtained from the semen of affected and non-affected animals does not restore the fertility levels of affected roosters, which still present a 41% reduction in the production of fertilized eggs (Janssen et al., 2000). Therefore, the decrease in fertility in roosters with lithiasis is likely due to alterations in the quality of spermatozoa that could reflect functional disruption of the affected epididymal region, especially in the efferent ductules.

What is the primary cause of epididymal lithiasis?

Although epididymal lithiasis results in severe reproductive impairment in affected animals, little is known about its etiology. Several hypotheses have been proposed to explain the cause of epididymal lithiasis. These hypotheses suggest that luminal calcified stones form within the efferent ductules due to (1) a pathological agent, (2) an autoimmune response, (3) dietary vitamin D3 and calcium intake or (4) genetic susceptibility.

Hypothesis 1. A pathological agent.

This hypothesis was proposed as a result of the common observation of abundant mononuclear cell infiltrates within the epididymal region of affected animals (Janssen et al., 2000; Mahecha et al., 2002; Boltz et al., 2006; Jackson et al., 2006). One possible cause for these infiltrates is the presence of a local infectious agent. Avian infectious bronchitis virus (AIBV), which shows a tropism for and replicates in ciliated epithelia such as in the respiratory tract and possibly the efferent ductules, has been suggested as a putative infectious cause of epididymal lithiasis (Dhinakar and Jones, 1997; Jackson et al., 2006; Cavanagh, 2007; Shen et al., 2010). Moreover, AIBV infection has been associated with other pathologies in which calcification of soft tissue is common, such as urolithiasis (Niznik et al., 1985; Brown et al., 1987; Fitz-Coy et al., 1988; Glahn et al., 1989). Based on these considerations, recent studies have focused on the association of AIBV infection with epididymal lithiasis. Systematic monitoring of roosters for circulating anti-AIBV antibodies showed that even roosters with undetectable levels of anti-AIBV antibodies developed epididymal lithiasis (Mahecha et al., 2002). In addition, studies in which animals were vaccinated with live attenuated or killed AIBV failed to prove that the virus or the anti-AIBV antibodies are the cause of the epididymal lithiasis, as the non-vaccinated control roosters also developed epididymal lithiasis (Boltz et al., 2006; Jackson et al., 2006); however, these studies demonstrated that the incidence of epididymal stones was greater in animals exposed to AIBV than in unexposed animals and that the onset of epididymal lithiasis was accelerated in exposed animals (Boltz et al., 2006; Jackson et al., 2006). Together, these data indicate that although AIBV has an influence on the development of epididymal lithiasis, it is not the primary factor involved in its etiology.

Recently, another epitheliotropic virus was associated with the rooster genital tract. The avian metapneumovirus (aMPV) was identified by RT-PCR in testis samples and showed a high prevalence in roosters with epididymal lithiasis (Villarreal et al., 2007). Despite these findings, it is not known whether aMPV plays a role in the origin of epididymal lithiasis; however, several other pathological agents have been associated with soft tissue calcification (Ennever et al., 1981; Cisar et al., 2000; Kosowski et al., 2000; Colpan et al., 2004; Reyes et al., 2009), indicating that the hypothesis of a pathological causes of epididymal lithiasis should not be overlooked.

Hypothesis 2. Autoimmune disease.

Another explanation for the observation of chronic inflammation in the epididymal region of roosters with luminal calcium stones is an autoimmune disease. Among the mammalian reproductive organs, the efferent ductules are the segment most permeable to luminal antigens and are thus the primary site of autoimmune responses (Suzuki and Nagano, 1978; Tung and Alexander, 1980). The autoimmunity hypothesis states that the blood-lumen barrier of the efferent ductules in affected roosters is somehow disrupted or at least leakier than in non-affected animals (Jackson et al., 2006). This leakage may result in the passage of luminal content to the connective tissue and the subsequent stimulation of the interstitial immune cells, including the antigen presenting cells and $CD4^+$ and $CD8^+$ T cells that are abundant in the areas adjacent to the efferent ductules (Yoshimura et al., 2005, 2006). These cells are the putative modulators of a local immune response against luminal antigens that results in the recruitment of other immune cells and in the production of antibodies that are secreted to the lumen. It is reasonable to speculate that these antibodies may promote sperm clumping and consequently the initiation of calcium stone formation as previously proposed (Jackson et al., 2006). Further investigations are necessary to determine the role of immune modulation in the development of epididymal lithiasis.

Hypothesis 3. Dietary vitamin D3 or calcium intake.

Based on the constitution of luminal calcium stones, epididymal lithiasis has been suggested to be related to dietary intake of calcium or vitamin D3 (Janssen et al., 2000; Mahecha et al., 2002), which is a secosteroid hormone well-known for its role in maintaining calcium metabolism (de Matos, 2008); however, no correlation has been found between the number of calcium stones or the weight of the testis/epididymal region and the concentration of calcium ingested (Mahecha et al., 2002). Furthermore, no correlation was observed between vitamin D3 consumption and the occurrence of epididymal lithiasis (Jackson et al., 2006). In support of this finding, some rooster strains do not consume food containing high levels of vitamin D3 but do develop calcium stones within the efferent ductules (Mahecha et al., 2002).

Hypothesis 4. Genetic susceptibility.

Another explanation for the origin of this reproductive disorder is genetic susceptibility. *Gallus gallus* is a species that has been genetically selected for about 3000 years (Etches, 1993), resulting in increased egg production and faster bone formation to shorten the slaughter period. Therefore, it is possible that the selection of animals that mobilize calcium more efficiently has resulted in the selection of roosters that express higher levels of proteins that transport or bind to calcium in different organs, including the efferent ductules, potentially resulting in the formation of luminal calcium stones (Mahecha et al., 2002).

Calcium homeostasis impairment: an alternative hypothesis for the origins of epididymal lithiasis

Although several plausible hypotheses have been proposed to explain the origin of epididymal lithiasis, none of them have proven to fully explain this reproductive anomaly. Nevertheless, considering that calcium is the major component of the stones formed within the lumen of the efferent ductules (Janssen et al., 2000; Mahecha et al., 2002), a segment that in birds is involved in the reabsorption of great amounts of calcium (Clulow and Jones, 2004), it is possible that epididymal lithiasis could result from local impairment of calcium homeostasis (Fig. 3) (Oliveira et al., 2008). As a consequence of this impairment, calcium concentrations would be higher in the lumen of the efferent ductules in affected roosters, forming nucleating centers for calcium aggregation (Oliveira et al., 2008).

Calcium reabsorption across the epithelium

Calcium can be reabsorbed by the epithelium through two distinct pathways: the paracellular route, which involves calcium movement through the intercellular space, and the transepithelial route, in which the ions are transported through the cell's cytoplasm. Transepithelial calcium transport occurs in three general steps, comprising (i) calcium entry through the apical plasma membrane, (ii) calcium translocation to the basolateral membrane and (iii) calcium extrusion to the extracellular environment and bloodstream (Hoenderop et al., 2005). Each of these steps depends on the expression of specific proteins. Calcium enters the cell through specific calcium channels located in the apical membrane, named TRPV5 or TRPV6 (**T**ransient **R**eceptor **P**otential **V**anilloid channel), following an inward electrochemical gradient (Hoenderop et al., 2002). Once inside the cytoplasm, calcium ions bind to calcium binding proteins (CaBP), such as CaBP-D9K or CaBP-D28K, which facilitate calcium diffusion through the cytoplasm to the basolateral membrane of the cell (Hoenderop et al., 2005; Bar, 2009). Finally, the extrusion of cytoplasmic calcium may be mediated by the action of at least two transporters present on the basolateral membrane: the Na^+/Ca^{2+} exchanger (NCX) and the **P**lasma **M**embrane calmodulin-dependent **C**alcium **A**TPase (PMCA) (Bindels et al., 1991; Van Baal et al., 1996; Hoenderop et al., 2005).

A transcriptome analysis of the rat epididymis identified the presence of TRPV6, but not TRPV5 in this tissue (Jelinsky et al., 2007). Further studies also demonstrated expression of calcium binding proteins and PMCA in the rat epididymis (Wilhelm et al., 2008; Hamzeh and Robaire, 2010). In contrast, information about the expression of the proteins involved in the transepithelial calcium transport within the avian male genital tract is still lacking. In the avian efferent ductules, calcium reabsorption occurs against a blood-to-lumen electrochemical gradient, as the luminal calcium concentration in these ductules is approximately 1.4 mM, whereas the circulating calcium concentration is about 2.4 mM (Clulow and Jones, 2004). Therefore, it is likely that the bulk of calcium transport in this segment occurs through the active transepithelial pathway rather than through the passive paracellular route (Fig. 3A) (Hoenderop et al., 2005).

Transepithelial calcium transport and its putative association with epididymal lithiasis

Transepithelial calcium transport is influenced by the hormones that modulate the expression and activity of the proteins involved in this process, including vitamin D3, androgens and estrogens. It is well-known

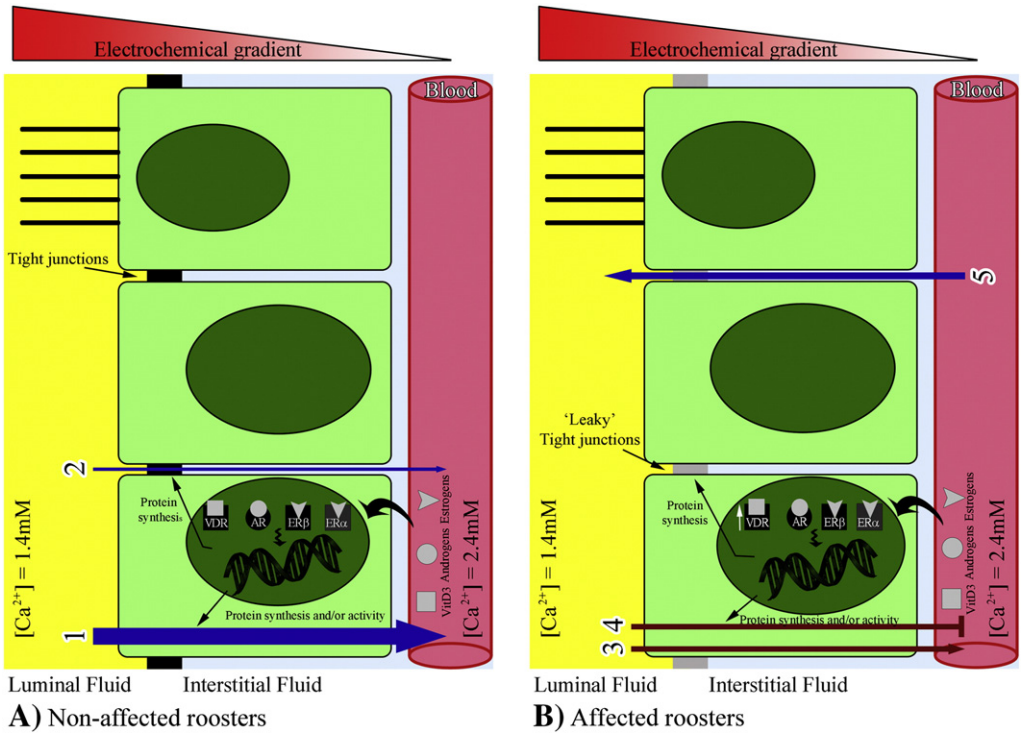

Fig. 3. Diagram showing possible mechanisms for luminal calcium stone formation in the efferent ductules of roosters. (A) In non-affected roosters, calcium is reabsorbed in the efferent ductules against an electrochemical gradient (Clulow and Jones, 2004) through two distinct pathways: the transepithelial pathway (1) and, to a lesser extent, the paracellular pathway (2). These processes are mediated by different proteins (i.e., TRPV5/TRPV6, CaBP-D28K, PMCA and NCX1, junctional complex proteins) and regulated by vitamin D3, estrogens and androgens and their receptors VDR, ERα/ERβ and AR, respectively, which control the synthesis and/or activity of these proteins (Hoenderop et al., 2005; Wada-Hiraike et al., 2006; Fujita et al., 2008; Kong et al., 2008; Braniste et al., 2009; Hsu et al., 2010). (B) In affected roosters, an imbalance in the system responsive to vitamin D3, estrogens and androgens may lead to reduced (3) or impaired (4) transepithelial calcium transport. Alternatively, a hormonal imbalance may result in alterations in tight junction assembly, causing tight junctions to become leaky and calcium to be transported to the lumen following the electrochemical gradient through the paracellular pathway (5). These events may occur individually or synergistically to create a favorable microenvironment for calcium aggregation into the efferent ductule lumen.

that vitamin D3 is one of the most important hormones involved in calcium reabsorption through the transepithelial pathway, as the expression of TRPV5, TRPV6, CaBP-D28K and PMCA are highly dependent on this hormone and its receptor VDR (Cai et al., 1993; Hoenderop et al., 2001, 2002, 2005; Meyer et al., 2007; Suzuki et al., 2008). Androgens and their nuclear receptor AR also influence calcium transport, as high levels of testosterone decrease the expression of TRPV5, CaBP-D28K and PMCA, whereas the opposite effect is observed when testosterone levels are low (Hsu et al., 2010).

Based on these facts and considering that VDR, AR and their ligands directly modulate transepithelial calcium transport, our laboratory investigated whether these receptors are expressed in the epididymal region of roosters and examined their distribution in this segment (Dornas et al., 2007, 2008). Higher levels of VDR were found in the efferent ductules, whereas AR was more highly expressed within the epididymal duct epithelium, suggesting that different segments of the epididymal region may be differentially regulated by vitamin D3 and androgens (Dornas et al., 2007, 2008). Roosters with epididymal lithiasis expressed significantly higher levels of VDR in the epithelium of the efferent ductules than did non-affected animals. In contrast, AR expression levels were not altered in this segment. These data suggest that vitamin D3/VDR signaling can be a key factor in the origin or development of epididymal lithiasis by diminishing or preventing the calcium reabsorption process (Fig. 3B) (Oliveira et al., 2008). On the other hand, nothing is known about the levels of the ligands of these receptors in tissues or circulation. Therefore, it is necessary to investigate the levels of vitamin D3 and androgens in roosters affected by epididymal lithiasis to better understand the physiological significance of the expression patterns of VDR and AR.

In addition to vitamin D3 and androgens, transepithelial calcium transport is also influenced by estrogens. Previous studies have shown that 17β-estradiol induces the expression of TRPV5, TRPV6 and CaBP-D28K proteins and increases the synthesis and activity of PMCA (Van Abel et al., 2002; Dick et al., 2003; Oz et al., 2007). It is well-known that the efferent ductules are the main target for estrogen action among the components of the male genital system, and that estrogen receptors ERα and ERβ are highly expressed within this male genital tract segment including in birds (Hess et al., 1997; Kwon et al., 1997; Oliveira et al., 2010). Thus, considering the high expression levels of ERα and ERβ in the efferent ductules of roosters (Oliveira et al., 2010), which are the site of formation of calcium stones, together with the participation of estrogens in the maintenance of local calcium homeostasis, it is important to address the expression of ERα and ERβ in the epididymal region of roosters with epididymal lithiasis in order to better understand the mechanism of calcium stone formation.

Paracellular calcium transport and its putative association with epididymal lithiasis

Calcium transport may also occur to a lesser extent through the paracellular pathway. If we consider only the paracellular pathway, calcium would preferentially be transported towards the efferent ductule lumen, following the blood-to-lumen electrochemical gradient, a process that — at least under normal conditions — would be avoided or finely regulated by the tight junctions present between the efferent ductule epithelial cells (Ozegbe et al., 2006), similar to the regulation found in mammalian tissues (Hoenderop et al., 2005). If the epithelial junctional complexes in efferent ductules affected by lithiasis became leaky, they would serve as a passage for calcium to the lumen, increasing the luminal concentration of this ion and consequently creating a microenvironment favorable to calcification (Fig. 3B). The presence of leakier junctional complexes in the efferent ductules of roosters affected by epididymal lithiasis has already been proposed as the source of luminal/mucosal substance exchange (Jackson et al., 2006).

The expression of proteins responsible for the assembly of the junctional complexes, including key proteins required for the maintenance of tight junctions, adherens junctions and desmosomes, is highly dependent on hormonal regulation. Not surprisingly, vitamin D3, estrogens and androgens and their respective receptors are important factors in this regulation. Vitamin D3/VDR signaling has been shown to alter the expression profiles of ZO-1, E-cadherin and claudins (Cldn), as Cldn2 and Cldn12, but not Cldn7 and Cldn15 (Fujita et al., 2008; Kong et al., 2008). Furthermore, VDR knockout mice present decreased expression of Cldn1, Cldn5, E-cadherin and ZO-1 and increased expression of Cldn3 (Fujita et al., 2008; Kong et al., 2008). Together, these alterations might explain the increased susceptibility of VDR knockout mice to mucosal injury (Kong et al., 2008) and the induction of paracellular calcium transport as seen in different intestinal segments (Fujita et al., 2008).

In addition, estrogens and androgens also play a role in the modulation of junctional complex assembly. Estrogens modulate the expression of occludins, α-catenin, plectin, α-spectrin and β-spectrin. Both the decrease in estrogen levels and the disruption in estrogen/ERβ signaling in βERKO knockout mice result in the impairment of cell–cell adhesion and the consequent increase in paracellular permeability (Wada-Hiraike et al., 2006; Braniste et al., 2009). Androgen levels are also related to the modulation of testicular Cldn proteins, as Cldn1 decreases after puberty, while Cldn11 increases after puberty, paralleling the increase in testosterone levels (Park et al., 2010).

Considering the complex dependence of junctional adhesion complexes on different hormones and the altered VDR and AR expression patterns within the epididymal region of roosters with epididymal lithiasis, one cannot rule out the possibility that paracellular calcium reabsorption is altered in these animals and contributes to the formation of luminal calcium stones.

Concluding remarks and future perspectives

Studies of epididymal lithiasis have revealed that this reproductive dysfunction is definitely more complex than originally thought. Currently, none of the hypotheses presented can fully explain the development of epididymal lithiasis. Each of the proposed mechanisms may contribute to the formation of luminal calcium stones, but a synergistic action cannot be ruled out. Future studies proposing to further investigate hormonal imbalances in affected roosters to address the roles of the epididymal alterations described to date, including mononuclear cell infiltration and altered VDR and AR expression patterns, and to assess the estrogen responsive system and calcium reabsorption in the efferent ductules of roosters are critical to understand this intriguing reproductive anomaly.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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ARTIGO 4

Oliveira, AG et al. Involvement of the Transepithelial Calcium Transport Disruption in the Formation of Epididymal Stones in Roosters. **Reproduction** *(submitted)*

Involvement of the Transepithelial Calcium Transport Disruption and the Formation of Epididymal Stones in Roosters

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Abstract

Epididymal lithiasis is a dysfunction of unknown origin characterized by the formation of calcium stones into the lumen of efferent ductules of roosters. Affected animals present an imbalance in the hormonal responsive systems that regulate the expression of proteins involved in the transepithelial calcium transport, as TRPV6, CaBP-D28K, NCX1 and PMCA. Because the efferent ductules are the major site of fluid and calcium reabsorption in excurrent ducts, it was hypothesized that impairment in local calcium homeostasis would lead to lithiasis. To test this hypothesis, we addressed the expression of these proteins in the epididymal region of affected animals. The study focused on the investigation of the occurrence, tissue distribution and physiological impact of the transepithelial calcium transport in roosters under normal and pathological conditions. The results showed that affected roosters presented a significant increase in TRPV6 and CaBP-D28k levels, whereas NCX1and PMCA were not changed. Such alterations were more conspicuous in the proximal efferent ductules, in which was also observed accumulation of calcium within the epithelial cells. These findings provided the first evidences for the involvement of alteration in the expression of proteins essential for calcium reabsorption as a plausible mechanism for the formation of calcium stones within efferent ductules.

Introduction

The epididymal region of roosters is located at the dorso-medial surface of the testis, being composed by the rete testis, efferent ductules, connecting ducts and epididymal duct (King 1975, Aire 1979a, Aire 2000, Oliveira & Oliveira 2011). Among these components, the efferent ductules are the most conspicuous segment as they compose about 25% to 60% of the epididymal region (Aire 1979b, Clulow & Jones 1988, Oliveira *et al.* 2011b). The efferent ductules of birds are involved in the reabsorption of the bulk of testicular fluid as well as calcium (Clulow & Jones 1988, Clulow & Jones 2004) and are considered to play important roles in the maintenance of fertility. Therefore, alterations in the functions of these ductules may negatively impact the reproductive success of roosters.

In this regard, the epididymal lithiasis is a reproductive dysfunction described in the epididymal region of roosters, which is characterized by the formation of abundant luminal calcium stones (Janssen *et al.* 2000, Mahecha *et al.* 2002). It has been consensual in the literature related to the epididymal lithiasis that the efferent ductules are the segment most affected by this anomaly, as calcium stones are formed and located in the lumen of the ductules, that also show more drastic morphological alterations (Janssen *et al.* 2000, Mahecha *et al.* 2002, Boltz *et al.* 2004, Boltz *et al.* 2006, Jackson *et al.* 2006, Oliveira *et al.* 2008, Oliveira *et al.* 2011b). Although the primary cause of epididymal lithiasis is not yet elucidated, considering that (1) calcium is the major component of the stones, and (2) the efferent ductules are major site for calcium reabsorption in the epididymal region, it has been hypothesized that an imbalance in local calcium homeostasis may be involved in the formation of the luminal calcium stones (Oliveira *et al.* 2008, Oliveira *et al.* 2011b, Oliveira & Oliveira 2011).

Calcium transport across the epithelium may occur by the movement of the ion between the intercellular spaces – the paracellular pathway – or throughout the epithelial cell cytoplasm – the transepithelial pathway. The transepithelial calcium reabsorption depends on proteins that mediate the apical calcium entry within the cell, named TRPV5 and TRPV6 (Transient Receptor Potential Vanilloid channel), the diffusion of the ion throughout the cytoplasm bound to calcium binding proteins (CaBP), such as CaBP-9K or CaBP-D28K, and its extrusion to the extracellular environment in the basolateral membranes of the cells by the Na^{+}/Ca^{2+} exchanger (NCX) or the Plasma Membrane calmodulin dependent Calcium ATPase (PMCA) (Bindels *et al.* 1991, Van Baal *et al.* 1996, Hoenderop *et al.* 2002, Hoenderop *et al.* 2005, Bar 2009). This process is orchestrated by several hormones that regulates the expression and activity of the aforementioned proteins, including vitamin D3, androgens and estrogens (Cai *et al.* 1993, Hoenderop *et al.* 2001, Hoenderop *et al.* 2002, Van Abel *et al.* 2002, Dick *et al.* 2003, Hoenderop *et al.* 2005, Meyer *et al.* 2007, Oz *et al.* 2007, Suzuki *et al.* 2008, Hsu *et al.* 2010, Yang *et al.* 2011).

Interestingly, the efferent ductules of roosters express high levels of vitamin D3 receptor (VDR), androgen receptor (AR) and estrogen receptors (ESR1 and ESR2 – also known as ERα and ERβ, respectively) (Kwon *et al.* 1997, Dornas *et al.* 2007, Dornas *et al.* 2008, Oliveira *et al.* 2011a). Roosters affected by epididymal lithiasis expressed significantly higher levels of VDR and ESR2 in the efferent ductules, whereas AR and ESR1 proteins were not altered (Oliveira *et al.* 2008, Oliveira *et al.* 2011b). Moreover, it was shown that affected animals also present alterations in the concentration profiles of hormones that bind to these receptors, as in the epididymal region of these animals estradiol

levels were increased whereas vitamin D3 and testosterone concentrations were decreased (Oliveira *et al.* 2011b).

Therefore, based on these information and considering that the responsive systems of hormones that are essential for the maintenance of calcium homeostasis are altered in the epididymal region of roosters, the present study aimed to investigate the expression of TRPV6, CaBP-D28K, NCX1 and PMCA, that directly mediate calcium transport across the epithelium, to further elucidate the cause of calcium stones formation within the lumen of efferent ductules.

Results

Western blotting assays of the total protein extract of the epididymal region of roosters showed major protein bands of 81 kDa, 28 kDa, 125 kDa and 120 kDa for TRPV6, CaBP-D28K, PMCA and NCX1, respectively (Fig. 1). These molecular weights are in agreement with those previously reported in the literature (de Talamoni *et al.* 1993, Hsu *et al.* 2010, Kennedy *et al.* 2010). When compared to non-affected animals, roosters with epididymal lithiasis presented altered expression of the calcium transporting proteins. The TRPV6 expression within the epididymal region of roosters affected by epididymal lithiasis increased significantly (75%), whereas CaBP-D28K levels were increased in 13% (Fig. 1A, B and E, F). On the other hand, no differences in PMCA and NCX1 expression were found in the epididymal region of affected animals (Fig. 1C, D and G, H).

Within the epididymal region, positivity for TRPV6 ($TRPV6^{+}$) was restricted to the apical membrane of the efferent ductule non-ciliated cells (Fig. 2). In the proximal efferent ductules, few $TRPV6^{+}$ cells were detected, contrasting with the distal efferent ductules in which most of non-ciliated cells were positive for this protein (Fig. 2A and C). The ciliated

cells of the efferent ductules as well as the epithelial cells lining the extratesticular rete testis (not shown) and epididymal duct were not immunoreactive for TRPV6 (Fig. 2E). The immunohistochemical analyses revealed a drastic increase in the frequency of $TRPV6^{+}$ cells in the proximal efferent ductules (Fig. 2B and H), thus confirming the Western blotting results. Whilst only about 10% of the non-ciliated cells of the proximal efferent ductules were stained for TRPV6 in non-affected animals, positivity for this protein was found in 75% of the non-ciliated cells of affected roosters (Fig. 2H). No apparent alterations in TRPV6 expression were detected in the rete testis, distal efferent ductules and epididymal duct (Fig. 2D and F).

CaBP-D28K was widely expressed within the epididymal region, as positivity for this protein was found in the cytoplasm of the epithelial cells lining the rete testis, efferent ductules and epididymal duct (Fig. 3). In the efferent ductules, strong positivity for CaBP-D28K was found in the ciliated cells, whereas non-ciliated cells were slightly stained (Fig. 3A and C). Within the epididymal duct, positive staining for CaBP-D28K was observed in the epithelial principal cells (Fig. 3E). Regarding CaBP-D28K expression, affected roosters showed evident alteration in the pattern of distribution within the epididymal region. In the efferent ductules of these animals, the non-ciliated epithelial cells expressed higher levels of CaBP-D28K, both in the proximal and distal region of these ductules (Fig. 3B and D), when compared to non-affected animals. No apparent alterations were found in the immunoreaction for this protein in the ciliated cells or in the epithelial cells of other segments composing the epididymal region (Fig. 3F). A noteworthy finding was that a high number of cells composing mononuclear cell infiltrates, commonly found in the connective tissue of affected animals, were positive for CaBP-D28K (Fig. 3, insert in D).

The expression of NCX1 was restricted to the proximal efferent ductule epithelia (Fig. 4). Among the epithelial cells, positivity was found in the basolateral membrane of some non-ciliated cells with an apparent higher concentration of this protein within the basal region (Fig. 4A). The non-ciliated cells of the distal efferent ductules and ciliated cells of both segments as well as the epithelia lining the rete testis and epididymal duct were unreactive for NCX1 (Fig. 4A, C and E). This pattern of expression was not altered in roosters affected by epididymal lithiasis (Fig. 4B, D and F).

Considering the alterations in the levels of proteins important for calcium transport across the epithelia in affected animals, alizarin red staining of epididymal region sections was used to investigate whether calcium could be depositing within the tissue. This histochemical analysis revealed alizarin red positive granules at the basal region in the non-ciliated cells of the affected proximal efferent ductules, contrasting with the slight stain observed in non-affected ductules (Fig. 5A and B). Also, as expected, the luminal calcium stones were strongly stained by alizarin red (Fig. 5C).

Discussion

Here, we showed that TRPV6, CaBP-D28K, PMCA and NCX1 proteins, all of them involved in the transport of calcium across the epithelia, were present in the epididymal region of roosters. Roosters with epididymal lithiasis presented distinct pattern of expression of these proteins, suggesting that an imbalance in calcium homeostasis may be related to the formation of the luminal calcium stones. These are the first findings pointing out a possible molecular mechanism to explain the origin of the epididymal lithiasis, a disorder that drastically decreases the fertility in roosters.

The present study described that the classical proteins involved in calcium reabsorption – TRPV6, CaBP-D28K, PMCA and NCX1 – are expressed within the epididymal region of roosters, providing, therefore, the putative molecular basis to guarantee calcium homeostasis in the excurrent ducts of birds. Under physiological conditions, it was observed that positivity for TRPV6 and NCX1 was restricted, respectively, to the apical and basolateral membrane of the non-ciliated cells of the efferent ductules among the ducts composing the epididymal region. The cytoplasm of these cells was also slightly positive for CaBP-D28K. These results corroborate the finding that the bulk of calcium reabsorption occurs in the efferent ductules (Clulow & Jones 2004) and reinforces the hypothesis that these cells are the major reabsorptive cells within the efferent ductules of birds (Aire 1980). The maintenance of the correct concentrations of calcium within the luminal fluid in the excurrent ducts is essential for male fertility. In this regard, transgenic male mice that encode TRPV6 with a mutated pore or that are knocked out for PMCA are infertile due to high concentrations of calcium within the epididymal lumen resulting in impaired capacity of fertilization or in the presence of low motile sperm (Schuh *et al.* 2004, Weissgerber *et al.* 2011).

Roosters affected by epididymal lithiasis had increased levels of TRPV6 and CaBP-D28K but not PMCA and NCX1. It is known that modulation of expression of these proteins are under control of different hormones, such as vitamin D3, estrogens and androgens (Cai *et al.* 1993, Van Abel *et al.* 2002, Kip & Strehler 2004, Hoenderop *et al.* 2005, Oz *et al.* 2007, Hsu *et al.* 2010). On this sense, we showed recently that roosters with lithiasis presented altered levels of vitamin D3, estradiol and testosterone that paralleled changes in their receptors (VDR, ESR2 and AR, respectively) within the epididymal region (Oliveira *et al.* 2011b). The vitamin D3 and estradiol responsive systems are directly

correlated with TRPV6 and CaBP-D28K expression levels (Hoenderop *et al.* 2001, Hoenderop *et al.* 2005, Oz *et al.* 2007). However, the hormonal effect on the calcium extrusion step is less clear, although it has been shown that vitamin D3 stimulates PMCA expression in the basolateral membranes of reabsorptive cells in the intestine and kidney (Cai *et al.* 1993, Kip & Strehler 2004). Recently, it was demonstrated that testosterone inhibits the expression of TRPV5, CaBP-D28K and PMCA (Hsu *et al.* 2010). Therefore, the hormonal imbalance observed in roosters affected by epididymal lithiasis may be related to the altered pattern of expression of the proteins involved in the transepithelial calcium transport (Oliveira *et al.* 2011b).

One hypothesis to explain the origin of the epididymal lithiasis was that disruption of local calcium homeostasis would culminate in the formation of the luminal calcium stones (Mahecha *et al.* 2002, Oliveira *et al.* 2011b, Oliveira & Oliveira 2011). The present results corroborate this hypothesis and provide a possible molecular mechanism that could be involved with the origin of such reproductive disorder (Fig. 6). According to the most accepted model for the calcium transport across the epithelia, calcium enters the cell through specific epithelial calcium channels (TRPV5 or TRPV6), moves throughout the cytoplasm bound to calcium binding proteins (CaBP-D9k or CaBP-D28K) and is extruded to the extracellular medium by the action of a membrane calcium ATPase (PMCA) and/or a sodium-calcium exchanger (NCX1) (Hoenderop *et al.* 2005). The first steps – calcium entry and diffusion – occur following an electrochemical gradient directed from the lumen to the cell cytoplasm and, therefore are considered as passive steps (den Dekker *et al.* 2003, Hoenderop *et al.* 2005). On the other hand, the extrusion step is an active process as it occurs against an electrochemical gradient (Hoenderop *et al.* 2005). On this sense, based on the fact that only TRPV6 and CaBP-D28K expression are increased within the epididymal

region, it is possible that the calcium transport is saturated, especially considering that the levels of the proteins involved in the extrusion of the ion from the cell do not increase in the same rate. In line with this interpretation is the presence of Alizarin red granules at the basal region of the cells, which suggests the concentration of calcium in this region of the cell. This concentration would result in the dissipation of the inward electrochemical gradient that drives calcium into the cell with the consequent reduction of calcium reabsorption in the efferent ductules (Fig. 6). Finally, calcium is possibly accumulating within the lumen of the efferent ductules and forming nucleating centers to its aggregation into the luminal stones.

Another interesting finding was the strong positivity for CaBP-D28K found in the ciliated cells of the efferent ductules and in the epididymal duct principal cells, both of which were unreactive for the membrane calcium transporters TRPV6 and NCX1. Calbindin-D28K is a protein known to buffer intracellular calcium concentrations but it also serves as a calcium source (Schwaller 2009, Schwaller 2010). This dual role of calbindins within the cell allows the participation of this protein in other biological processes, such as movement of cilia and secretory function, which may be the case for the efferent ductule ciliated cells and epididymal principal cells, respectively (Oz *et al.* 2007, Schwaller 2009, Schwaller 2010).

In conclusion, this study provides evidence for the involvement of alterations in the expression of key proteins for calcium reabsorption in the formation of luminal calcium stones within the efferent ductules of roosters. The alterations presently found in the calcium handling may be a result of the local hormonal imbalance, such as overexpression of VDR and estrogen/ESR2 and reduction in testosterone, previously demonstrated in the epididymal region of rooster affected by the lithiasis.

Materials and methods

Animals and tissue preparation

The study was performed on the epididymal region of 18 adult cross breed roosters (*Gallus gallus*), between 1 to 2 years old, in reproductive activity. The animals were obtained from commercial sources and housed at the Universidade Federal de Minas Gerais facilities under natural conditions of light, temperature and humidity. Water and food (Socil III Guyomarc, Belo Horizonte, Brazil) were administered *ad libitum*. The experimental procedures were approved by the local ethical committee published by the CETEA/UFMG (http://www.ufmg.br/bioetica/coep).

The roosters were weighted, anesthetized (i.p. lethal dose of sodium pentobarbital 50 mg/Kg of body weight) and perfused intracardially with saline and 10% neutral buffered formalin (NBF) or only with saline solution for immunohistochemical and Western blotting analysis, respectively.

Diagnostic of epididymal lithiasis

Epididymal lithiasis was diagnosed by clearing fragments of the epididymal region with potassium hydroxide (KOH) at 0.5% (w/v) followed by immersion in glycerin solutions (1:2, 1:1 and pure glycerin) as previously described and validated (Oliveira *et al.* 2008, Oliveira *et al.* 2011b). Animals were classified as affected or non-affected based on the presence or absence of stones within the epididymal tissue, respectively.

Western Blotting

For Western blotting assays, fragments of the epididymal region of non-affected and affected animals (n = 5 per group) were frozen in liquid nitrogen and macerated with dry

ice to address the expression of the proteins of interest: TRPV6, CaBP-D28K, PMCA and NCX1. We focused on investigating TRPV6 and CaBP-D28K, instead of TRPV5 and CaBP-D9K, respectively, because the formers have been previously identified in the reproductive system, including in bird tissues (den Dekker *et al.* 2003, Jelinsky *et al.* 2007, Bar 2008, Bar 2009).

After total extraction, proteins were separated by continuous electrophoresis using 10% or 12.5% sodium dodecyl sulphate-polyacrilamide gels (for proteins with molecular weight between 140-70 kDa or less than 50 kDa, respectively). Then, the proteins were transferred to nitrocellulose membranes and non-specific binding sites in the membranes were blocked by using normal goat serum (NGS) diluted at 10%. The blots were incubated with the primary antibodies rabbit anti-human TRPV6 (Sigma-Aldrich, Saint Louis, USA), rabbit anti-rat CaBP-D28K (Sigma-Aldrich, Saint Louis, USA), mouse anti-human PMCA (Santa Cruz Biotechnology, Santa Cruz, USA) and mouse anti-rabbit NCX1 (Thermo Scientific, Rockford, USA) diluted at 1:500, 1:200, 1:500 or 1:1000 respectively. Following this step, the membranes were incubated with goat anti-rabbit (for TRPV6 and CaBP-D28K) or goat anti-mouse (for PMCA and NCX1) secondary biotinilated antibodies (Dako, Glostrup, Denmark) at 1:1000 and 1:5000 (for NCX1) and then with the avidin-biotin complex conjugated with peroxidase (Vector Laboratories, Burlingame, USA). After several washes with PBS-Tween 0.05% and pure PBS, the immunolabeling was visualized with a solution of 0.1% (w/v) 3,3' diaminobenzidine in PBS containing 0.05% (w/v) chloronaphtol , 16.6% (v/v) methanol and 0.04% (v/v) H_2O_2.

Immunohistochemistry

NBF fixed fragments of the epididymal region of roosters affected and non-affected by epididymal lithiasis were embedded in paraffin, sectioned at 5.0 µm and used for immunohistochemical analysis. The technique was performed in duplicates and, for comparison between animals and groups, the staining was run in parallel. After endogenous peroxidase activity blocking with 0.6% (v/v) H_2O_2 in methanol, sections were submitted to standard protocol of antigen retrieval in 0.01 M sodium citrate buffer pH 6.0 and microwave heating. Non-specific binding were blocked by incubation of the sections with avidin and biotin blocking kit (Vector Laboratories, Burlingame, USA) and 10% NGS. The slides were incubated with the primary rabbit anti-human TRPV6 (Sigma-Aldrich, Saint Louis, USA), rabbit anti-rat CaBP-D28K (Sigma-Aldrich, Saint Louis, USA) and mouse anti-rabbit NCX1 (Thermo Scientific, Rockford, USA) antibodies at the dilution of 1:500, 1:200 and 1:100, respectively. Then, sections were incubated with goat anti-rabbit (for TRPV6 and CaBP-D28K) and goat anti-mouse (for NCX1) secondary biotinilated antibodies (Dako, Glostrup, Denmark) diluted at 1:100 and then with the avidin-biotin complex conjugated with peroxidase (Vector Laboratories, Burlingame, USA). Negative controls were obtained by omitting the primary antibodies and incubation with the appropriate anti-IgG. The reaction was visualized by immersion in 0.05% 3,3'-diaminobenzidine solution containing 0.01% H_2O_2 in 0.05 M Tris-HCl buffer, pH 7.4. Finally, the sections were counterstained with Hematoxylin, dehydrated, washed in xylene and mounted.

Morphometry

The frequency of TRPV6 positive cells within the epididymal region was determined according to the literature (Oliveira *et al.* 2006). For this purpose, it was

counted the immunoreactive cells per 100 epithelial non-ciliated cells in 10 randomly selected longitudinal sections of the proximal efferent ductules.

Alizarin Red Stain

To investigate the occurrence of possible calcium deposits within the epididymal region, 5 µm sections of NBF fixed tissues were incubated with alizarin red stain for 3 minutes. The Alizarin red interaction with calcium results in an orange/red product that can be visualized in the microscope (McGee-Russell 1958, Mori *et al.* 2000).

Statistical analysis

Data about the expression of TRPV6, CaBP-D28K, PMCA and NCX1 as well as the frequency of TRPV6 positive cells in the epididymal region of roosters non-affected and affected by epididymal lithiasis were statistically analyzed to detect possible differences between groups. For this purpose, the normal distribution of the data and the homogeneity of variances were tested prior to the realization of the Student's t-test. Differences were considered significant at $P \leq 0.05$.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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473 **Figure legends**

474

475 **Figure 1:** Western Blotting analysis of TRPV6 **(A)**, CaBP-D28K **(B)**, PMCA **(C)** and
476 NCX1 **(D)** within the epididymal region of roosters non-affected and affected by
477 epididymal lithiasis. Graphical representation of the image analysis of TRPV6, CaBP-
478 D28K, PMCA and NCX1 expression **(E-H**, respectively**)**. n = 5; * $P \leq 0.05$.

479

480 **Figure 2:** Immunodetection of TRPV6 in the epididymal region of roosters non-affected
481 **(A, C, E)** and affected **(B, D, F)** by epididymal lithiasis. PED = proximal efferent ductules;
482 DED = distal efferent ductules; EP = epididymal duct; black arrows = TRPV6 positive non-
483 ciliated cells; white arrows = TRPV6 negative non-ciliated cells; arrowheads = ciliated
484 cells; bar in A-F = 50 µm. Inserts in A-D = details of the positivity of non-ciliated cells and
485 the negative ciliate cells of the efferent ductules. **(G)** Negative control of the
486 immunostaining highlighting the non-ciliated cells (arrow) and ciliated cells (arrowhead).
487 **(H)** Frequency of TRPV6 positive and negative cells ($TRPV6^+$ and $TRPV6^-$, respectively)
488 in the proximal efferent ductules of roosters affected and non-affected by lithiasis.

489

490 **Figure 3:** Immunodetection of CaBP-D28K in the epididymal region of roosters non-
491 affected **(A, C, E)** and affected **(B, D, F)** by epididymal lithiasis. PED = proximal efferent
492 ductules; DED = distal efferent ductules; EP = epididymal duct; arrows = non-ciliated cells;
493 arrowheads = ciliated cells; Bar in A-F = 50 µm. Insert in C = negative control of
494 immunostaining. Insert in D = detail of the positivity of cells within the mononuclear
495 infiltrates.

496

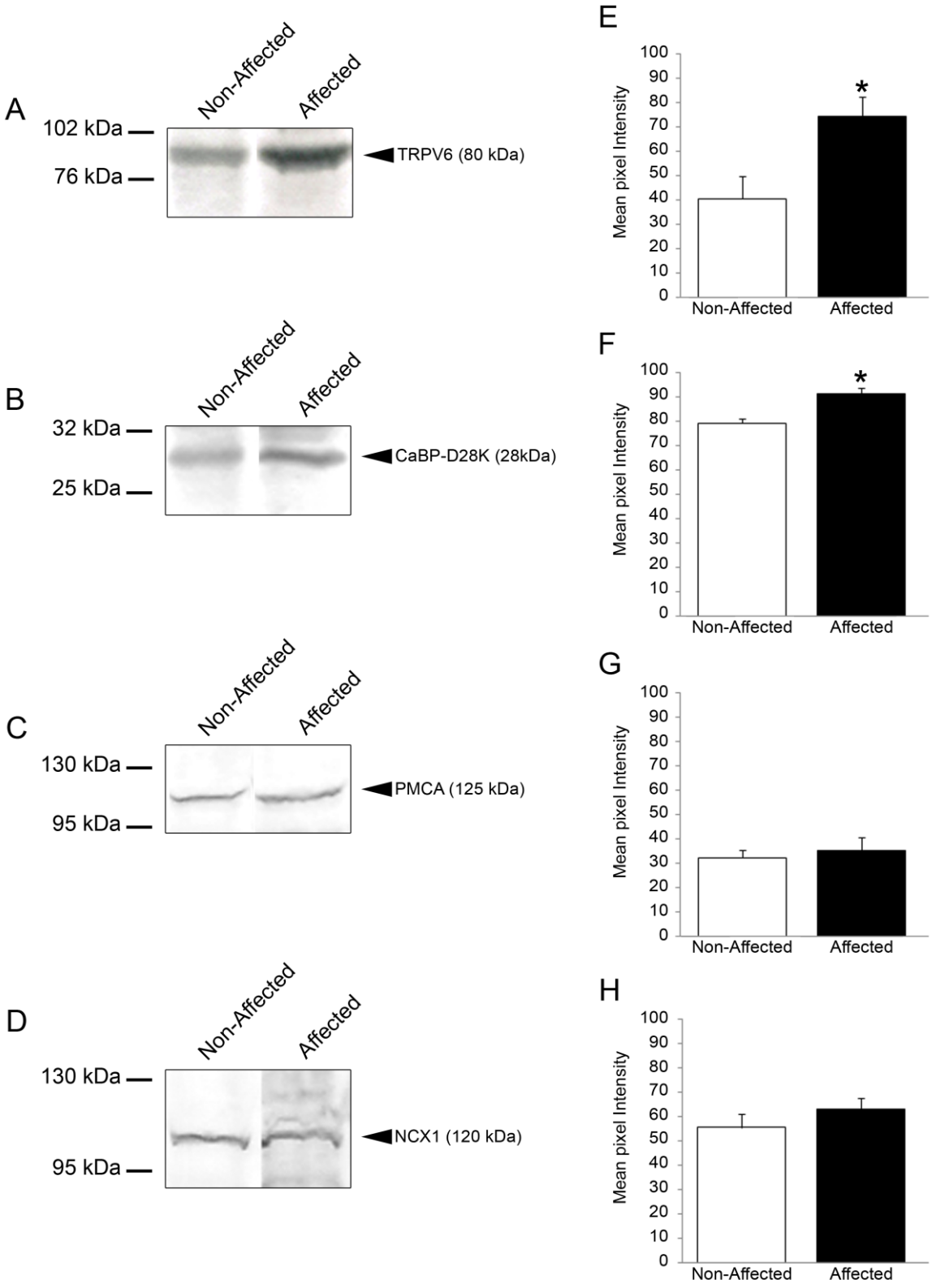
Figure 4: Immunodetection of NCX1 in the epididymal region of roosters non-affected **(A, C, E)** and affected **(B, D, F)** by epididymal lithiasis. PED = proximal efferent ductules; DED = distal efferent ductules; EP = epididymal duct; black arrows = NCX1 positive non-ciliated cells; white arrows = NCX1 negative non-ciliated cells; Bar in A-F = 50 µm. Insert 1 in A = negative control of immunostaining; Insert 2 in A and Insert in B = details of the positivity for NCX1 in the non-ciliated cells of the proximal efferent ductules.

Figure 5: Alizarin red stain of the epididymal region of roosters non-affected **(A)** and affected **(B)** by epididymal lithasis. A positive calcium stone is shown **(C)**. Arrowheads = alizarin-red positive granules; * = calcium luminal stone within the efferent ductule. Inserts in A and B show the dashed area in higher magnitude and highlights the occurrence and localization of the alizarin-red positive granules within the basal region of the non-ciliated cells of affected animals.

Figure 6: Working hypothesis for luminal calcium stone formation in the efferent ductules of roosters. In roosters non-affected by epididymal lithiasis (cell on left), normal calcium reabsorption across the proximal efferent ductule epithelium (arrow 1) is mediated by TRPV6, CaBP-D28K, NCX1 and/or PMCA (not shown). Roosters affected by epididymal lithiasis showed higher levels of TRPV6 and CaBP-D28K, but not NCX1 and PMCA proteins. Therefore it is possible that there is an increase in calcium entry within the cell without a parallel increase in its extrusion (arrow 2). This alteration would result in the saturation of the transepithelial calcium transport with consequent decrease in overall calcium reabsorption (arrow 3). This hypothesis is reinforced by the observation of calcium accumulation within the basal region of epithelial cells (orange). Finally, luminal calcium

521 concentrations would be higher in affected animals, serving as nucleating centers for its
522 aggregation into stones.
523

524 **Figure 1**



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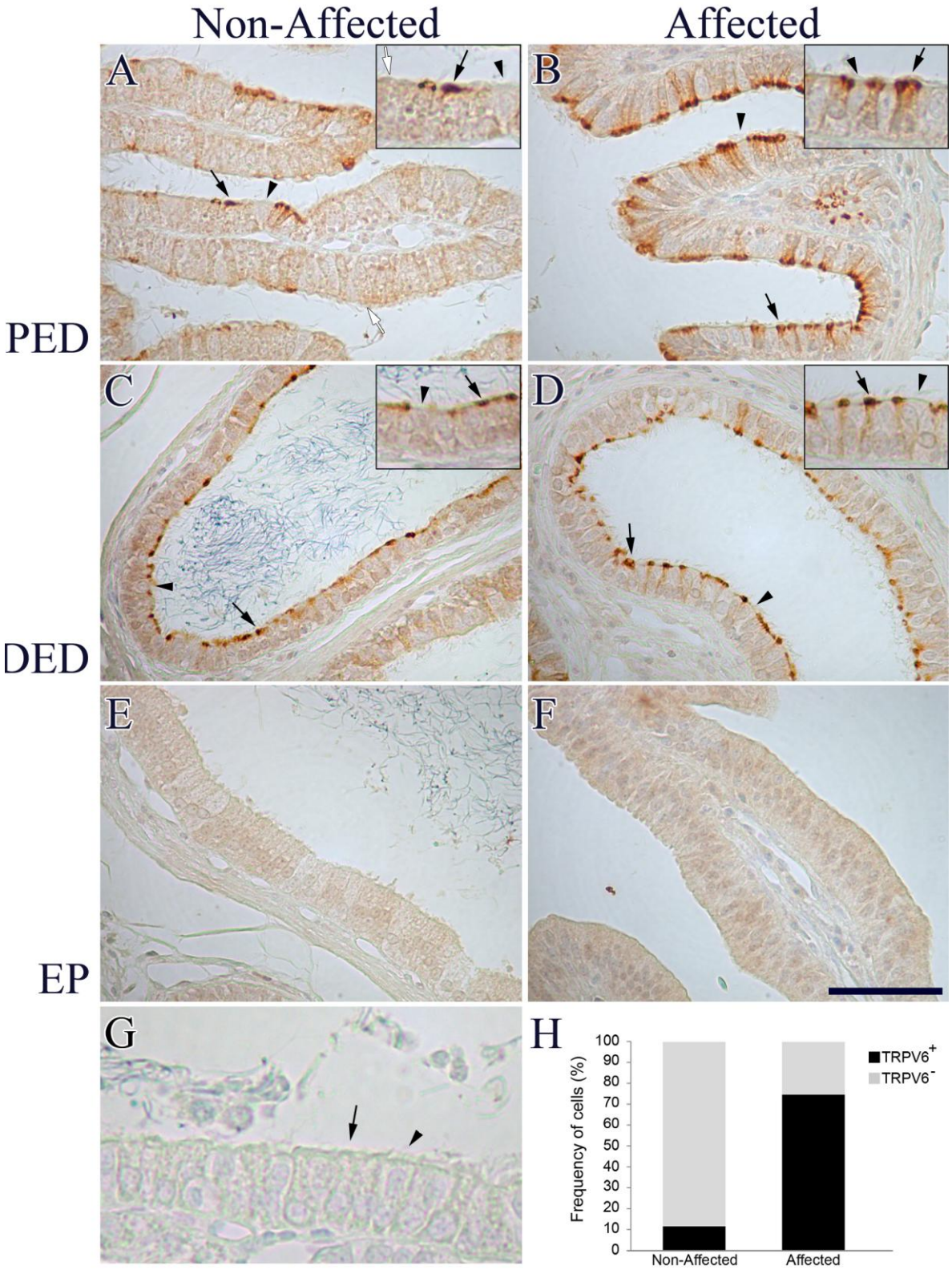
526

527 **Figure 2**



528

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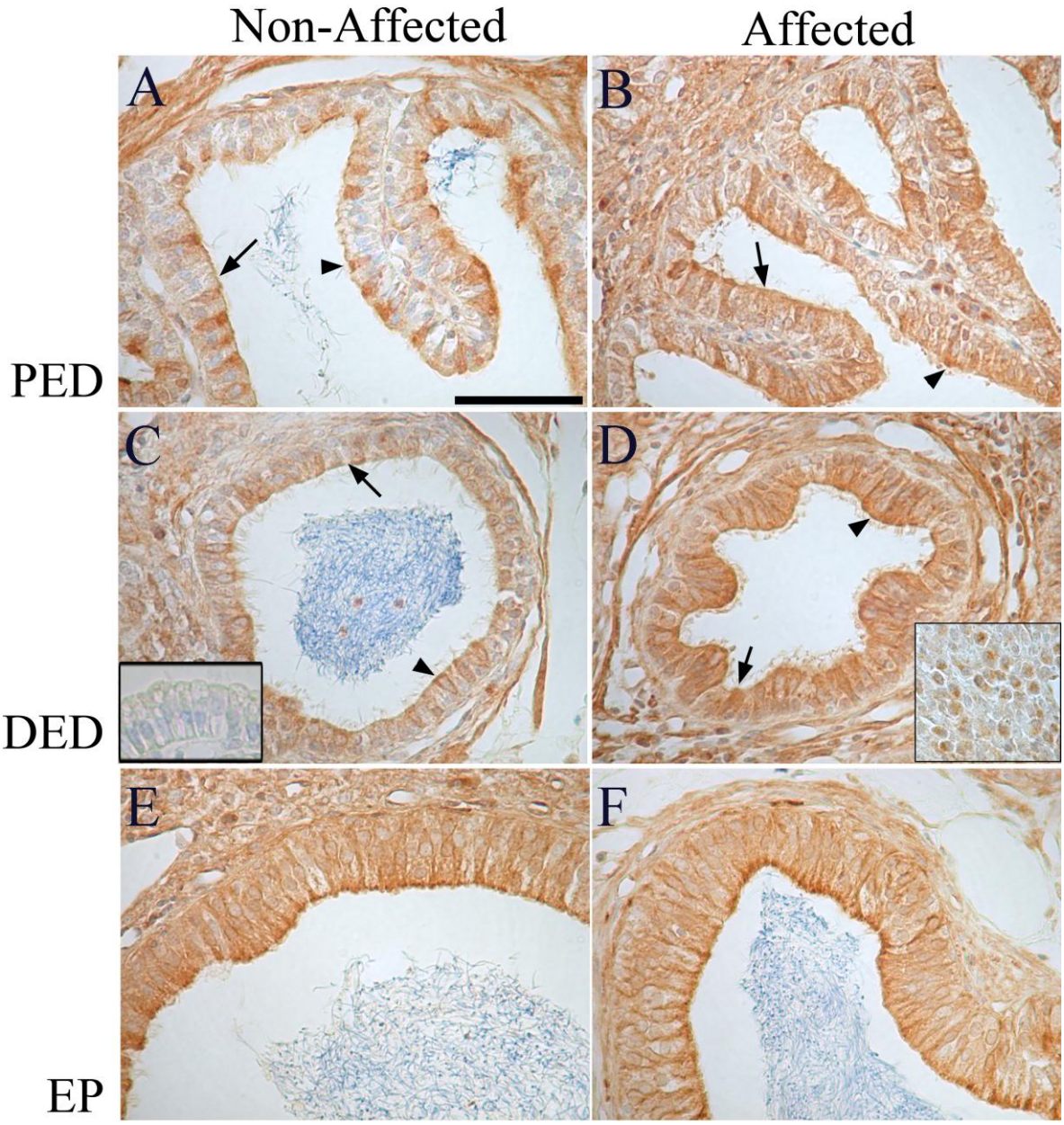
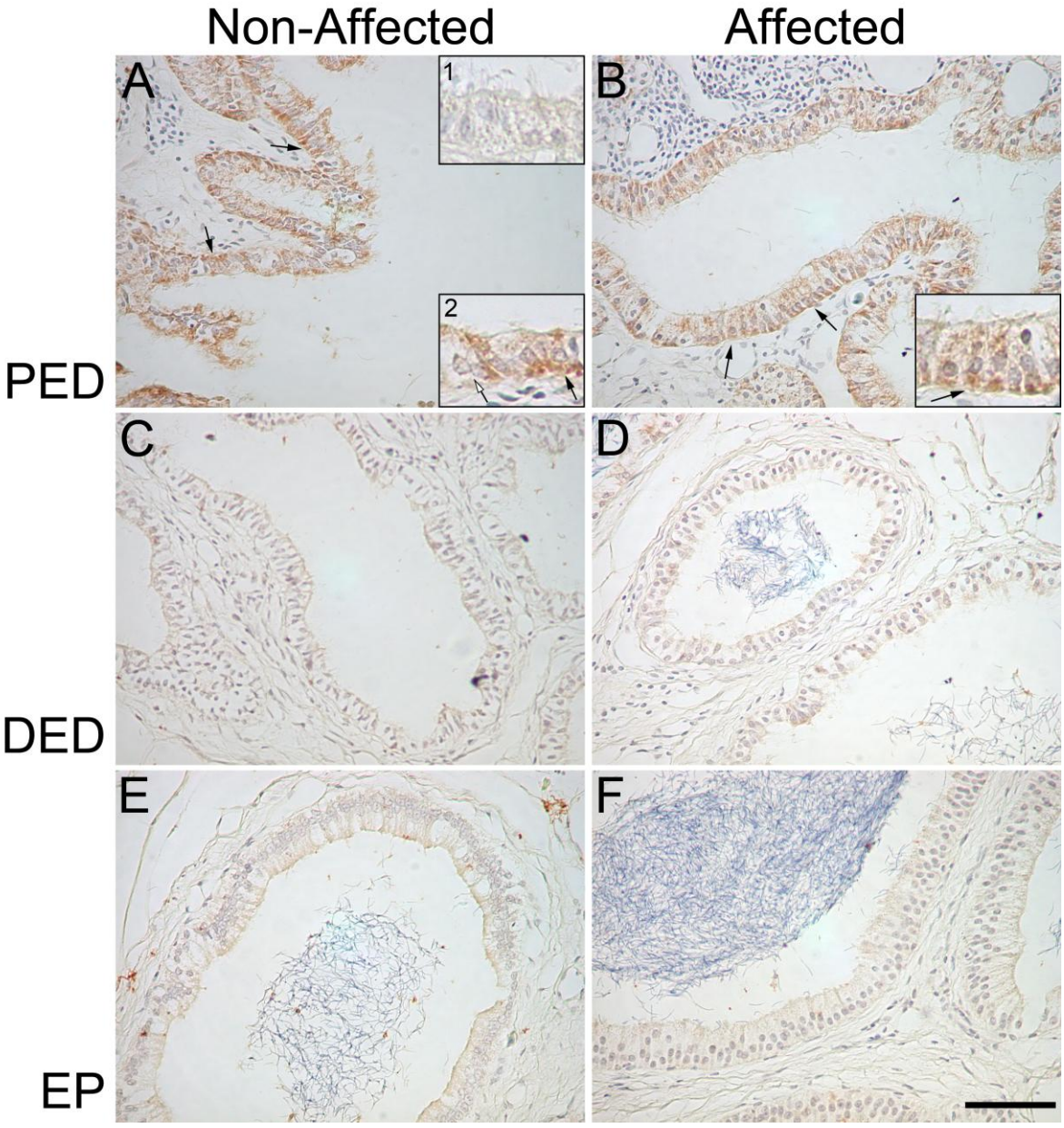
Figure 3



533 **Figure 4**



534

535

536 **Figure 5**

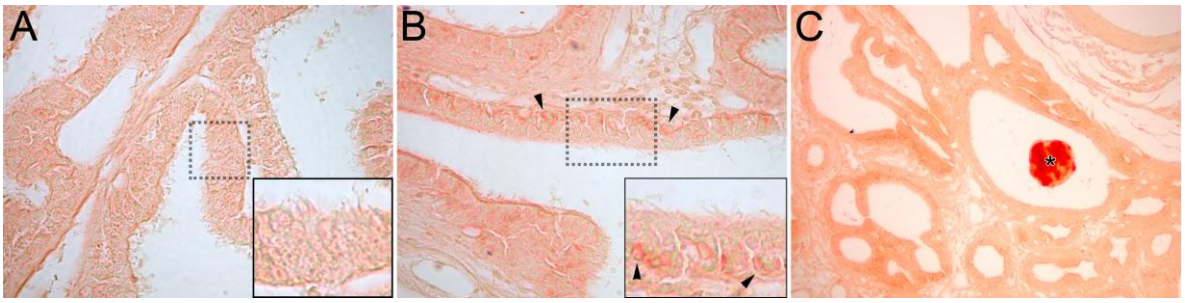


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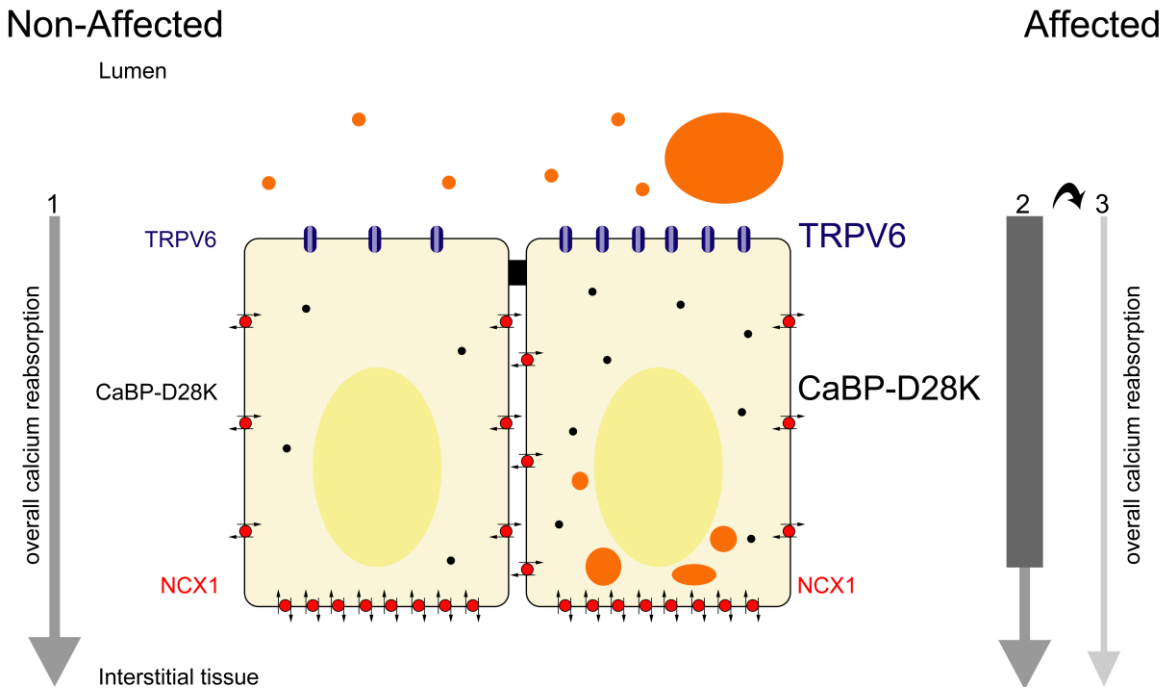
Figure 6


Non-Affected
Affected
Lumen
1
2
3
TRPV6
TRPV6
CaBP-D28K
CaBP-D28K
NCX1
NCX1
overall calcium reabsorption
overall calcium reabsorption
Interstitial tissue


IV
Discussão e Conclusão

IV- DISCUSSÃO E CONCLUSÃO

O presente estudo demonstrou que os receptores de estrógenos ERα e ERβ (também conhecidos como ESR1 e ESR2, respectivamente) são expressos no testículo e região epididimária de galos, porém apresentando diferente distribuição dependendo do tipo celular e da região considerada. Esses resultados sugerem que tanto os testículos quanto as vias extratesticulares podem apresentar respostas diferentes quanto à sinalização mediada via receptores de estrógenos. O papel exato dos estrógenos na manutenção da morfofisiologia da região epididimária de galos ainda não é conhecido, mas é possível que o sistema responsivo a estrógenos possa regular a reabsorção do fluido testicular pelos dúctulos eferentes pela regulação de proteínas chave para o processo reabsortivo, como aquaporinas e o trocador Na+/H+ (NHE3), em um processo semelhante ao descrito em mamíferos eutérios (Zhou et al, 2001; Lee et al, 2001; Oliveira et al, 2002; Zaniboni et al, 2004; Oliveira et al, 2005; Bahr et al, 2006; Picciarelli-Lima et al, 2006; Ruz et al, 2006).

Em animais afetados pela litíase epididimária, foram detectadas alterações no padrão de expressão de receptores de estrógenos, visto que os níveis de ERβ foram aumentados, enquanto os de ERα foram semelhantes ao de animais não-afetados. Paralelamente a essas alterações, foi observado aumento nas concentrações de estradiol na região epididimária em animais afetados. Em adição, os níveis tissulares de vitamina D3 e testosterona apresentaram reduzidos nesses animais. Considerando que uma característica da região epididimária de galos é seu envolvimento na reabsorção do fluido testicular e também de íons cálcio (Clulow & Jones, 1988; Clulow & Jones, 2004), é possível que o desequilíbrio hormonal em animais afetados pela litíase epididimária revelado por alterações significativas nos sistemas responsivos a estrógenos, vitamina D3 e andrógenos, possa estar relacionado à formação dos cálculos ricos em cálcio no lúmen dos dúctulos eferentes. Essa hipótese é reforçada pelo importante papel desempenhado por esses hormônios e seus receptores na modulação de proteínas importantes que atuam no transporte transepitelial de cálcio (Cai et al, 1993, Hoenderop et al, 2001, Kip & Strehler, 2004; Hoenderop et al, 2005, Oz et al, 2007; Hsu et al, 2010; Yang et al, 2011).

Dessa forma, visando compreender o impacto fisiológico da desregulação hormonal supracitada, bem como investigar a hipótese da alteração na homeostase de

cálcio como possível origem da formação dos cálculos no lúmen dos dúctulos eferentes de animais afetados pela litíase, estudamos a expressão das proteínas que medeiam o transporte transepitelial de cálcio, a saber: TRPV6, CaBP-D28K, NCX1 e PMCA. Todas essas proteínas foram expressas na região epididimária de galos. Em condições fisiológicas, as expressões de TRPV6 e NCX1 foram restritas, respectivamente, às membranas apicais e basolaterais das células não-ciliadas dos dúctulos eferentes. Por outro lado, a proteína CaBP-D28K apresentou distribuição mais ampla, sendo detectada no epitélio da rede testicular, dúctulos eferentes e ducto epididimário. Esses dados reforçam a hipótese de que a região epididimária de galos, sobretudo o segmento dos dúctulos eferentes, é o principal local de reabsorção de cálcio (Clulow & Jones, 2004), uma função de grande importância para a manutenção da fertilidade masculina (Schuh et al, 2004; Weissgerber et al, 2011).

Animais afetados pela litíase epididimária apresentaram aumentos significativos na expressão de TRPV6 e CaBP-D28K, sobretudo na região dos dúctulos eferentes proximais. Por outro lado, a expressão dos transportadores NCX1 e PMCA não foi alterada. Em adição, animais afetados apresentaram grânulos positivos ao vermelho de Alizarina localizados na região basal dos dúctulos eferentes proximais, sugerindo acúmulo de cálcio nessas células. Uma das hipóteses postuladas para explicar a origem da litíase epididimária é que a alteração na homeostase local de cálcio poderia culminar na formação dos cálculos luminais (Oliveira et al, 2008). De acordo com o modelo de transporte transepitelial com maior aceitação, os íons cálcio entram na célula através de canais de cálcio específicos (TRPV6), são transportados através do citoplasma da célula por proteínas ligadoras de cálcio (como a CaBP-D28K) e liberados no meio extracelular por transportadores específicos (NCX1 e PMCA) (Hoenderop et al, 2005). As etapas de entrada e difusão pelo citoplasma são passivas, enquanto a extrusão do cálcio para o meio extracelular é ativa (Hoenderop et al, 2005). Dessa forma, considerando que animais afetados pela litíase epididimária apresentam aumento na expressão de TRPV6 e CaBP-D28K e níveis normais de NCX1 e PMCA, nossa hipótese corrente é que o processo de transporte de cálcio pelo epitélio pode estar saturado. A presença de grânulos de vermelho de Alizarina no epitélio corrobora essa hipótese. Essa concentração resultaria na dissipação do gradiente eletroquímico que move o cálcio para dentro da célula, com a consequente redução no transporte desse íon. Consequentemente, esse íon poderia se acumular no lúmen dos dúctulos eferentes formando centros de nucleação para a formação dos cálculos de cálcio luminais.

Em suma, os resultados indicam que alterações na homeostase de cálcio na região epididimária de galos afetados pela litíase epididimária, decorrentes da desregulação hormonal observada nesses animais, estão diretamente implicadas com a formação de cálculos de cálcio no lúmen dos dúctulos eferentes.

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