MARIANA MORAES DE CASTRO

DESCRIÇÃO MORFOLÓGICA, HISTOMORFOMÉTRICA E ULTRAESTRUTURAL DO EPITÉLIO EPIDIDIMÁRIO DO MORCEGO VAMPIRO *Desmodus rotundus*

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Biologia Celular e Estrutural, para obtenção de título de *Doctor Scientiae*.

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(Orientadora)

“ Que a importância de uma coisa não se mede com fita métrica nem
com balanças nem barômetros etc.
Que a importância de uma coisa há que ser medida pelo
encantamento que a coisa produza em nós.”
Manoel de Barros

Aos meus grandes amores, Teresina,
André Luiz e José Ronaldo.

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RESUMO

CASTRO, Mariana Moraes de, D.Sc., Universidade Federal de Viçosa, abril de 2016. **Descrição morfológica, histomorfométrica e ultraestrutural do epitélio epididimário do morcego vampiro *Desmodus rotundus***. Orientador: Mariana Machado Neves. Coorientadores: Mariella Bontempo de Freitas e Maria do Carmo Queiroz Fialho.

Existem poucos estudos sobre a caracterização do sistema reprodutor masculino, especialmente o epidídimo, em morcegos neotropicais. Estudos prévios em morcegos, *Molossus molossus* e *Eumops glaucinus*, mostraram que o epitélio epididimário apresenta características similares a de outros mamíferos, quando analisados sob microscopia de luz. Porém, não foi encontrado nenhum trabalho caracterizando o epidídimo do morcego vampiro comum, *Desmodus rotundus*. O epidídimo exerce diversas funções como concentração, maturação, transporte e estocagem do espermatozoide. Esse órgão é composto por epitélio pseudoestratificado, podendo ser dividido em quatro regiões, segmento inicial, cabeça, corpo e cauda. Assim, o objetivo desse trabalho foi caracterizar o epidídimo do morcego vampiro comum com base nos aspectos morfológicos, histomorfométricos e ultraestruturais, através do uso de microscopia de luz, epifluorescência e eletrônica. Para a imunohistoquímica, foram usados marcadores de diferentes tipos celulares, como aquaporina 9, citoqueratina 5 e H^+ V-ATPase, e proteínas de *tight junction*, como claudina 1, claudina 3, claudina 4 e zônula de oclusão 1. No presente estudo foi possível identificar a região proximal do epidídimo, segmento inicial e cabeça, e a região distal, corpo e cauda. Todas as regiões apresentaram epitélio pseudoestratificado composto por células principais, basais e claras. A altura do epitélio epididimário diminuiu a partir da região proximal para a distal, enquanto o diâmetro do ducto e o de interducto aumentaram. O compartimento do ducto foi o principal componente do epidídimo do morcego vampiro comum. Células principais apresentaram formato colunar com estereocílio na membrana apical da célula em contato com o lúmen e foram as mais frequentes.Ao longo de todo o epidídimo, foi observado marcação PAS-positiva, formando o glicocálice, e expressão da proteína aquaporina 9 no estereocílio. Em todas as regiões observamos grânulos PAS-positivo no citpolasma, além de características ultraestruturais relacionadas às funções de absorção e secreção. O segundo tipo celular mais observado foi a célula basal. Essas foram identificadas pela posição do núcleo, localizado na porção basal do epitélio, adjacente à membrana basal. Em todas as regiões do epidídimo as células basais expressaram citoqueratina 5 e formaram uma rede de comunicação, através de prolongamentos citoplasmáticos laterais, na base do epitélio.

Células claras foram as células menos observadas no epitélio epididimário do morcego vampiro comum. Essas apresentaram formato caliciforme, com núcleo na região apical da célula, baixa frequência na região da cauda e expressaram H^+ V-ATPase. Ultraestruturalmente, os tipos celulares mostraram características similares ao longo do epidídimo. No morcego vampiro comum, observamos a expressão de proteínas de *tight junction*, claudinas e zônula de oclusão 1, na porção apical das membranas laterais das células adjacentes que compõem o epitélio epididimário. Entretanto, enquanto zônula de oclusão 1 foi observada somente em *tight* junction, as claudinas foram observadas na região de *tight junction*, e na membrana basolateral das células adjacentes, incluindo células basais. Houve diferença na distribuição das claudinas quando comparado o epitélio das regiões proximais com as distais do epidídimo e entre as estações de chuva e seca. Conclui-se que o conhecimento das características do epitélio epididimário do morcego comum aumenta as informações sobre o aparelho reprodutor masculino de espécies de morcegos neotropicais, uma vez que o epidídimo de animais dessa espécie apresenta algumas peculiaridades, como o formato caliciforme das células claras em todas as regiões e baixa quantidade dessas na região da cauda, e a ausência de prolongamento citoplasmático em direção ao lúmen das células basais. Entretanto, mais estudos são necessários para o entendimento de como ocorre a maturação espermática nessa espécie, e se variações hormonais e sazonais podem interferir de alguma forma nesse órgão.

ABSTRACT

CASTRO, Mariana Moraes de, D.Sc., Universidade Federal de Viçosa, April, 2016. **Morphological, histomorphometric and ultrastructural description of the epididymal epithelium of the vampire bat *Desmodus rotundus***. Adviser: Mariana Machado Neves. Co-advisers: Mariella Bontempo de Freitas and Maria do Carmo Queiroz Fialho.

There are few studies about the characterization of the male reproductive system, especially the epididymis, in neotropical bats. Previous studies in bats, *Molossus molossus* and *Eumops glaucinus*, showed that the epididymal epithelium presents similar characteristics to the other mammals, when analyzed by light microscopy. However, there is not any study focused on the morphological features of the epididymis in common vampire bat *Desmodus rotundus*. The epididymis is responsible for several functions such as concentration, maturation, transport and storage of sperm. This organ is composed of pseudostratified epithelium and may be divided into four regions, initial segment, caput, corpus and cauda. Therefore, the aim of this study was to characterize the common vampire bat's epididymis based in morphological, histomorphometric and ultrastructural aspects using light, epifluorescence and electron microscopy. For the immunohistochemistry, were used different cell types markers, such as aquaporin 9, cytokeratin 5 and H^+ V-ATPase, and tight junction proteins, as claudin 1, claudin 3, claudin 4 and zonula occludens 1. In this current study, we observed the proximal region of the epididymis, initial segment and caput, and the distal region, corpus and cauda. All the regions showed a pseudostratified epithelium composed of principal, basal and clear cells. The epithelium height decreased from the proximal to the distal region, while the duct and interduct diameter increased. The duct compartment was the main component of the common vampire bat epididymis. Principal cells showed a columnar shape with stereocilia in the cell apical membrane in contact with the lumen, and were the most frequent cell type. Along the epididymis, was observed PAS-positive staining, forming the glycocalix, and expression of aquaporin 9 in the stereocilia. In all regions, we observed PAS-positive granules in the cytoplasm, besides of ultrastructural characteristics related to absorption and secretion functions. The second most frequent cell type was basal cells. These cells were identified by the nuclei position, located in the basal portion of the epithelium, adjacent to the basement membrane. In all epididymal regions, the basal cells expressed cytokeratin 5, and formed a dense network, through cytoplasmatic lateral extensions, at the basal portion of the epithelium. Clear cells were the less frequent cell type observed in the epididymal epithelium of common vampire bat. These cells showed a goblet-shaped body, with nuclei in the apical region, low frequency in the cauda region, and expressed H^+ V-ATPase. Ultrastructurally, the cell types showed the same cellular characteristics along the epididymis. In the common vampire bat, we observed expression of tight junction proteins, claudins and zonula occludens 1, at the apical portion of lateral membranes of the adjacent epididymis epithelial cells. However, only zonula occludens 1 was observed in the tight junction, while the claudins were observed in the tight junction and in the basolateral membrane of adjacent epithelial cells, including basal cell. There was difference in the claudins distribution when compared the epithelium of the proximal with the distal regions, and between rainy and dry seasons. In conclusion, the knowledge of the epididymal epithelium of the common vampire bat increases the information about the male reproductive system in neotropical bat species, since the epididymis of this specie shows peculiarities, as the shape of clear cells in all regions and low frequency in the cauda, and absence of the luminal-reaching body into the lumen in basal cells. Moreover, more studies are necessary to understand the process of sperm maturation in this species, and if hormonal and seasonality variations could interfere in this organ.

INTRODUÇÃO GERAL

Os morcegos pertencem à ordem Chiroptera e constituem um dos grupos de mamíferos mais diversificados do mundo, com 18 famílias, 202 gêneros e 1116 espécies descritas (Simmons, 2005). A família Phyllostomidae está restrita à região neotropical e é formada por diversas espécies que apresentam diferentes hábitos alimentares, como insetivoria, carnivoria, frugivoria, nectarivoria, onivoria e hematofagia (Peracchi et al., 2007). A subfamília Desmontinae é formada por três espécies com hábito alimentar hematófago, sendo elas *Desmodus rotundus* (E. Geoffroy, 1810), *Diphylla ecaudata* (Spix, 1823) e *Diaemus youngi* (Jentink, 1893) (Simmons, 2005).

Desmodus rotundus (Figura 1), também conhecido como morcego vampiro comum, não é considerado uma espécie ameaçada de extinção e apresenta ampla distribuição geográfica, ocorrendo desde o México até a Argentina, Chile e Uruguai, incluindo o território brasileiro (Peracchi et al., 2007; IUCN, 2016). Os indivíduos desta espécie se alimentam durante as primeiras horas da noite, geralmente de sangue de animais domésticos como gado e equinos (Greenhall et al., 1983).

Figura 1: Exemplar de um indíviduo macho adulto de *Desmodus rotundus*. (Foto gentilmente cedida por Luciano Carlos Heringer P. Puga).

Morcegos vampiros apresentam comportamentos sociais interessantes, como a catação e o compartilhamento de alimentos através de regurgitação de sangue entre fêmeas adultas e animais jovens (Greenhall et al., 1983; Wilkinson, 1986; Wilkinson, 1988). A regurgitação pode estar relacionada ao aumento de chances de sobrevivência de animais que não saíram para se alimentar, uma vez que animais dessa espécie apresentam sensibilidade à privação alimentar por períodos maiores que duas a três noites de jejum (Freitas et al., 2003). *D. rotundus* mostrou-se incapaz de manter o nível de glicose circulante estabelecido para mamíferos, e também não secreta altas concentrações de insulina após o aumento da glicemia tanto *in vitro* quanto *in vivo*, como ocorre em outros mamíferos (Freitas et al., 2003; Freitas et al., 2013).

D. rotundus tem grande importância epidemiológica nas áreas tropicais e subtropicais da América Latina, por ser um dos reservatórios silvestres da zoonose de etiologia viral conhecida como raiva, que pode ser transmitida pelo contato com a saliva contaminada de morcegos infectados para animais domésticos e humanos (WHO, 2013). A predação de herbívoros domésticos por *D. rotundus* foi facilitada, nas últimas décadas, devido ao aumento de áreas de desmatamento, expansão das áreas de criação de gado ou remoção dessas, migração do homem para áreas inexploradas e abandono de abrigos artificiais como casas, túneis e minas inutilizadas (Schneider et al., 2001; Kotait et al., 2007). Assim como alguns países da América Latina, o Brasil sofre com prejuízos econômicos causados pela infecção da raiva em herbívoros, com a morte de até 40.000 cabeças de gado por ano (Kotait et al., 1998). Visando a diminuição dos prejuízos econômicos, várias ações sanitárias, como vacinação pré-exposição do gado e controle da população de morcegos através do uso de pasta vampiricida, foram implementadas na América Latina a partir da década de 1980 (Kotait et al., 1998; WHO, 2013).

Para a predação, *D. rotundus* apresenta modificações nos dentes caninos e incisivos superiores, que são grandes, cortantes e em forma de estilete para facilitar a mordedura. Os indivíduos da espécie possuem ainda lábio inferior e língua sulcados, além de substâncias anticoagulantes na saliva, que possibilitam o fluxo contínuo de sangue da presa durante a alimentação (Greenhall et al., 1983; Fernandez et al., 1999; Peracchi et al., 2007).

Indivíduos de *D. rotundus* vivem em colônias, com variação entre 20 a 100 indivíduos, geralmente localizadas em abrigos naturais, como cavernas, fendas em formações rochosas e ocos de árvores, ou abrigos artificiais, como construções abandonadas, pontes e bueiros (Gomes e Uieda, 2004). Apesar da literatura sobre o comportamento reprodutivo de *D. rotundus* ser escassa, a estrututura social característica desta espécie se baseia em um grupo com um macho dominante e várias fêmeas. Machos podem ser classificados como sexualmente ativos quando apresentam os testículos na bolsa escrotal, e sexualmente inativos quando os testículos se localizam na cavidade abdominal (Greenhall et al., 1983).

Em geral, morcegos apresentam baixo potencial reprodutivo, gerando apenas um filhote em cada gravidez, por isso, várias espécies de morcegos de áreas tropicais aumentaram seu potencial reprodutivo apresentando estro pós-parto (Crichton e Krutzch, 2000), o que não foi o caso de *D. rotundus*, que apresenta gestação longa de cerca de sete meses, com nascimento de apenas um filhote, seguido de um longo período com alto grau de investimento parental durante aproximadamente um ano. Durante o primeiro mês os filhotes somente amamentam, e em seguida, o sangue é introduzido via regurgitação pelas mães. Ao fim do quarto mês os animais já acompanham a mãe até a presa para aprenderem a se alimentar (Greenhall et al., 1983). *D.* rotundus apresenta ciclo reprodutivo do tipo poliéstrico, onde as fêmeas ciclam ao longo do ano sem período definido de reprodução (Alencar et al., 1994). Em um estudo realizado no estado de São Paulo durante a estação seca, de Abril a Setembro, foi observada presença de machos sexualmente ativos e aparente ausência de atividade reprodutiva na maioria das fêmeas. Assim, os autores acreditam que o nascimento dos filhotes ocorra preferencialmente na estação chuvosa (Gomes e Uieda, 2004).

Em *D. rotundus*, assim como em outros morcegos neotropicais, o conhecimento sobre aspectos histofisiológicos do aparelho reprodutor masculino ainda é escasso. Existem trabalhos que caracterizam a espermatogênese, a morfometria testicular (Beguelini et al., 2009; Duarte e Talamani, 2010; Morais et al., 2012; Morais et al., 2013; Morais et al., 2014) e a morfologia epididimária (Beguelini et al., 2010; Oliveira Neto, 2013; Oliveira et al., 2013) de algumas espécies encontradas no Brasil, sendo estas geralmente sazonais. No entanto, não foram encontrados trabalhos caracterizando morfologicamente órgãos reprodutivos, em especial o epidídimo, de *D. rotundus*.

O epidídimo é um único, longo e convoluto ducto, e é o órgão responsável pela maturação e estocagem espermática. Espermatozoides produzidos nos testículos adquirem capacidade fecundante e de se moverem durante a interação com proteínas secretadas pelo epitélio durante a passagem pelo ducto epididimário, sendo estocados até o momento da ejaculação (Orgebin-Crist, 1969; Robaire e Hermo, 1988; Turner, 1995; Toshimori, 1998; Cooper, 2007; Sullivan e Saez, 2013).

O epidídimo pode ser dividido em diferentes regiões de acordo com a forma anatômica, localização e aspectos microscópicos, como altura do epitélio epididimário, diâmetro tubular e variação na frequência dos diferentes tipos celulares epiteliais (Robaire e Hermo, 1988; Hermo e Robaire, 2002; Robaire e Hinton, 2015). Na maioria das espécies de mamíferos é possível a identificação de quatro regiões no epididímo, segmento inicial, cabeça, corpo e cauda, podendo cada uma delas ser subdivida em segmentos intra regionais por septos do tecido conjuntivo. Acredita-se que essa separação seja funcional, uma vez que cria microambientes controlados que permitem a produção de proteínas específicas que irão auxiliar no processo de maturação espermática (Orgebin-Crist, 1969; Turner et al., 2003; Tomsig et al., 2006; Turner et al., 2007). Entretanto, o número de subdivisões do epidídimo pode variar de acordo com diferentes espécies. Este número pode chegar a 10 em camundongo, 19 em rato (Turner et al., 2007), nove em javali (Syntin et al., 1996) e seis em gato doméstico (Axnér et al., 1999).

O espermatozoide torna-se fértil à medida que atinge a região distal do corpo do epidídimo. Para isso, a região proximal do epidídimo, segmento inicial e cabeça, está envolvida com os processos iniciais da maturação espermática, enquanto a região distal, corpo e cauda, tem diferentes funções. Enquanto na região do corpo ocorre os processos tardios da maturação espermática, a região da cauda é o local de estocagem dos espermatozoides funcionalmente maduros (Orgebin-Crist, 1967; Orgebin-Crist, 1973; Robaire e Hermo, 1988; Hermo e Robaire, 2002). Sabe-se que o armazenamento dos espermatozoides na região da cauda pode ocorrer por um longo período em morcegos. Em um estudo com morcegos hibernantes (*Myotis lucifugus* e *M. septentrionalis*) percebeu-se que o prolongamento da vida do espermatozoide na região da cauda dá-se pelo aumento da osmolaridade no ambiente de estocagem, o qual gera desidratação e estado de quiescência no espermatozoide (Crichton et al., 1994).

O epidídimo, em todas as regiões, é constituído por um epitélio pseudoestratificado composto por diversos tipos celulares que podem estar presentes ao longo do epitélio, como células principais e basais, ou por células com localização específica, como células estreitas no segmento inicial, e células claras na cabeça, corpo e cauda (Shum et al., 2009; Robaire e Hinton, 2015). No epidídimo de morcegos das espécies *Eumopus glaucinus* e *Molossus molossus*, foram observadas células principais, basais, apicais, halo e claras (Beguelini et al., 2010).

Células principais são as células mais abundantes em todo o epitélio epididimário de mamíferos. Apresentam formato colunar, estendendo-se da membrana basal do ducto até o lumen, e são responsáveis pela secreção e absorção de água, íons, proteínas e lipídios no fluido epididimário (Hermo e Robaire, 2002; Robaire e Hinton, 2015). A aquaporina 9 é usada como marcador específico para esse tipo celular (Pastor-Soler et al., 2001), devido sua expressão na porção apical da membrana dessas células. Células principais do epidídimo do morcego *Artibeus literatus* também expressam aquaporina 9 (Oliveira et al., 2013).

Células basais são o segundo tipo celular mais encontrado no epidídimo, apresentam núcleo alongado e estão localizadas na região basal do epitélio (Hermo e Robaire, 2002). Essas células podem ser identificadas pela expressão de dois marcadores, claudina 1 na membrana e citoqueratina 5 no citoplasma (Shum et al., 2008; Shum et al., 2013; Shum et al., 2014; Kim et al., 2015). Apesar das células epiteliais serem geralmente consideradas estáticas em relação às células vizinhas, sabe-se que elas apresentam alto grau de plasticidade no epidídimo, uma vez que auxiliam na regulação do transporte de água e eletrólitos pelas células principais e do pH luminal pelas células claras (Leung et al., 2004; Cheung et al., 2005; Shum et al., 2008; Shum et al., 2013). No caso da acidificação luminal, células basais emitem uma projeção citoplasmática estreita, denominada axiopodia, em direção ao lúmen que funciona como um sensor do ambiente luminal. No lumen, a partir da detecção de angiotensina luminal II, células basais comunicam-se com células claras, desencadeando uma série de eventos que irá culminar no aumento da acidificação luminal, processo essencial para a maturação e armazenamento adequado dos espermatozoides (Shum et al., 2008; Kim et al., 2015; Roy et al., 2016).

Outros dois tipos celulares frequentemente encontrados no epitélio epididimário são as células estreitas e claras. Enquanto as células estreitas estão presentes na região do segmento inicial, as células claras são observadas na região da cabeça, corpo e cauda. Ambos os tipos celulares são responsáveis pela liberação de íons H^+ para o interior do lúmen, sendo responsáveis pelo controle do pH luminal (Adamali e Hermo, 1996; Shum et al., 2008) e manutenção dos espermatozoides em estado quiescente (Shum et al., 2009). A proteína H^+-ATPase vacuolar é utilizada como marcador para esse tipo celular (Da Silva et al., 2007a; Da Silva et al., 2007b; Shum et al., 2009; Shum et al., 2011; Breton e Brown, 2013).

Todas as células constituintes do epitélio epididimário são mantidas unidas por um complexo de junções celulares que regulam a passagem de moléculas e células pelo epitélio. Essas junções celulares formadas por *tight junction* são presentes na extremidade apical das células principais, sendo denominada de barreira hematoepididimária (Cyr et al., 2007).

A *tight junction* é composta por proteínas transmembranas e periféricas, sendo que a interação entre elas promove o fechamento de espaços entre as células epiteliais adjacentes, impedindo o contato entre o compartimento luminal do ducto epididimário com o interducto (Cyr et al., 2007). Dessa forma, essas proteínas garantem a manutenção da composição do fluido epididimário e a proteção dos espermatozoides contra o sistema imunológico (Cyr et al., 2007). Além disso, essas junções também participam do estabelecimento da polaridade celular, permitindo que haja transporte paracelular de íons, água e solutos das células do epididímo (Kim e Breton, 2016).

Ocludinas são proteínas transmembrana que compõem a *tight junction*. São presentes nas regiões de cabeça, corpo e cauda, e ausentes na região do segmento inicial de ratos e camundongos adultos (Furuse et al., 1993; Cyr et al., 1999; Cyr et al., 2007; Kim e Breton, 2016). Já as claudinas fazem parte de uma grande família de proteínas responsáveis pela modulação da permeabilidade paracelular a íons e água (Lingaraju et al., 2015; Markov et al., 2015). Diversas claudinas, como claudina 1, 3 e 4 já foram observadas no epidídimo de humanos (Dubé et al., 2010), ratos (Gregory et al., 2001; Gregory e Cyr, 2006; Shum et al., 2008) e camundongos (Kim e Breton, 2016). As proteínas periféricas conhecidas como zônulas de oclusão também fazem parte da *tight junction*, uma vez que essas realizam a ligação entre a porção citosólica de claudina e o citoesqueleto de actina (Stevenson et al., 1986; Haskins et al., 1998; Ruan et al., 2014; Lingaraju et al., 2015; Markov et al., 2015).

Não são conhecidos a morfologia, localização, distribuição e prováveis marcadores dos tipos celulares presentes no epitélio epididimário de *D. rotundus*. Além disso, também não há registros sobre quais proteínas podem estar relacionadas à formação da *tight junction* nessa espécie. Por isso, o objetivo desse trabalho foi descrever o epitélio epididimário de *D. rotundus* considerando I) Aspectos morfológicos e histomorfométricos usando microscopia de luz e eletrônica; II) Presença e imunolocalização de Aquaporina 9, citoqueratina 5 e H^+ V-ATPase; III) Provável influência da sazonalidade na presença eimunolocalização de proteínas que compõem a *tight junction.*

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CAPÍTULO I

Microscopic and histomorphometric features of the epididymis of the common vampire bat

Microscopic and histomorphometric features of the epididymis of the common vampire bat

Abstract

Little is known about morphophysiological aspects of the epididymis of neotropical bats, specially vampires. It is only during transit throughout the epididymis that spermatozoa undergo maturation and acquire progressive motility and the ability to fertilize oocytes. Therefore, the aim of this study was to characterize the main features of the epididymis regions by light and electronic microscopy that can fulfill the lack of information about the *Desmodus rotundus* epididymis. We performed histological, volumetric and histomorphometric analysis by light microscopy, to estimate the relative distribution of the cellular types observed in the epithelium of the epididymis. All the cell types were characterized by their ultrastructural aspects using electron transmission microscopy. The results showed the epididymal duct lined by a columnar pseudostratified epithelium. The epithelium height decreased from the proximal segment to the distal, whereas the duct and interduct diameters increased. The duct compartment was the main component of the epididymis (proximal region, 90%; corpus, 88%; cauda, 80%). Principal cells (PCs) were more frequent in the epithelium, followed by basal cells (BCs) and clear cells (CCs), respectively. Principal cells showed a columnar shape, with PAS-positive granules in the cytoplasm, PAS-positive staining in the stereocilia, and ultrastructural characteristics related to absorption and secretion functions. Basal cells were observed in the basal portion of the epithelium, adjacent to the basement membrane. Clear cells showed a goblet-shaped body, unlike of was described in rats and mice, with nuclei in the apical region of the cell along the entire epididymis. Also, showed a low frequency in the cauda region, and no endocytic apparatus. Ultrastructural aspects of all cells types showed similar characteristics between the same cell type in different regions of the epididymis.

Key words: *Desmodus rotundus*, post-testicular reproductive tract, epididymal cells, morphology, electronic transmission microscopy.

1. Introduction

The Desmodontinae subfamily is found in the New World and consists of three species with hematophagous feeding habits, *Desmodus rotundus* (E. Geoffroy, 1810), *Diphylla ecaudata* (Spix, 1823) and *Diaemus youngi* (Jentink, 1893) (Simmons, 2005). In regard to *D. rotundus*, also known as common vampire bat, they can feed commonly in domestic herbivores and less in dogs and humans due to their proximity with them, which may provide an unlimited food source (Greenhall et al., 1983; Schneider et al., 2001; Kotait et al., 2007; WHO, 2013). This species, as other wild mammals such as *Procyon lotor*, *Callithrix* spp., *Cerdocyon* spp., has an epidemiologic importance, as it is a wild and primary hosts and agent of rabies virus (WHO, 2013).

D. rotundus is not considered an endangered species and has a wide geographical distribution in Latin America, including Brazil (Peracchi et al., 2007). These animals live in large colonies located in natural or artificial shelters (Gomes and Uieda, 2004). Although the available information about pregnancy and breeding features in *D. rotundus* (Greenhall et al., 1983; Alencar et al., 1994; Gomes and Uieda, 2004) the literature on its reproductive behavior is still scarce. Moreover, there is a lack of information on morphological and physiological aspects in reproductive organs of this species when compared to other Neotropical species of bats (Beguelini et al., 2010; Beguelini et al., 2013; Oliveira et al., 2013; Beguelini et al., 2015).

Studies in bat species such as *Artibeus lituratus*, *Molossus molossus* and *Eumops glaucinus* (Beguelini et al., 2010; Oliveira et al., 2013) have been focused on the epididymis, an important organ of male reproductive tract and responsible for the sperm maturation, which involves the acquisition of sperm motility and their ability to fertilize oocytes (Cornwall, 2009). The epididymal duct is lined by an epithelium with different cell types. Principal and basal cells are located throughout the duct, while others, such as narrow and clear cells, are found in specific regions depending on the species (Arrighi, 2014; Robaire and Hinton, 2015). However, the role of each cell type in the sperm maturation and storage is still unclear and many studies have been conducting in order to clarify their regulatory mechanisms in epididymal function (Serre and Robaire, 1998; Belleannée et al., 2011; Belleannée et al., 2012; Kim and Breton, 2016; Roy et al., 2016). Currently it is known that principal cells are responsible for fluid and nutrient exchange and protein secretion (Pastor-Soler et al., 2001), while clear cells regulate the luminal pH (Pastor-Soler et al., 2005; Da Silva et al., 2007; Shum et al., 2008; Breton and Brown, 2013). On the other hand, basal cells establish crosstalk between epithelial cells, participating on the regulation of adjacent epithelial cells (Leung et al., 2004; Shum et al., 2008).

Therefore, due to the lack of information about its reproductive histophysiology and the importance it has for the reproductive knowledge of this species, the aim of this study was to characterize the epididymal duct of *D. rotundus* considering morphological, volumetric and histomorphometric features.

2. Material and Methods

2.1 Animal capture and ethics statement

Five adult male *D. rotundus* (30,17 – 34,69 g) were captured in Itamarati de Minas, Minas Gerais, Brazil (21°23'55.3" S; 42°51'53" W; elevation 585 m). Bats were housed in cage and transported to the Laboratory of Structural Biology at the Federal University of Viçosa (UFV), Viçosa, Minas Gerais, Brazil, where they were protected from light. Permits for field collection were provided by Chico Mendes Institute for Biodiversity Conservation (ICMBio; number 40629-1). All experimental procedures were in accordance with the ethical principles for animal research adopted by the Animal Ethics Committees (CEUA protocol nº 55/2013).

The animals were weighed, anesthetized with sodium pentobarbital (40 mg/kg/intraperitoneal) and euthanized by guillotining (Freitas et al., 2013). Epididymides were removed, dissected and weighed. The epididymosomatic index (ESI) was obtained by the ratio between epididymis weight (EW) and body weight (BW), where ESI = EW/BW x 100. The results were expressed as mean ± standard error mean (SEM).

2.2 Histology and histomorphometry

Entire left epididymides (n=5) were immersed in Karnovisky fixative (Karnovisky, 1965) for 24 hours and subsequently dehydrated in crescent ethanol series (70%, 80%, 90%, and 100%), and embedded in 2-hydroxyethyl methacrylate (Historesin®, Leica Microsystems, Nussloch, Germany). Sections with a thickness of 3 µm were obtained using a rotary microtome (RM 2255, Leica Biosystems, Nussloch, Germany) and stained with toluidine blue-sodium borate (1%) for histological analysis, whereas other sections were stained with periodic acid-Schiff (PAS) method to identify the presence of neutral glycoproteins in the epithelium. All the sections were qualitatively analyzed using Olympus CX40 optical microscope (Olympus, Tokyo, Japan). The regions were identified according to macroscopic appearance and histological aspects, such as epithelium height and diameter of the epididymal duct (Robaire and Hinton, 2015).

For morphometric analysis, digital images of the regions were obtained by photomicroscope Olympus BX-53 (Olympus, Tokyo, Japan) and analyzed with Image-Pro Plus® 4.5 (Media Cybernetics, Silver Spring, USA) software. The mean of the duct diameter of each epididymal region was obtained by randomly measuring 20 tubular cross sections of the duct, as circular as possible, per animal. These sections were also used to measure the luminal diameter and the epithelium height, which was taken from the basement membrane to the lumen. The epithelium height was the average of four diametrically opposed measurements.

The volumetric proportion of the epididymal regions was obtained by counting 2,660 points projected onto 10 images captured in histological slides per animal. Coincident points were registered in duct components (epithelium and lumen) and interduct components (blood vessels and connective tissue). The percentage of points in each component was calculated using the formula: volumetric proportion (%) = (number of points in the component / 2,660 total points) x 100.

The relative distribution of different cell types in the regions was estimated by cell counting in different sections of the duct (Beu et al., 2009). Cells were counted in 10 sections per region per animal. The result of the crude cell count was corrected by applying the formula of Amann (1962): [cells number] x [section thickness/ section thickness + $\sqrt{}$(nuclear diameter of the cell/2)^2 - $\sqrt{}$(nuclear diameter of the cell/4)^2]. This calculation provides the corrected value of the number of cells counted in the histological sections. The results were expressed as percentual.

2.3 Electron Microscopy

Fragments from the proximal, medium and distal epididymis were immersed in 2.5% glutaraldehyde in sodium cacodylate buffer 0.1 M pH 7.2 during 24 hours at room temperature, followed by washes with cacodylate buffer 0.1 M pH 7.2 sodium and post-fixation in osmium tetroxide 1% during 2.5 hours. After two washes in cacodylate buffer, samples were dehydrated in a crescent series of ethanol and embedded in LR white resin (LR White Resin, London Resin Company, England) mixed with absolute ethanol in the ratio of 1:1, followed by 2:1 ratio for 1 hour each. Thereafter, samples were embedding in pure resin overnight at 4 °C and polymerized in gelatin capsules (Electron Microscopy Sciences) at 60 °C during 24 hours. Ultrathin sections were contrasted for 20 minutes with 1% aqueous uranyl acetate, and then with lead citrate (Reynolds, 1963) during 8 minutes. Sections were analyzed and photographed under a Zeiss EM 109 transmission electron microscope at Nucleus of Microscopy and Microanalysis (UFV).

2.3 Statistical analysis

One-way analysis of variance followed by Student T Test was employed to detect significant differences between regions for histomorphometry and relative cell distribution. The level of significance was taken as $P < 0.05$. The results were expressed in mean ± standard error mean (SEM).

3. Results

Epididymis weight and epididymosomatic index in *D. rotundus* were, respectively, 0.057 ± 0.019 g and 0.173 ± 0.055 %. The epididymis of *D. rotundus* is a single highly convoluted duct covered by a dense connective tissue. It was possible identify three portions of the epididymis by anatomic (Figure 1A) and microscopic features (Figure 1B-D). The first one, classified as proximal region was the large part nearest to the testis and efferent ducts, followed by the corpus region, characterized by elongated and thin shape in the middle of the organ. The third and largest portion was the cauda, which was connected to the vas deferens (Figure 1A).

Mean values for duct and luminal diameters and epithelium height are shown in Table 1. The proximal region presented higher epithelium height than the other regions, besides the lowest luminal diameter, whereas the cauda region presented higher duct diameter ($P < 0.05$). Those differences were possible to identify when we observed histological sections of each region under light microscopy (Figure 1B-D).

The duct compartment was composed of pseudostratified epithelium and lumen filled with sperm in all regions. It comprised more than 80 % of the total epididymis tissue (Table 1). The interduct compartment, showed fibroblasts immersed in the extracellular matrix (Figure 1B-D), besides smooth muscle fibers involving the duct and blood vessels. The percentual of epithelium decreased from the proximal region to the cauda, and the mean values were different between regions (Table 1). The percentual of lumen increased toward the cauda, and the proximal region showed the lowest mean value among the regions (Table 1). The connective tissue increased its extension from the proximal to the cauda region, which presented the highest value when compared to the other regions. In regard to the percentual of blood vessels, the proximal region showed lower mean value than cauda region (Table 1).

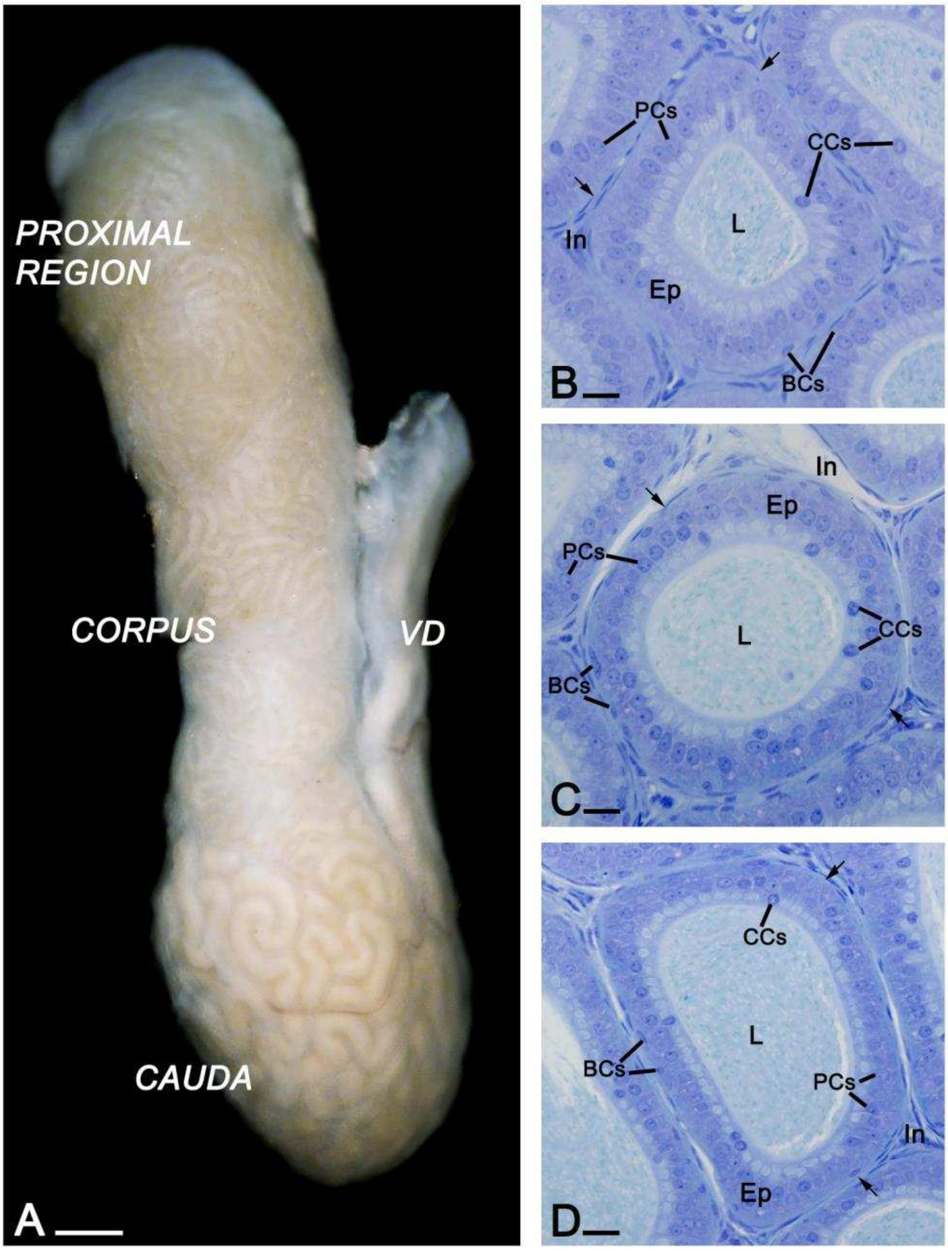

Figure 1. A) Epididymis of *Desmodus rotundus* with three regions identified macroscopically and vas deferent (VD); Epididymal duct sections showing B) proximal region, C) corpus and D) cauda regions stained by Toluidine blue. Ep, epididymal epithelium; PCs, Principal cell; BCs, Basal cell; CCs, Clear cell; L, lumen; arrows, basement membrane; In: interduct compartment. Scale bars: 1mm (A) (Photo: José Lino Neto), 50 µm (B-D).

Table 1: Morphometry and volumetric proportion of the duct and interduct compartments in epididymis of *Desmodus rotundus*.

Parameters (n=5)	Proximal region	Corpus	Cauda
Duct diameter (µm)	127.66 ± 3.04	135.60 ± 3.09	180.80 ± 13.33*
Luminal diameter (µm)	62.32 ± 2.88*	89.31 ± 9.14	132.58 ± 16.44
Epithelium height (µm)	32.66 ± 0.69*	28.18 ± 0.97	24.11 ± 1.64
Epithelium (%)	74.61 ± 2.56*	63.64 ± 1.84*	48.17 ± 4.91*
Lumen (%)	15.52 ± 1.55*	24.79 ± 1.68	31.84 ± 2.56
Connective tissue (%)	8.99 ± 0.92	10.57 ± 1.39	18.75 ± 2.30*
Blood vessels (%)	0.88 ± 0.33*	1.00 ± 0.28	1.24 ± 0.33*

Mean ± SEM. *Superscript asterisk in the same row indicate significant differences among regions ($p < 0.05$) by T Test.

In general, the epithelium along the epididymal duct was composed of three cell types (Figure 1B-D; Figure 2). The relative distribution of epithelial cell types showed that principal cells were the most predominant throughout the epididymis, followed by basal and clear cells (Figure 3). The percentual of principal cells was statistically different between proximal region and cauda regions, whereas no differences were observed to clear cells between regions. Cauda region showed lower percentual of basal cells than the other two regions ($P < 0.05$). All of those cells showed the same histological features in all regions analyzed.

Principal cells showed a columnar shape with their cytoplasm extending from the basement membrane to the lumen (Figure 2A-2C; Figure 4). The nuclei was located in their mid-basal portion of the cytoplasm (Figure 2A-2C; Figure 5A and 5B). The apical portion of principal cell presented lightly stained areas (Figure 2A-2C). Basal cells were located in the basal portion of the epithelium adjacent to the basement membrane (Figure 2A-2C; Figure 4). This cell type presented scarce cytoplasm area and elongated nuclei (Figure 2A-2C; Figure 6C). Finally, clear cells were observed between principal cells (Figure 2A-2C; Figure 4). They showed an oval shape with a small cytoplasm area and nuclei located in their mid-apical portion (Figure 2A-2C; Figure 6A). PAS-positive granules were observed in the apical, mid and basal cytoplasm of principal cells (Figure 2D-2F), besides the glycocalix in the stereocilia.

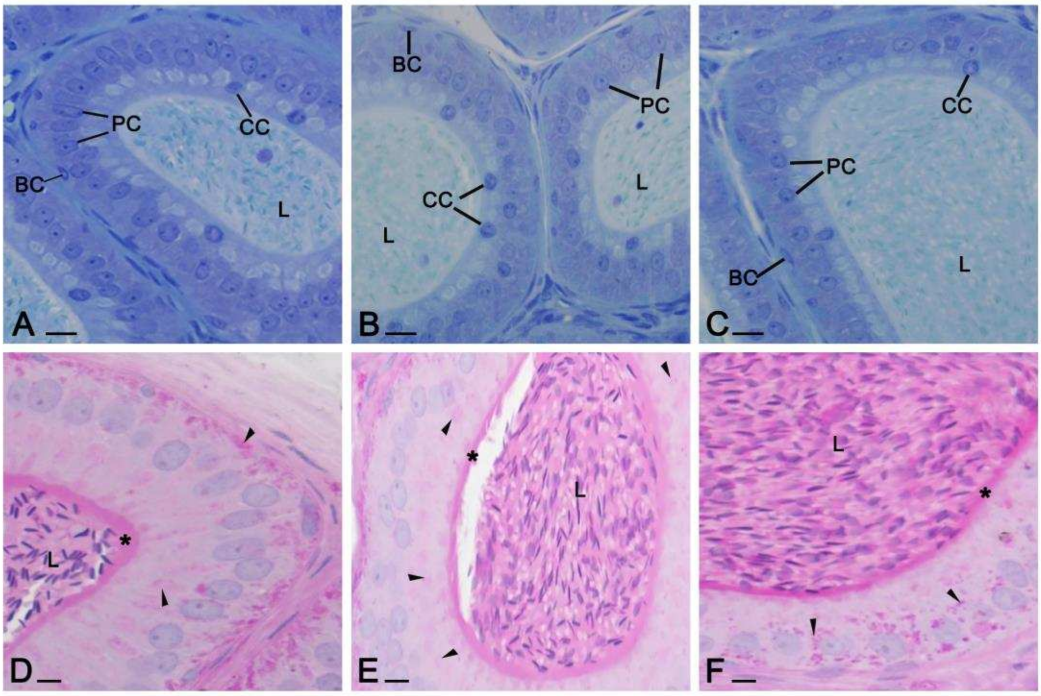

Figure 2. Photomicrographs of *Desmodus rotundus* epididymis showing principal (PC), basal (BC) and clear (CC) cells in the proximal region (A), corpus (B) and cauda (C) regions; PAS-positive granules (arrowhead) in the cytoplasm of principal cells in the proximal region (D), corpus (E) and cauda regions (F). PAS-positive staining in the glycocalix of the stereocilia of principal cell were indicated by *. L, lumen. Scale bars: 50 µm (A-C), 10 µm (D-F).

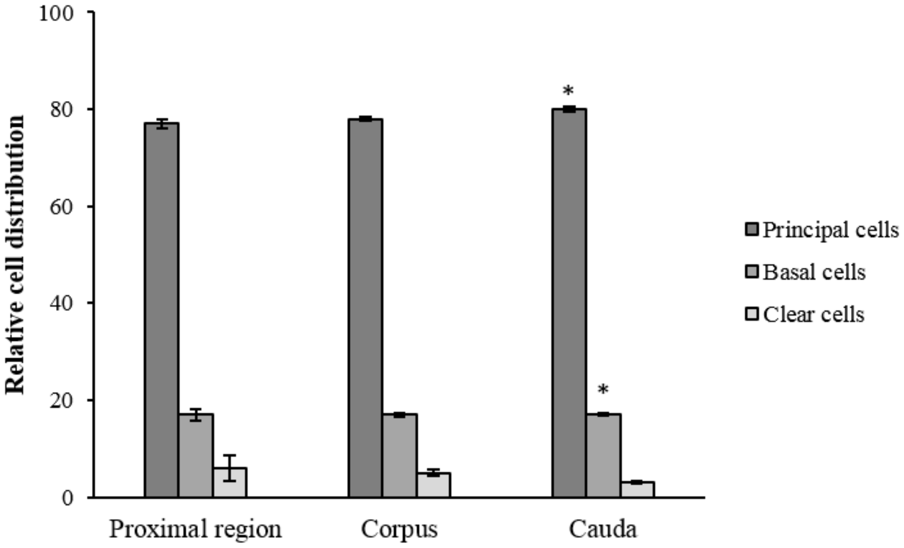

Figure 3. Relative cell distribution in three regions of the *Desmodus rotundus* epididymis (% of total). Bars represent mean ± SEM. * $p < 0.05$.

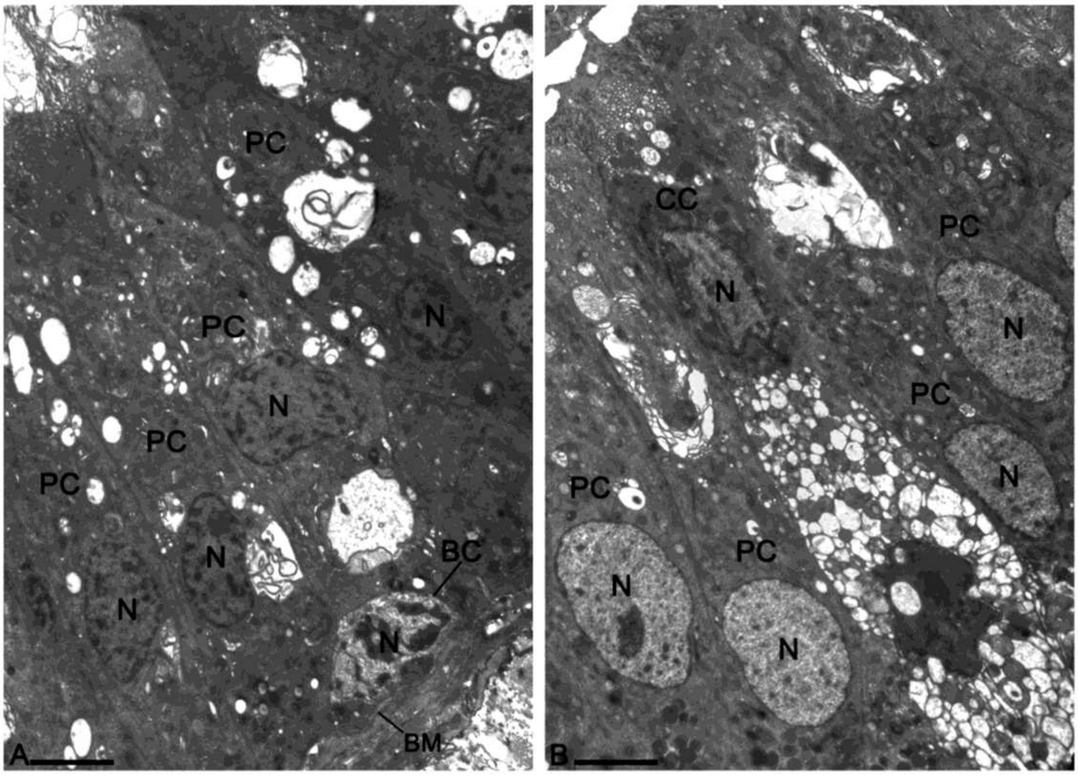

Figure 4. Photomicrographs of electron transmission of the epididymal duct of *Desmodus rotundus* from the proximal region showing epithelial cells in A) PC, Principal cell, BC, Basal cell; B) PC, Principal cell, CC, Clear cell. N, nuclei; BM, basement membrane. Scale bars: 5 µm.

Principal, clear and basal cells showed similar ultrastructure along the entire epididymis as shown in figure 4. In the cytoplasm of principal cells were observed several number and different size of vesicles with membranous content and electron-dense granules in the basal portion (Figure 5A). The nucleus showed a descondensed chromatin (Figure 5A and 5B). Myelin bodies were observed in cytoplasm in the apical portion (Figure 5B) and small vesicles near to the apical border (Figure 5C). Stereocilia was observed in contact with the lumen (Figure 5C). A highly developed Golgi complex was observed preferentially in the apical portion of principal cells (Figure 5D). Whilst electron-dense granules and a highly developed endoplasmic reticulum were observed in the perinuclear portion (Figure 5E), mitochondria were visualized mostly near the cell boundary with small size (Figure 5F).

The nuclei of clear cells showed irregular shape and heterochromatin at its periphery (Figure 6B). In addition, we observed electron-lucid vesicles of different sizes and mitochondria in the apical cellular portion (Figure 6B). Basal cells nuclei showed elongated with clumps of condensed chromatin and small cytoplasm region (Figure 6C).

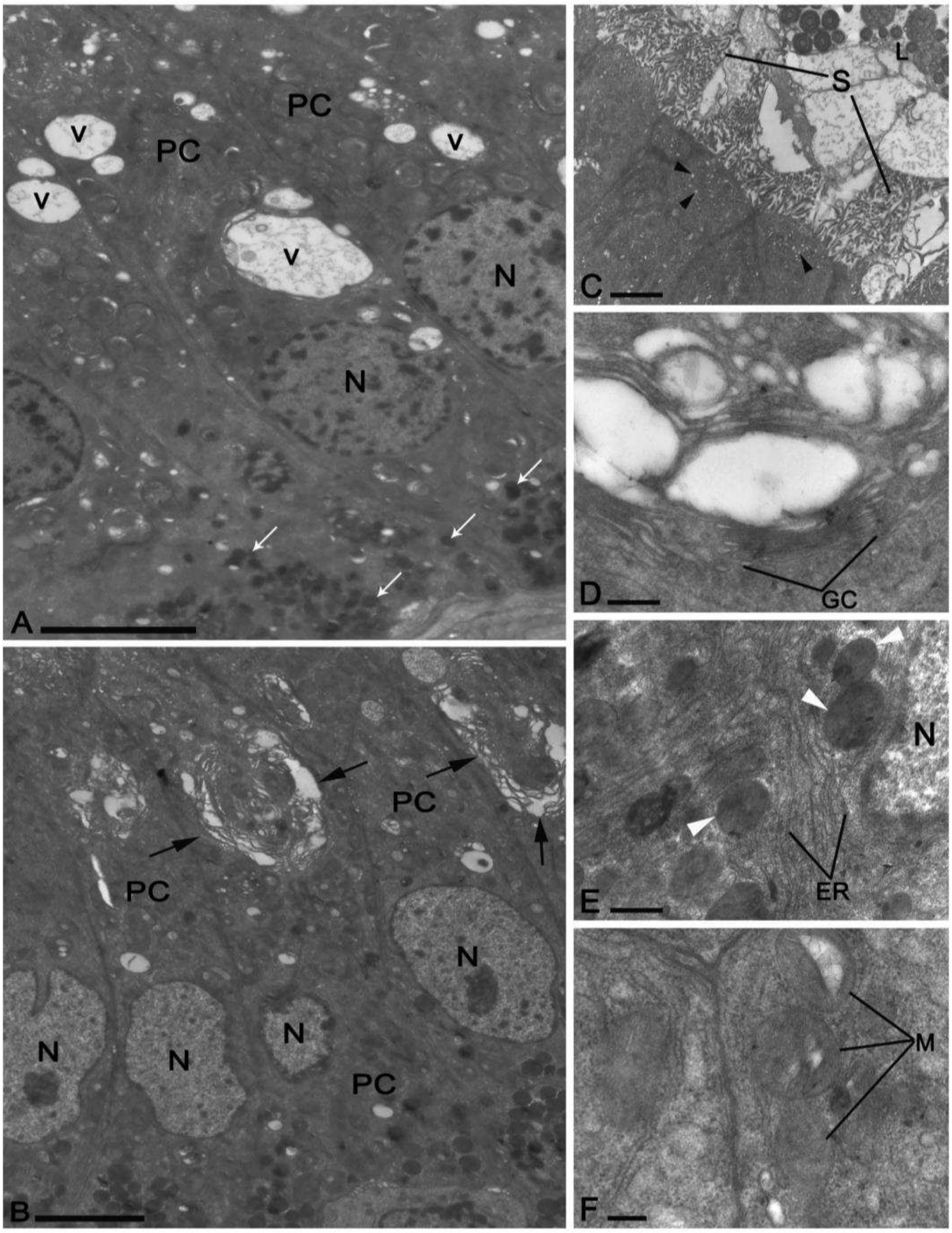

Figure 5. Ultrastructure of the principal cells (PC) of the *Desmodus rotundus* epididymal duct, showing in A) its prismatic shape and the presence of electron-dense granules (white arrow), vesicles (v); B) myelin bodies (black arrow); C) Several small vesicles (black arrowhead) near to the apical border, besides of stereocilia (S) in contact with the lumen; D) Golgi complex (GC); E) Perinuclear region with endoplasmic reticulum (ER) and electron–dense granules (white arrowhead); F) Mitochondria (M). L, lumen; N, nuclei. Scale bars: 5 µm (A), 2 µm (B), 200 nm (C, D, F), 500 nm (E).

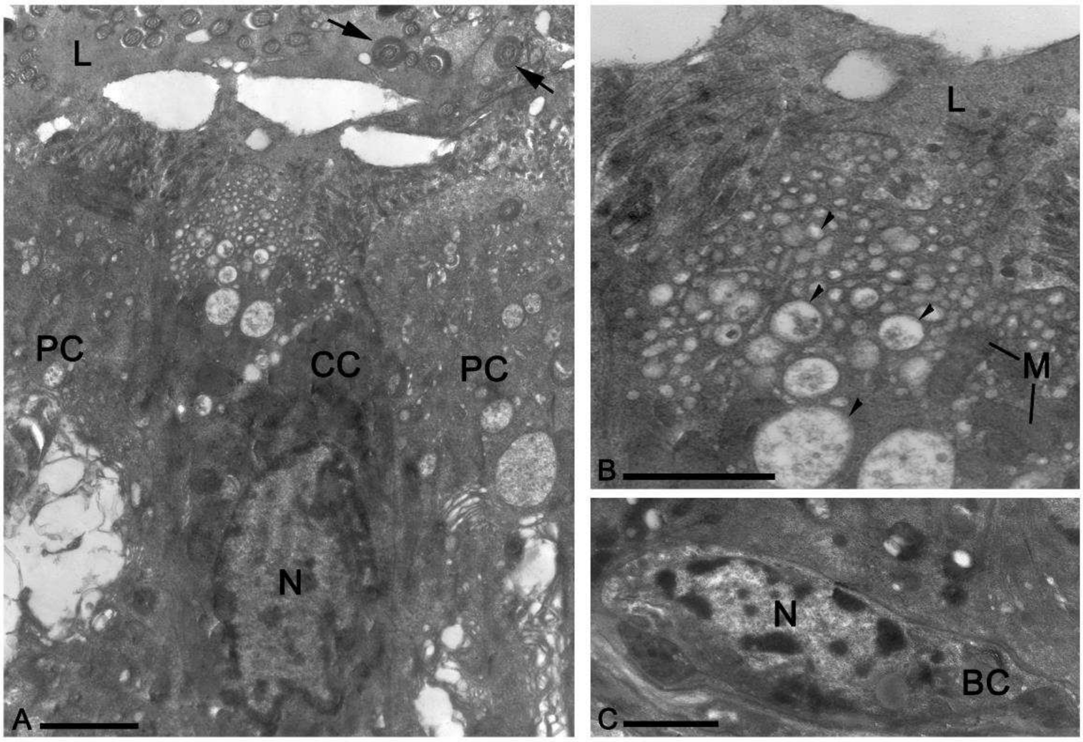

Figure 6. Electron transmission photomicrographs of the *Desmodus rotundus* epididymal duct showing in A) Nucleus of clear cell (CC) in the apical region of the epididymal epithelium; B) Apical portion of the CC, with vesicles of different sizes (arrowheads) and mitochondria (M). C) Basal cell (BC) in the basal region of the epithelium, with nucleus (N). PC, principal cell; L, lumen; arrow, sperm. Scale bars: 2 µm, (A), 500 nm (B), 1 µm (C).

4. Discussion

The current study provides morphological and histomorphometric description of *D. rotundus* epididymis. Herein we focused our analysis in three epididymal regions, the proximal region, corpus and cauda, as performed in other mammals (Syntin et al. 1996; Serre and Robaire, 1998; Axnér et al. 1999; Domeniconi et al. 2007; Turner et al. 2007; Beguelini et al. 2010). The differences observed between regions regarding to their morphology and the histomorphometry of the common vampire bat epididymal duct sections were similar to the results described in rodents (Serre and Robaire, 1998), cat (Axnér et al., 1999), gerbil (Domeniconi et al., 2007) and bats (Beguelini et al., 2010).

The abundance of principal cells in the epithelium was observed in *D. rotundus* and in other two species of Neotropical bats, *Eumops glaucinus* and *Molossus molossus* (Beguelini et al. 2010). It is known that this cell type is responsible for the transepithelial flow of water, urea and non-charged solutes, due to the presence of aquaporin 9 in their apical membrane (Tsukaguchi et al., 1998; Pastor-Soler et al., 2001). Moreover, principal cell synthesize and secrete proteins into the lumen and perform an active endocytosis of proteins along the epididymis, which helps in the creation of a specialized luminal microenvironment (Turner, 1991; Hermo et al., 1994; Cornwall, 2009; Hermo and Robaire, 2002). Ultrastructural characteristics of principal cells, such as presence of vesicles with membranous content, electron-dense granules, myelin bodies, well-developed Golgi complex, endoplasmatic reticulum, and the stereocilia indicate secretory and absorption functions of principal cells in common vampire bat epididymis, as well as described in other mammals (Flickinger, 1985; Domeniconi et al., 2007; Lorenzana et al., 2007; Shimming and Vicentini, 2008).

Furthermore, the location of Golgi complex and endoplasmatic reticulum observed in the cytoplasm of principal cells in *D. rotundus* and in rodents (Oke et al., 1989; Domeniconi et al., 2007) was the same. The only difference found in this study to others was related to mitochondrial aspects in this cell type. Principal cells of *D. rotundus* showed mitochondrias with small size and located near the cell boundary, whereas African rat presented clumps of mitochondria in their supranuclear and infranuclear region (Oke et al., 1989). Although we had no difference between regions to the principal cell ultrastructure, Robaire and Hinton (2015) cited that it is possible to identify different appearance and organization of the secretory and endocytic apparatus of principal cells depending of the region analyzed.

In the present study, PAS-positive granules observed in the cytoplasm of principal cells along the entire epididymis may suggest that glycoproteins are secreted into the lumen, and their presence might reflect the high activity of this cell type. Moreover, an extensively PAS-positive staining was observed in the stereocilia of principal cells, which indicate there are glycoproteins associated to cellular membrane.

D. rotundus showed similar percentual of the basal cells distribution, with the lowest value in the cauda region. Studies have shown that basal cell has a high plasticity along the entire epididymis, especially related to the emission of a long and narrow projection toward the lumen named as axiopodia, which was observed in rats and mice (Shum et al., 2008; Shum et al., 2013; Kim et al., 2015; Roy et al., 2016), and wild rodents (Menezes et al., no prelo), but was not in *D. rotundus*. Axiopodia may be related to basal cell function as it is involved in the crosstalk between basal and clear cells that helps to increase luminal acidification by detection of luminal angiotensin II in the epididymis (Shum et al., 2008). The nuclear morphology observed in basal cells here was similar to the description in rat for this cell type (Oke et al., 1989).

In regard to the clear cells, they were identified along the entire epididymis in *D. rotundus* as described in rat (Serre and Robaire, 1998), and in contrast to descriptions made in gerbils and golden hamster, which showed clear cells only in the cauda region (Domeniconi et al., 2007; Beu et al., 2009). Clear cells presented a low percentual in the epithelium of the epididymis corpus in *M. molossus* and in the initial segment and caput in *E. glaucinus* (Beguelini et al. 2010). As well known, clear cells are responsible for luminal acidification (Pastor-Soler et al., 2005; Da Silva et al., 2007; Shum et al., 2008; Breton and Brown, 2013) in order to create an optimal environment for the maturation and storage of spermatozoa in a quiescent state for long periods (Carr et al., 1985; Shum et al., 2009). Therefore, it is usual to observe high frequency of clear cells especially in the cauda region (Shum et al. 2009). However, the opposite was observed in *D. rotundus* epididymis. The low frequency of this cell type in the cauda region may suggest that even with low number of clear cells in the epididymis, they are able to maintain the acid pH in this region or there is another regulatory mechanism in this species that need to be clarified.

Moreover, *D. rotundus* epididymis showed the clear cells with a goblet shape that extends from the basement membrane to the lumen, and nuclei at the apical portion of the epithelium. This shape was similar to apical cells described in *M. molossus* and *E. glaucinus* epididymis, which showed a slender shape in the basal and parabasal region, with a large cytoplasmic apical region with the nuclei (Beguelini et al., 2010). As cells with those features in *D. rotundus* were positive labeled to H^+-ATPase (unpublished data), we considered them as clear cells and not apical cells, since there is no information about the relation of apical cells and H^+-ATPase.

Although clear cells are also related to endocytic function (Da Silva et al., 2007; Domeniconi et al., 2007; Shum et al., 2008; Breton and Brown, 2013), we did not observe any PAS-positive staining in *D. rotundus*, neither multivesicular bodies and lysosomes in it cytoplasm as described in gerbil (Domeniconi et al., 2007). Otherwise, it was observed vesicles of different sizes, and many mitochondria in the cytoplasm of the apical portion.

In summary, the results of this investigation indicate that clear cells of the *D. rotundus* epididymis is the cell type with more particularities in the epithelium. The other morphological and histomorphometric findings related to the epididymal and epithelial duct are similar to described in other mammals. Thus, these results should increase the knowledge of the epididymis features in neotropical bats, helping in the understanding of reproductive biology of the Desmodontinae subfamily.

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CAPÍTULO II

The expression patterns of aquaporin 9, vacuolar H^+-ATPase, and cytokeratin 5 in the epididymis of the common vampire bat

The expression patterns of aquaporin 9, vacuolar H^+-ATPase, and cytokeratin 5 on the epididymis of the common vampire bat

Abstract

Desmodus rotundus is a very important species for public and animal health due to the fact that it is a primary reservoir of diseases like rabies. However, some basic aspects of this species' biology are still unknown, as the histophysiological characteristics of the male reproductive tract. The epididymis is an important organ for performing a variety of functions, especially the sperm maturation and storage. The aim of this study was to identify principal, narrow, clear and basal cells using cell specific markers such as aquaporin 9 (AQP9), vacuolar H^+-ATPase (V-ATPase) and cytokeratin 5 (KRT5). All epididymides were fixed in PLP and evaluated by immunofluorescence. For AQP9 labeling, we also used two additional techniques: DAB staining for bright field and electronic transmission microscopy. Principal cells were labeled by AQP9 in their stereocilia, clear cells by V-ATPase, and basal cell by KRT5 along the entire epithelium. Clear cells were shown with a goblet-shaped body, including the initial segment of the proximal epididymis region. A dense network formed by lateral cytoplasmatic projections of basal cells was observed at the basal portion of the epithelium. In addition, no luminal-reaching basal cells were observed in the vampire bat epididymis. In conclusion, these results demonstrate that the epithelial cells of the vampire bat epididymis may are identified by specific markers typically used to study the epididymis in laboratory animals.

Key words: *Desmodus rotundus*, epididymal cells, AQP9, V-ATPase, KRT5.

1. Introduction

In mammals, the epididymis is a single highly coiled duct, with a large epithelial-luminal interface, where the spermatozoa, produced in the testis, acquire their functional maturity and are stored until ejaculation (Jones 1998; Hermo and Robaire 2002; Patel et al. 2013; Robaire and Hinton 2015). This important post-testicular organ may be divided into four major anatomical regions, the initial segment, the caput, the corpus and the cauda. Those regions are composed of pseudostratified epithelia that contain several cell types, with some cells located throughout the duct, whereas others are found exclusively in specific regions (Hermo and Robaire 2002; Shum et al. 2013; Shum et al. 2014; Robaire and Hinton 2015; Kim et al. 2015).

In general, the principal and basal cells can be found along the entire length of the epididymal epithelium in most mammals (Robaire and Hinton 2015). The principal cells, responsible for fluid and nutrient exchange, and protein secretion, are identified by their apical stereocilia, where the water channel aquaporin 9 (AQP9) is present (Elkjær et al. 2000; Pastor-Soler et al. 2001). Basal cells, identified by cytokeratin 5 (KRT5) labeling, are located adjacent to epithelial cells, with their bodies at the base of the epithelium and an axiopodium reaching toward the lumen (Hermo and Robaire 2002; Shum et al. 2013; Shum et al. 2014; Kim et al. 2015; Roy et al. 2016). Narrow cells are located in the initial segment, while clear cells are located along the caput, corpus, and cauda regions. Both these cell types express high levels of vacuolar proton pumping H^+-ATPase (V-ATPase) and secrete protons to regulate the luminal pH, which allows them to play a critical role in the maturation and viability of spermatozoa (Da Silva et al. 2007a, b; Shum et al. 2009; Shum et al. 2011; Breton and Brown 2013).

Desmodus rotundus, the common vampire bat, is one out of three vampire bat species among more than 1,000 bats species occurring in all continents. This strict hematophagous bat inhabits Latin America (Peracchi et al. 2007), and feeds exclusively on blood, mainly from cattle, pigs and horses (Kotait et al. 2007). Vampire bats usually live in colonies with dominant males forming a harem mating system (Gomes and Uieda 2004). They are reproductively active year around and most females have only one pregnancy per year, and the gestation period is about seven months, the longest gestation period for a bat (Gomes and Uieda, 2004; Peracchi et al. 2007).

The morphological characteristics of the male reproductive tract in neotropical bats are little studied (Beguelini et al. 2010) The aim of this study was to describe histological aspects of the common vampire bat epididymis, through its epithelial cells characterization, using cell specific markers such as AQP9, V-ATPase and KRT5.

2. Material and Methods

2.1 Animals and tissue collection

Five adult males were captured using mist nets, positioned near a cave in Itamarati de Minas, MG, Brazil (21°23'55"S and 42°51'53"W; 585 m altitude). The animals were transported to the Laboratory of Structural Biology at the Federal University of Viçosa (UFV), where they were placed in cages protected from light. Euthanasia was performed by intra-peritoneal administration of sodium pentobarbital at a dose of 40 mg/kg of body weight, followed by guillotining (Freitas et al., 2013).

Bat capture was authorized by the Chico Mendes Institute for Biodiversity Conservation (ICMBio, license number 40629-1). This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All the experimental procedures were reviewed and approved by the Committee on the Ethics and Use of Animal Experiments of UFV (CEUA process number 55/2013).

2.2 Immunofluorescence and antibodies

Entire epididymides were fixed by immersion in periodate-lysine-paraformaldehyde (PLP) solution containing 4% paraformaldehyde for 4 h at room temperature, and rinsed three times in phosphate-buffered saline (PBS). Tissues were then incubated in PBS with 30% sucrose. Tissues were embedded in OCT compound (Tissue-Tek; Sakura Finetek, Torrance, CA, USA), mounted on a cutting block, and frozen. Tissues were then cut at 5 µm thickness using a Leica 3050 cryostat (Leica Microsystems, Bannockburn, IL, USA) and sections were placed onto Superfrost Plus microscope slides (Fisher Scientific, Pittsburgh, PA, USA), and stored at 4^{o} C until use (Kim et al. 2015). Sections were hydrated in PBS for 10 min. Tissues were incubated in 1% w/v SDS for 4 min, washed three times in PBS for 5 min and blocked with 1% bovine serum albumin for 30 min. Affinity purified rabbit polyclonal AQP9 antibody was used to identify principal cells, as previously described (Pastor-Soler et al. 2001). A rabbit polyclonal KRT5 antibody (Abcam, Cambridge, MA, USA) was used for basal cell identification (Shum et al. 2014), while chicken V-ATPase B1 subunit antibody was used for clear cell identification (Pietrement et al. 2006).

A rabbit polyclonal claudin 1 antibody and a rat monoclonal ZO1 antibody were used as markers of the basolateral membrane and tight junction, respectively (Kim and Breton 2016). The secondary antibodies used in this study (goat anti-rabbit IgG FITC-conjugated, CY3-conjugated donkey and FITC-conjugated donkey anti-chicken IgG) were affinity purified and obtained from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). They were diluted in Dako antibody diluent (Dako, Carpinteria, CA, USA). Confocal images were acquired on a Nikon A1R confocal microscope with NIS Elements (Nikon Instruments), followed by analyses with NIS Elements, Volocity 6 (v.6.3.1, Perkin Elmer) and Adobe Photoshop.

To validate the specificity of the antibody produced in rabbit and chicken for use in bats, we performed negative control labeling in the common vampire bats epididymis

2.3 AQP9 DAB staining for bright field and electronic transmission microscopy

Four entire epididymides were fixed by immersion in PLP for 4 h followed by three washes in PBS, incubated in a solution of 30% sucrose in PBS, embedded in OCT compound, mounted on a cutting block and frozen. Sections of 5 µm thickness were placed onto Superfrost Plus microscope slides, and stored at 4 ^{o}C until use. The endogenous peroxidase activity was quenched with a 0.3% H_2O_2 solution in 1 x PBS for 10 min at room temperature, followed by incubation in 1% w/v SDS for 4 min. The sections were then washed three times in PBS for 5 min, and blocked with 1% bovine serum albumin for 15 min.

Affinity purified rabbit polyclonal AQP9 antibody was used as previously described (Pastor-Soler et al. 2001) followed by three washes in PBS and specific biotinylated secondary. After three washes in PBS, the AQP9 staining was intensified by using diaminobenzidine (DAB). DAB concentrate plus reaction buffer was applied for 1h at room temperature, followed by three washes in 0.05M Tris pH 7.6/7.5% sucrose. DAB solution (200 µl) was applied to each section for 10 min, followed by 0.2 µl of 1% H_2O_2, three washes in PBS, and mounted with vectashield. Sections were examined on a Nikon 90i microscope with NIS Elements (Nikon Instruments), followed by analyses with NIS Elements and Adobe Photoshop.

Two epididymides were used to perform the AQP9 staining for electronic transmission microscopy. Cryosections of 70 µm thickness were collected into 1x PBS/0.02% NaAzide, and the endogenous peroxidase activity was quenched with a 0.3% H_2O_2 solution in 1x PBS for 10 min at room temperature, followed by SDS washing for 4 min, and three washes with permeabilization buffer (1x PBS/1% PBS BSA/ 0.05% Saponin) for 5 min each. The sections were then quenched in the same permeabilization buffer for 1 h at room temperature and incubated with the AQP9 antibody overnight at 4^{o} C. The sections were washed three times (1x PBS + 0.05% Saponin) before the secondary antibody, and then washed again. DAB concentrate plus reaction buffer was applied for 2h at room temperature, followed by 3 washes in 0.05M Tris pH 7.6/7.5% sucrose. DAB solution (1 mL) was applied to the sections, which were then incubated for 10 min, followed by the addition of 10 µL of 1% H_2O_2 to each section. After three washes in 0.05M Tris pH 7.6, the sections were fixed in 1x PBS/ 1% glutaraldehyde/ 5% sucrose at room temperature for 30 min, followed by five washes in 0.05M Tris pH7.6/7.5% sucrose, and then washed in 0.1M NaCacodylate. The sections were incubated with 1% OsO4/0.1 M NaCacodylate on ice for 30 min and washed three times with 0.1M NaCacodylate. The samples were then dehydrated in an ascending series of ethanol, incubated overnight at room temperature in 1:1 mixture of Epon and EtOH, and then incubated in Epon resine (EMbed 812 Kit). Grids were examined using a JEM-1011 transmission electron microscope (JEOL, Tokyo, Japan) at 80kV, and images acquired using an AMT XR60 digital imaging system (Advance Microscopy Techniques, Danvers, MA) were subsequently imported into Adobe Photoshop CS5. The AQP9 DAB analysis was evaluated in three regions of the epididymis: caput, corpus and cauda.

3. Results

The expression patterns of AQP9, V-ATPase, and cytokeratin 5 on the epididymis of common vampire bats.

To determine the characteristics of epithelial cell types in the epididymis of bats, tissues were labeled with AQP9, V-ATPase, and KRT5, markers of principal cells, clear cells, and basal cells, respectively. Confocal immunofluorescence images from initial segment, caput, corpus and cauda regions showed principal cells labeled positively for AQP9 (Fig. 1, green), whereas clear cells were positively labeled for V-ATPase (Fig. 1, red).

AQP9 was abundantly expressed on the apical membrane and stereocilia of principal cells that were present in the epididymal epithelium along the entire duct. The presence of AQP9 was confirmed by DAB staining for bright field and electronic transmission microscopy (Fig. 2). Unlike the apical membrane, basolateral membranes of these cells were negative for AQP9 labeling (Fig. 1 and 2). Moreover, principal cells presenting a columnar shape, and were the most abundant cells lining the epithelium (data not shown).

Clear cells were identified by expression of V-ATPase (B1 subunit) at their apical membrane (Fig. 3). These cells were observed intercalated among principal cells along the epididymis. To identify the clear cells' shape in each region, we used claudin 1 (Cldn1), which is highly expressed in the membranes of epididymal epithelial cells. The nucleus was positioned at their middle apical region and the goblet-shaped clear cells were observed in all segment regions (Fig. 3). No narrow-shaped and cuboidal-shaped clear cells were observed in the initial segment and cauda regions, respectively, in the vampire bat epididymis, unlike in mice or rats.

KRT5 was expressed in basal cells located at the basal portion of the epithelium, along the entire duct (Fig. 4). To observe whether basal cells projections extend towards the lumen, we used a tight junction marker, ZO1. We did not observe basal cells extending projections toward the lumen in any regions analyzed (Fig. 4). Basal cells extended lateral body projections underneath adjacent epithelial cells, and formed a remarkable dense network (Fig. 5).

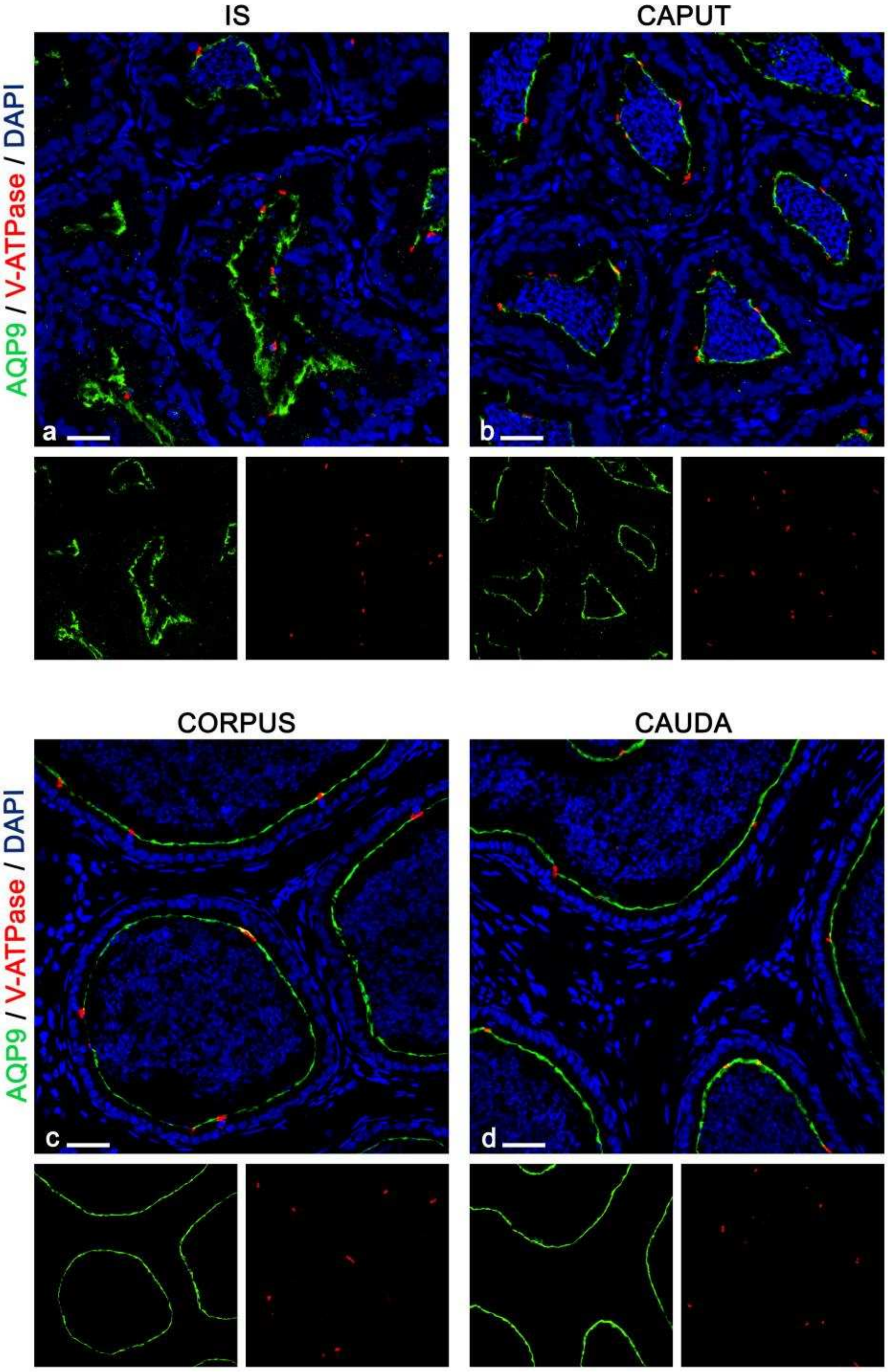

Figure 1. Double-immunolabeling of AQP9 (green) and V-ATPase B1 subunit (red) in the initial segment (IS), caput, corpus, and cauda of the *Desmodus rotundus*' epididymis. In all regions, AQP9 staining (green) was restricted to apical membrane of principal cells. Adjacent cells showed positive staining to V-ATPase (red) in the apical portion, a marker of clear cells. Sperm and nuclei were labeled with DAPI (blue). Scale bars: 50 µm (a-d).

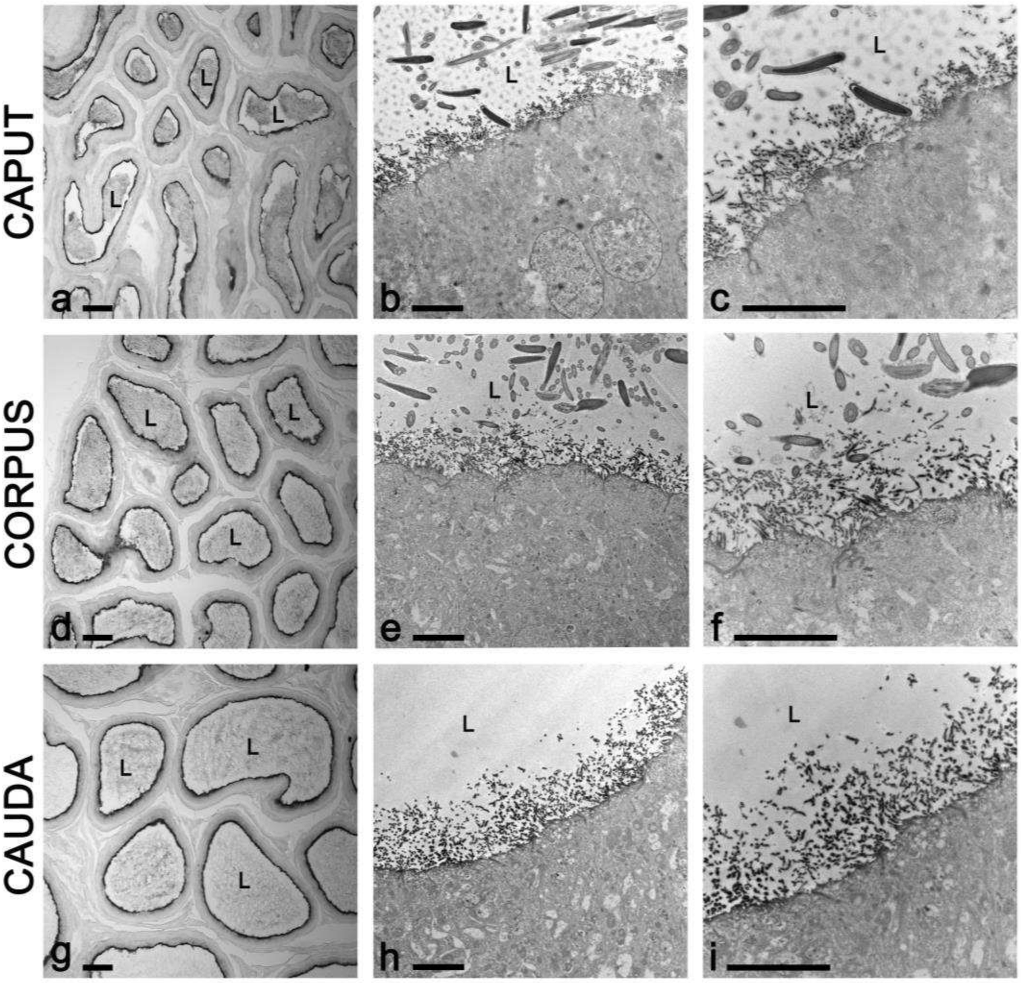

Figure 2: Aquaporin 9 (AQP9) DAB staining for bright field (a, d and g) and electronic transmission microscopy (b, c, e, f, h and i) in the caput, corpus and cauda regions of *Demodus rotundus*' epididymis. In all regions, AQP9 staining (black) was restricted to apical membrane and stereocilia of principal cells. L: lumen. Scale bars: 10 µm (a, d and g); 2 µm (b, c, e, f, h and i).

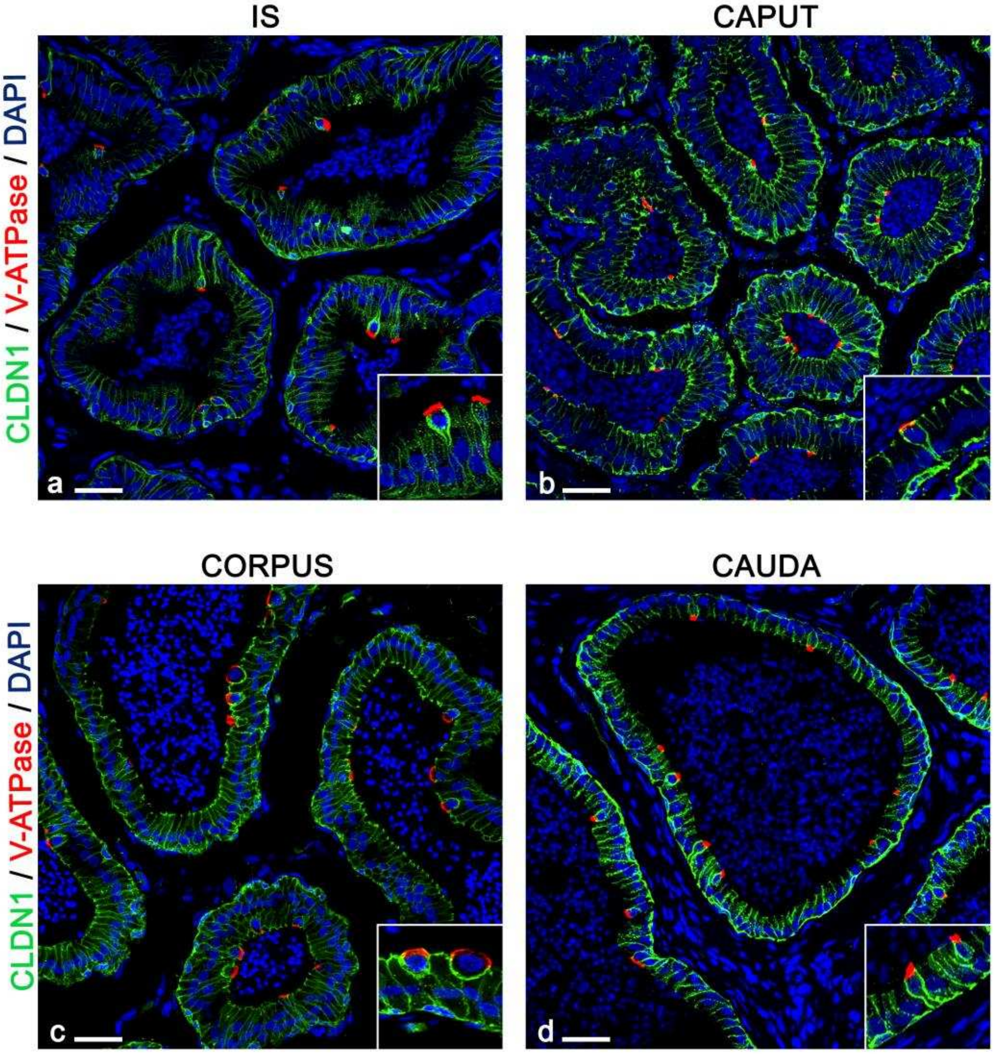

Figure 3: Double-immunolabeling of V-ATPase B1 (red) and claudin 1 (green) in the *Desmodus rotundus*' epididymis. V-ATPase positive cells were distributed between principal cells in all regions, and showed the same morphology from initial segment (IS) to cauda (a-d). Sperm and nuclei were labeled with DAPI (blue). Scale bars: 50 µm.

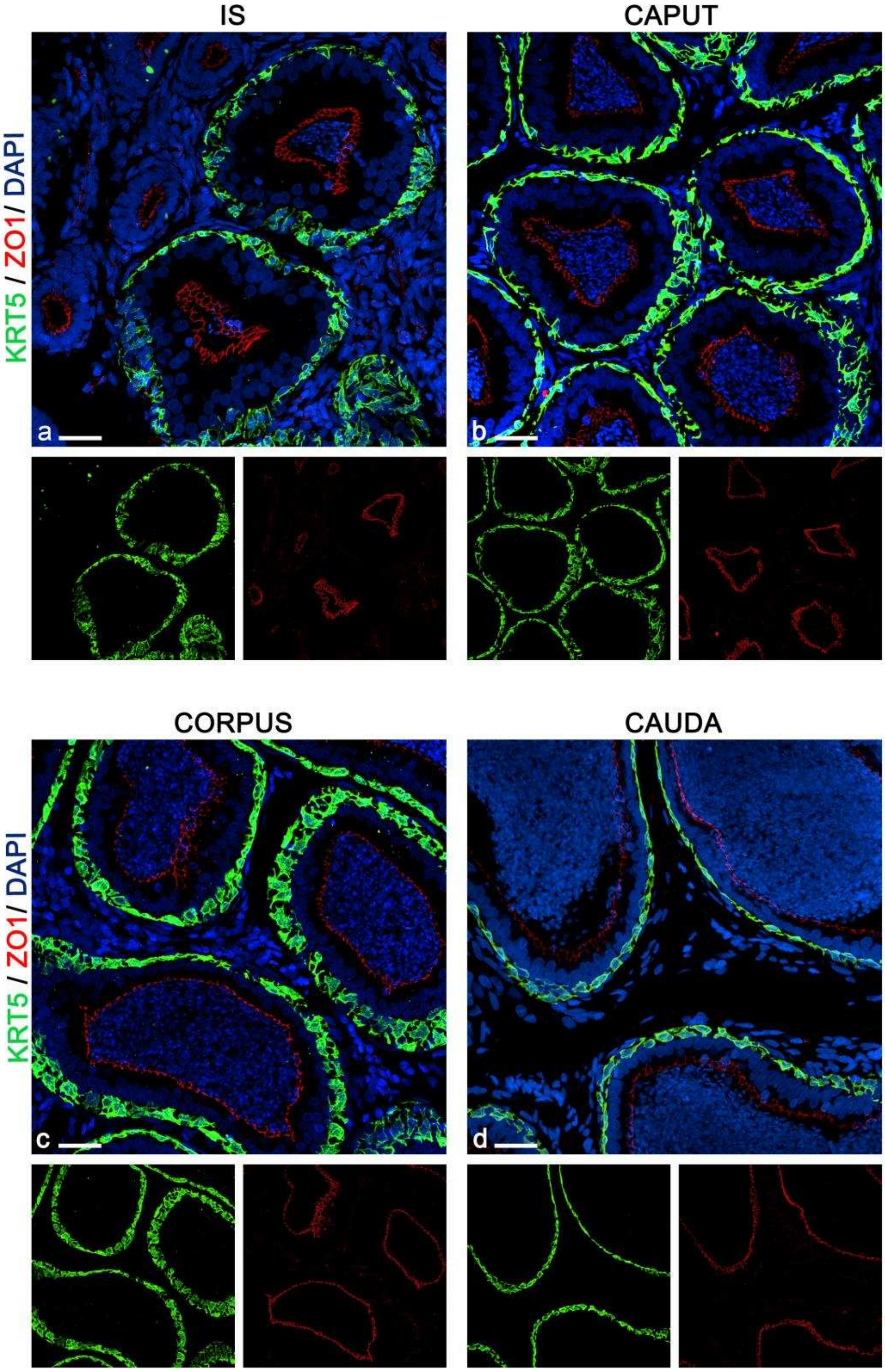

Figure 4. Double-immunolabeling of KRT5 (green), a basal cell marker, and ZO1 (red), a tight junction marker, in the *Desmodus rotundus*' epididymis. Basal cells were strongly labeled at the base of the epithelium without luminal reaching axiopodia in all regions of the epididymis, the initial segment (IS), caput, corpus and cauda regions (a-d). Sperm and nuclei were labeled with DAPI (blue). Scale bars: 50 µm.

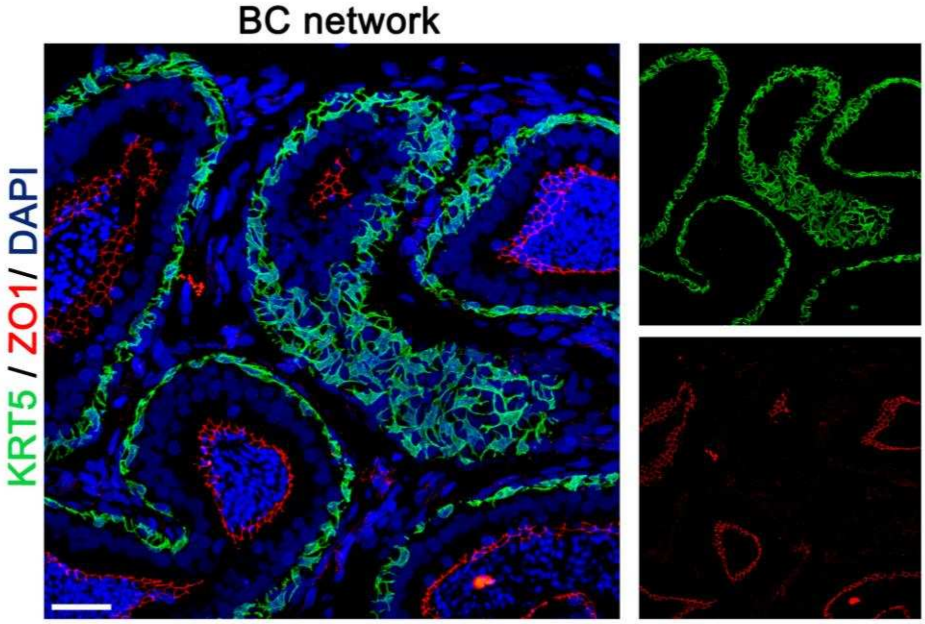

Figure 5. Double-immunolabeling of KRT5 (green), a basal cell marker, and ZO1 (red), a tight junction marker, in the *Desmodus rotundus*' epididymis showed the basal cells network at the base of the epithelium. Sperm and nuclei were labeled with DAPI (blue). Scale bars: 50 µm.

4. Discussion

In this study, we described the occurrence, distribution and expression pattern of AQP9, V-ATPase B1 subunit and KRT5 as cell specific markers of principal, narrow/clear and basal cells, respectively, in the initial segment, the caput, the corpus and the cauda regions of the vampire bats epididymis. The epididymal epithelium cells control the exchange of fluids, solutes, ions, and proteins between the basolateral and luminal compartments, by an extensive communication between adjacent cells and among different cells, in order to create specialized microenvironments needed to concentrate, mature and store sperm throughout the epididymis (Leung et al. 2004; Cheung et al. 2005; Cornwall et al. 2007; Shum et al. 2008; Belleannée et al. 2012; Robaire and Hinton 2015).

The positive labeling for AQP9 in the apical membrane at the stereocilia of principal cells observed here in *D. rotundus* was also reported in the epididymis of another bat species *Artibeus lituratus* (Oliveira et al. 2013) and in other species such as rats (Elkjær et al. 2000; Pastor-Soler et al. 2001) and small wild rodents (unpublished data). The AQP9 expression in the principal cells along the entire epididymis in this study, allows us to believe that those cells might presents a possible role in the secretion and reabsorption of water, urea and non-charged solutes in the common vampire bat, as described previously in rodents (Tsukaguchi et al. 1998; Pastor-Soler et al. 2001). The luminal fluid in the male excurrent duct is modified progressively after leaving the testis due the water reabsorption and secretion of different protein along the epididymis by principal cells (Wong and Yeung 1978; Clulow et al. 1998; Pastor-Soler et al. 2001; Hermo and Robaire 2002; Cornwall et al. 2007). In the caput and corpus regions, the luminal secretions contribute to the acquisition of spermatozoa motility and fertilizing ability, whereas the secretions from the cauda region are beneficial for the spermatozoa storage (Orgebin-Crist 1969; Turner 1995; Jones and Murdoch 1996).

Clear cells were present along the entire epididymal duct, and were positively labeled for V-ATPase B1 subunit in their apical membrane. The V-ATPase is a complex enzyme composed of several subunits that are assembled into transmembrane and cytosolic domains (Breton and Brown 2013). The subunit B from the V-ATPase has two isoforms, B1 and B2. The B1 subunit is expressed in the apical membrane of proton secreting cells, such as the clear cells, whereas the B2 subunit is ubiquitously expressed and is responsible for the acidification of intracellular organelles. In WT mice, B1 is the predominant plasma membrane subunit in clear cells, but in B1 KO mice, an increased amount of the B2 subunit at the plasma membrane compensates for the lack of B1 (Păunescu et al. 2004; Da Silva et al. 2007b). The V-ATPase complex hydrolyzes ATP to generate the energy required to pump ions into the lumen, and is thus essential for maintaining the low luminal pH, and bicarbonate concentration (Breton and Brown 2013). However, more studies are necessary to investigate as clear cells are involved in the luminal acidification in the common vampire bats.

Furthermore, clear cells showed a goblet-shape in vampire bats epididymis, which differs from the cuboidal-shape observed in caput and corpus regions of rat epididymis, and from the large clear cells observed in the rat cauda region (Hermo et al. 2005; Pietrement et al. 2006; Robaire and Hinton 2015).

Clear cells were observed in the initial segment of vampire bats instead of narrow cells. Narrow cells, which have been described as a pencil-shaped cell with elongated nuclei located only in the initial segment (Robaire and Hinton 2015; Schimming et al. 2015), were not observed in the epididymal epithelium of vampire bats. Although narrow cells have been cited present in this region in many species, such as ram (Schimming et al. 2015) and rats (Serre and Robaire 1998), and their absence in other animals has also been described in gerbil (Domeniconi et al. 2007), and in two species of neotropical bats, *Molossus molossus* and *Eumops glaucinus* (Beguelini et al., 2010).

Narrow and clear cells act similarly for the maintenance of the luminal acidification (Breton et al. 1999; Pietrement et al. 2006; Da Silva et al. 2010) due to the high expression of plasma membrane V-ATPase. Otherwise, both these cells exhibit differences in terms of morphological aspects.

Basal cells in *D. rotundus* epididymis can be identified by KRT5 expression as described in rats and mice (Shum et al. 2013; Shum et al. 2014; Kim et al. 2015), and wild rodents (data unpublished). In rats, basal cells have a role in regulating electrolyte and water transport by the principal cells (Leung et al. 2004). Moreover, they can emit cytoplasmatic projections toward to the lumen, establishing a new tight junction with adjacent cells, which allow contact with luminal substances increasing the proton secretion by adjacent clear cells (Shum et al. 2008). In the present study, we did not observe any basal cell axiopodia along the entire epididymis, suggesting that basal cells in the vampire bat epididymis may use a different mechanism to establish the crosstalk between basal and clear cells (Shum et al. 2008). On the other hand, basal cells presented a dense network formed by the lateral body projections in the basal epithelial portion and along the epididymal duct as described in mice (Shum et al. 2014). We suggest that these projections probably contribute to the communication establishment between basal cells.

In summary, we could observe some particularities in the common vampire bat epididymis epithelium regarding to the presence of clear cells in the initial segment instead of narrow cells, and the clear cells goblet-shape along the epididymis epithelium.

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CAPÍTULO III

Immunolocalization of tight junction proteins in the epididymis of common vampire bat at rainy and dry seasons

Immunolocalization of tight junction proteins in the epididymis of common vampire bat at rainy and dry seasons

Abstract

Desmodus rotundus, also known as common vampire bat, has an important epidemiological role in Latin America due to the fact that it is a primary reservoir of diseases like rabies. Common vampire bats usually live in colonies with dominant males, and may reproduce along the year. In several species, the epididymis is lined by a pseudostratified epithelium containing several cell types that are connected by tight junction (TJs), which form the blood-epididymis barrier (BEB). The BEB helps to create an optimal environment for the sperm maturation. Little is known about morphological and physiological characteristics of the BEB in neotropical bats. In this study, we characterized their post-testicular reproductive tract by examining the expression of TJs markers in the epididymis of the common vampire bats at rainy and dry season in Brazil. Cryostat sections of PLP-fixed epididymis were labeled for the TJs proteins, claudin 1 (Cldn1), claudin 3 (Cldn3), claudin 4 (Cldn4) and zonula occluden 1 (ZO1). TJs expression quantification was determined by ELISA. ZO1 was strictly located in TJs in all regions, during both seasons. Claudins showed a variation in the expression pattern, being located in the TJs, and in the basolateral membrane of epithelial cells. The respective labeling intensity varied depending on the region and season examined. Cldn1 showed an intense expression pattern in TJs and basolateral membrane at rainy season, which was different of observed at dry season, where Cldn1 was more intense in the basolateral membrane than in TJs in all regions. Cldn3 and Cldn4 showed different expression pattern in the proximal region of the epididymis. In both seasons, we observed an intense labeling in the TJs, however in the dry season we could observe Cldn3 and Cldn4 labeling in the basolateral membrane too. Cldn1, Cldn3 and Cldn4 labeling in TJs and basolateral membrane also were observed in the distal regions of the epididymis, the corpus and the cauda. In addition, Cldn1 labeling was observed in basal cells membrane in all regions, while Cldn3 showed labeling in the corpus and cauda, and Cldn4 was enriched in basal cells membrane of the caput, corpus and cauda regions in both seasons. Statistical difference for Cldn3 expression in the caput region and ZO1 in the corpus were observed.

Key words: *Desmodus rotundus*, male reproductive tract, seasonality, blood-epididymis barrier, epithelial cells.

1. Introduction

Tight junction (TJs) are a multiprotein complex formed by transmembrane and cytosolic proteins, which interaction is essential for the molecular, cellular and physiological regulation of many organs, especially the epididymis (Cyr et al., 2007; Angelow et al., 2008; Kim and Breton, 2016). The epithelial cells that line the epididymal duct are connect together by apical TJs, located at the apical end, luminal surface, of the lateral membrane of epithelial cells forming a continuous intercellular physical barrier named as blood-epididymis barrier (BEB) (Cyr et al., 1995; Cyr et al., 2007; Dubé et al., 2010; Robaire and Hinton, 2015; Kim and Breton, 2016). The BEB consists in a physical, physiological and immunological barrier that regulate the microenvironment within the epididymal duct, participating in the protection of sperm cells from immune system, regulating the composition of the luminal environment and also participating in the establishment and maintaining of cell polarity (Anderson and Van Itallie, 1995; Cyr et al., 1995; Robaire et al., 2006; Cyr et al., 2007; Dubé et al., 2010; Lingaraju et al., 2015; Robaire and Hinton, 2015). All these factors are important to drive the sperm physiology from a non-motile and infertile status, when they leave the testes, to a motile and fertile gamete during the sperm maturation process in the epididymis (Orgebin-Crist, 1969; Turner, 1995; Toshimori, 1998; Cooper, 2007; Sullivan and Saez, 2013).

TJs are composed of transmembrane proteins, such as occludins and claudins, and cytosolic proteins zonula occludens. Occludin is a region specific protein in mice epididymis, which is absent in the initial segment (Cyr et al., 1999; Cyr et al., 2007; Kim and Breton, 2016). In regard to claudins, they usually have aproximately 22 kDa, and they can show different expression pattern depending on the tissue or region. As these proteins are able to interact with other members of the claudin family it is possible to identify many types of claudins expressed in the same tissue (Furuse et al., 1999; Gregory and Cyr, 2006; Angelow and Yu, 2007; Dubé et al., 2010; Kim and Breton, 2016). Several claudins including Claudin 1 (Cldn1), Claudin 3 (Cldn3), and Claudin 4 (Cldn4) have been described in human, rat and mouse epididymis (Gregory et al., 2001; Gregory and Cyr, 2006; Cyr et al., 2007; Shum et al., 2008; Dubé et al., 2010; Kim and Breton, 2016). Moreover, zonula occludens (ZO) are peripheral membrane proteins that are located in the cytoplasm, which connect TJs proteins to the actin cytoskeleton and other cytosolic proteins. The ZO protein family is composed of three members, ZO1, ZO2, and ZO3 (Stevenson et al., 1986; Haskins et al., 1998; Ruan et al., 2014; Lingaraju et al., 2015; Kim and Breton, 2016).

Although numerous TJs proteins have been identified so far in several organs, our understanding about their functions in the epididymis remains unclear, especially in their participation in the formation of the BEB in wild animals. In this context, *Desmodus rotundus*, the common vampire bat, is one out of three vampire bat species among more than 1,000 bats species occurring in all continents. This strict hematophagous bat has a wide geographical distribution in Latin America (Simmons, 2005; Peracchi et al., 2007). Due to its feeding habits based exclusively on blood, the vampire bat population was favored by the introduction of domestic herbivores in Latin America, especially cattle, pigs and horses, which become their mainly food source (Kotait et al., 2007). There is little information about the reproductive behavior of this species. It is known that vampire bats usually live in colonies with dominant males forming a harem mating system, and are reproductively active along the year, resulting in variation in the birth records (Greenhall et al., 1983; Alencar et al., 1994, Gomes and Uieda, 2004; Peracchi et al., 2007). In contrast with some studies in other Neotropical bat species (Beguelini et al., 2010; Beguelini et al., 2013; Oliveira et al., 2013; Beguelini et al., 2015), there is no information of morphological features of the epididymis in this species.

Thus, the aim of the present study was to describe the expression pattern according to the presence, distribution and quantification of four TJs markers, Cldn1, Cldn3, Cldn4, and ZO1, in the common vampire bat epididymis during Brazilian rainy and dry seasons.

2. Material and Methods

2.1 Study area and animals

Sixteen adult males were captured using mist nets, positioned near a cave in Itamarati de Minas, MG, Brazil (21°23'55"S and 42°51'53"W; 585 m altitude) during the rainy (n=8; September to February; animals were collected in November) and dry (n=8; from March to August; animals were collected in June) seasons. The animals were transported to the Laboratory of Structural Biology at the Federal University of Viçosa (UFV), where they were placed in cages protected from light. Euthanasia was performed the following day by intra-peritoneal administration of sodium pentobarbital at a dose of 40 mg/kg of body weight, followed by guillotining (Freitas et al., 2013). Bat capture was authorized by the Chico Mendes Institute for Biodiversity Conservation (ICMBio, license number 40629-1). This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory

Animals of the National Institutes of Health. All the experimental procedures were reviewed and approved by the Committee on the Ethics and Use of Animal Experiments of UFV (CEUA process number 55/2013).

2.2 Tissue fixation and immunofluorescence

Entire epididymides from animals collected in each season were processed for immunofluorescence analysis. Samples were fixed by immersion in periodate-lysine-paraformaldehyde (PLP) solution containing 4% paraformaldehyde for 4 h at room temperature, and rinsed three times in phosphate-buffered saline (PBS). Tissues were then incubated in PBS with 30% sucrose, for at least 24 h, followed by embedding in OCT compound (Tissue-Tek; Sakura Finetek, Torrance, CA, USA), mounting on a cutting block, and then frozen. Tissues were cut at 5 µm thickness using a Leica 3050 cryostat (Leica Microsystems, Bannockburn, IL, USA) and all sections were placed onto Superfrost Plus microscope slides (Fisher Scientific, Pittsburgh, PA, USA), and stored at 4 ^{o}C until use (Kim et al., 2015).

Sections were hydrated in PBS for 10 min, and heated by microwaving in an alkaline buffer (Vector Laboratory, Burlingame, CA) four times for 1 min each time, with 5 min intervals for antigen retrieval. To block nonspecific binding, 1% bovine serum albumin in PBS was applied for 30 min at room temperature. The sections were incubated with primary polyclonal antibodies of Cldn1 (1:200; Invitrogen, Grand Island, NY, USA), Cldn3 (1:200; Invitrogen, Grand Island, NY, USA), and Cldn4 (1:200; Invitrogen, Grand Island, NY, USA) (Kim and Breton, 2016), in a moist chamber overnight at 4 ^{o}C. The samples were washed in PBS and incubated with secondary antibodies for 60 min at room temperature. Double-staining was performed with anti-ZO1 rat monoclonal antibody (1:40; Gift from Dr. Eveline Schneeberger, Department of Pathology, Massachusetts General Hospital, MA, USA). The secondary antibodies (goat anti-rabbit IgG FITC-conjugated, 1:200; CY3-conjugated donkey, 1:800) used in this study were affinity purified and were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). All antibodies were diluted in Dako antibody diluent (Dako). To evaluate the pattern expression of TJs markers along the common vampire bat's epididymis, we considered five different segments. The initial segment region was divided in two regions, the proximal initial segment, region near to the efferent duct, and the distal initial segment, between the proximal initial segment and the caput, the caput, the corpus and cauda. Confocal images were acquired on a Nikon A1R confocal microscope with NIS Elements (Nikon

Instruments), followed by analyses with NIS Elements, Volocity 6 (v.6.3.1, Perkin Elmer) and Adobe Photoshop.

3. Results

The expression patterns of TJs proteins in the common vampire bat's epididymis

In this study, we observed positive labeling for Clnd1, Clnd3, Clnd4, and ZO1 along the epididymal epithelium of common vampire bat. The evaluation was performed in the epithelium of five regions, the proximal initial segment, the distal initial segment, the caput, the corpus and the cauda. Differences in relation to the TJs proteins labeling expression were observed between regions when considered the dry and rainy seasons.

ZO1 showed to be strictly located in TJs at the apical end of the lateral membrane of epithelial cells throughout the epididymis in both seasons, as observed in Figure 1. Figure 2 and Figure 3. In regard to the claudin proteins expression, they were observed in the TJs and in the basolateral membrane of epithelial cells. However, the expression pattern of Cldn1 (Fig 1), Cldn3 (Fig. 2) and Cldn4 (Fig. 3) varied according to region and seasonal period.

Cldn1 showed an intense expression pattern in TJs and basolateral membrane of epithelial cells, principal and clear cells, at rainy season in all regions (Fig. 1). This labeling was more intense in the basolateral membrane of principal and clear cells than in TJs along the entire epididymal epithelium from animals captured at the dry season. Moreover, Cldn1 was positive-labeled in the basolateral membrane of basal cells in all regions at dry and rainy seasons (Fig. 1).

In regard to Cldn3, we observed differences on its expression between proximal (initial segment and caput) and distal epididymal regions (corpus and cauda) regardless of the season. We observed an intense Cldn3 labeling in the TJs at the proximal and distal initial segments and caput region (Fig. 2). However, a lower expression in the basolateral membrane of epithelial cells in the caput region at rainy season, and in all proximal epididymal regions at dry season were observed. Otherwise, distal epididymal regions presented an epithelium positive labeled to Cldn3 in the TJs and in the basolateral membrane in both seasons. However, Cldn3 labeling in the corpus and cauda regions from the rainy season was more intense in the TJs than the TJs from the dry season (Fig. 2). As observed for Cldn1, Cldn3 labeling was also observed in the basal cells membrane, but just in the corpus and cauda regions in both seasons.

We observed Cldn4 labeling in the TJs in the proximal and distal initial segments from both seasons. However, Cldn4 labeling was also observed in the basolateral membrane of those regions at the dry season (Fig. 3). Differently of Cldn3, Cldn4 showed an intense labeling in the basolateral membrane of principal and clear cells in the caput region at dry and rainy seasons, besides TJs. Corpus and cauda regions showed the same Cldn4 labeling pattern, in the TJs and basolateral membrane, for both seasons (Fig. 3). As Cldn1 and Cldn3, Cldn4 labeling was also observed in the basal cells membrane in the caput, corpus and cauda regions in both seasons.

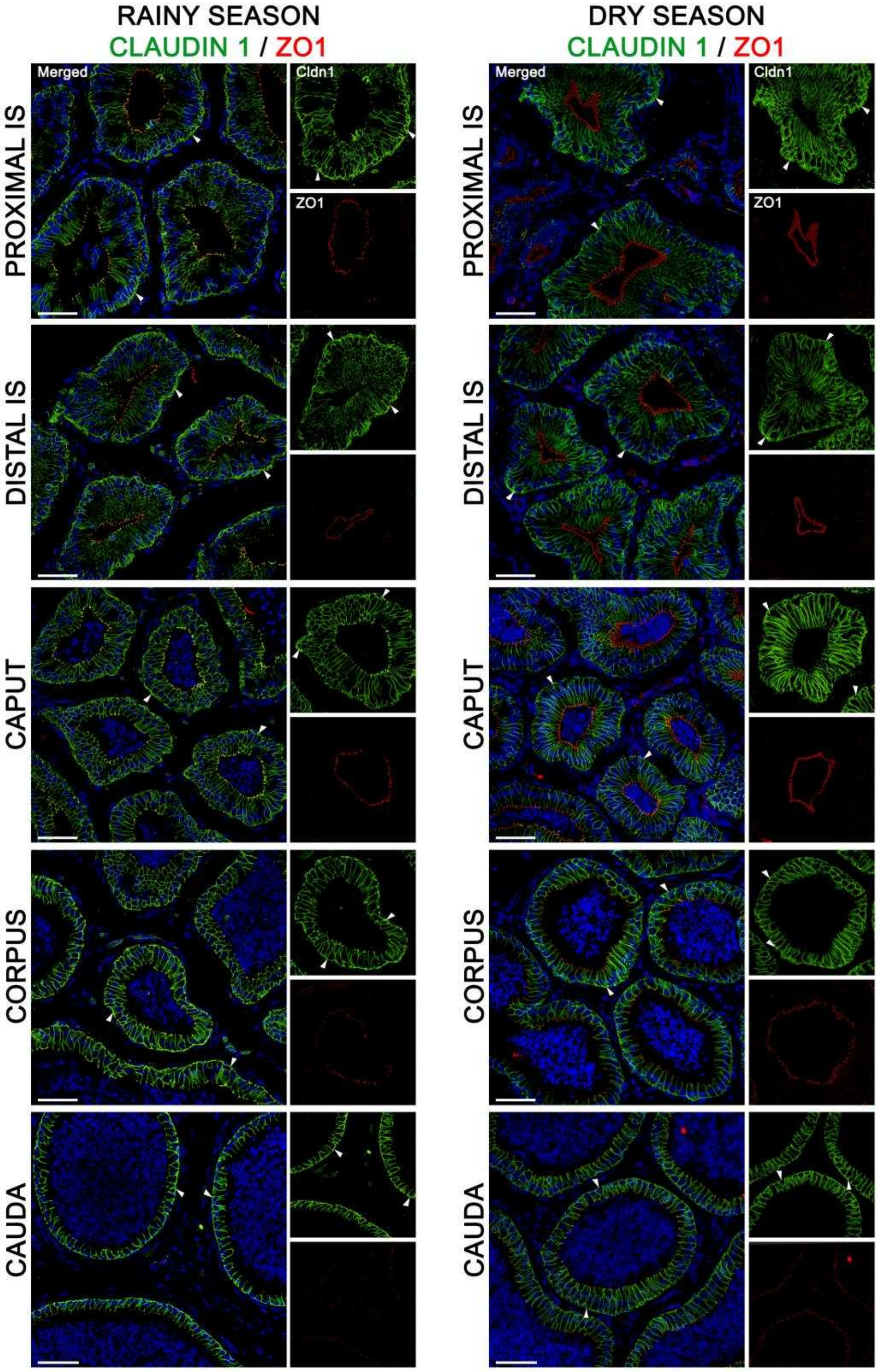

Fig. 1: Expression of claudin 1 (green) and ZO1 (red) in the epididymis of *Desmodus rotundus* during the rainy and dry season. Sperm and nuclei are labeled with DAPI (blue). Claudin 1 labeling was observed in basal cell plasma membrane (arrowheads) in all epididymis regions at rainy and dry season. Scale bars: 50 μm.

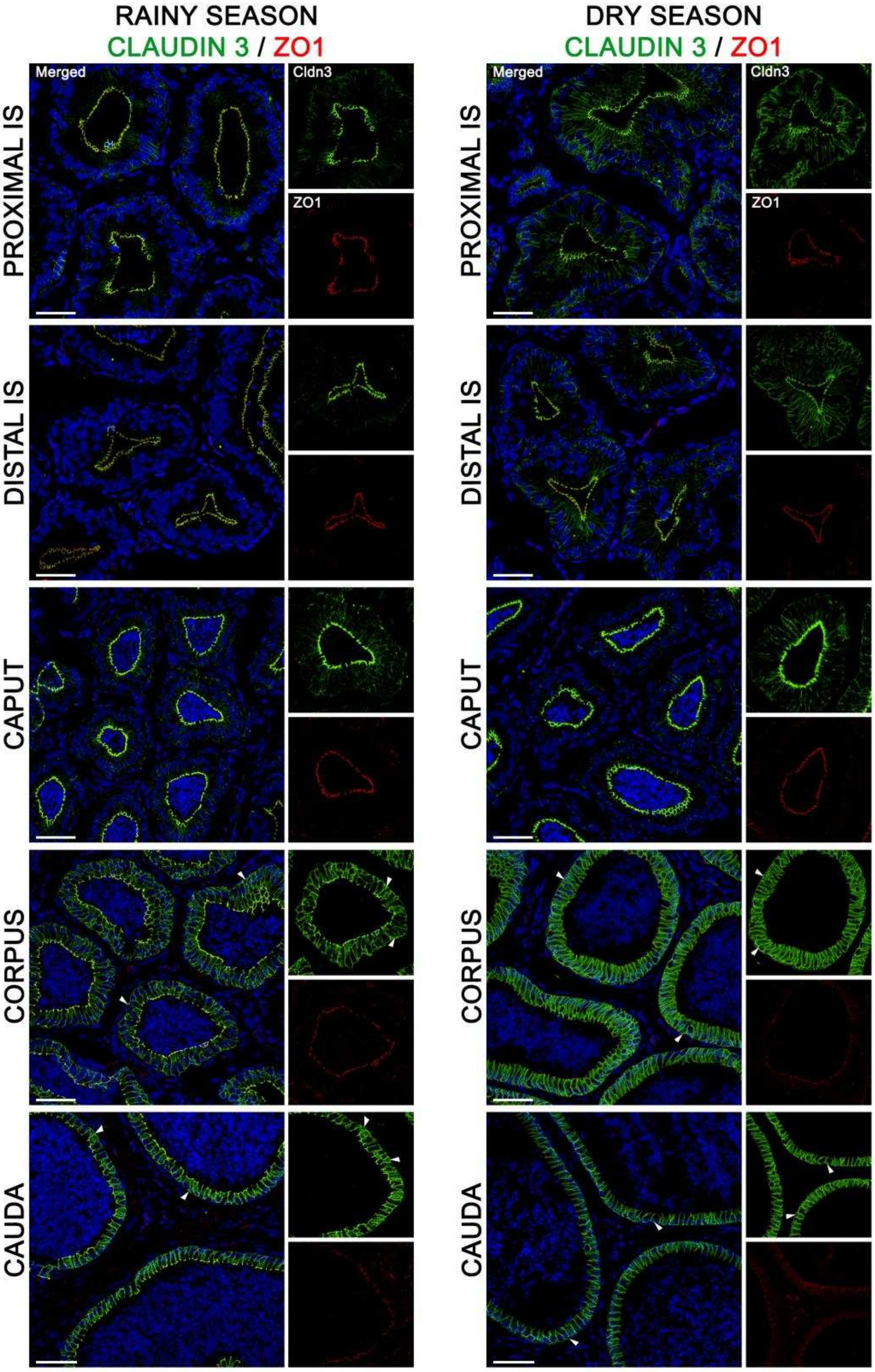

Fig. 3: Expression of claudin 3 (green) and ZO1 (red) in the epididymis of *Desmodus rotundus* during the rainy and dry season. Sperm and nuclei are labeled with DAPI (blue). Claudin 3 labeling was observed in basal cell plasma membrane (arrowheads) in the corpus and cauda regions at rainy and dry season. Scale bars: 50 µm.

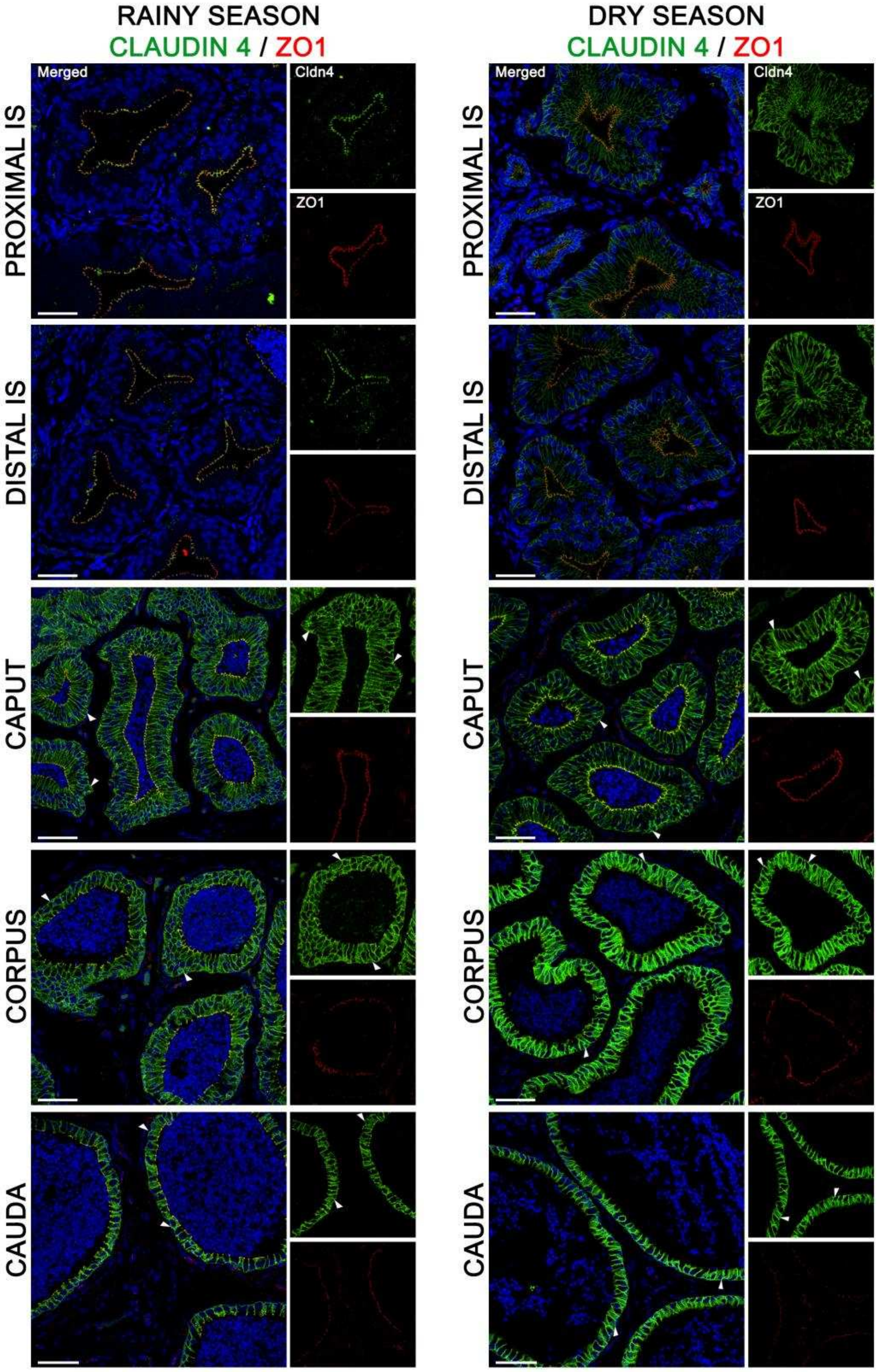

Fig. 3: Expression of claudin 4 (green) and ZO1 (red) in the epididymis of *Desmodus rotundus* during the rainy and dry season. Sperm and nuclei are labeled with DAPI (blue). Claudin 4 labeling was observed in basal cell plasma membrane (arrowheads) in the caput, corpus and cauda regions at rainy and dry season. Scale bars: 50 µm.

4. Discussion

In this current study, we described the expression pattern of four TJs proteins (Cldn1, Cldn3, Cldn4 and ZO1) according to their presence, distribution and quantification along the epididymis of common vampire bat captured during rainy and dry seasons in Brazil.

Herein we observed the presence of four TJs proteins in the epididymal epithelium, which is composed of three cell types, such as principal, basal and clear cells (unpublished data). Therefore, we might suggest the epithelial cells are connected together by TJs in the vampire bat's epididymis as other mammals (Gregory et al., 2001; Gregory and Cyr, 2006; Cyr et al., 2007; Dubé et al., 2010; Kim and Breton, 2016). TJs are located in the apical portion of the membrane of epithelial cellsforming a physical barrier in the epididymis. This barrier contributes to the establishment and maintenance of a specific luminal microenvironment for sperm maturation, protects the sperm against immune system activity, and participates in the cell polarity (Anderson and Van Itallie, 1995; Cyr et al., 1995; Cyr et al., 2007; Dubé et al., 2010; Robaire and Hinton, 2015).

We observed three transmembrane proteins of the claudin family, Cldn1, Cldn3 and Cldn4, and one cytosolic protein, ZO1, along of the epididymis of the vampire bat. Several claudins, including Cldn1, Clnd3, and Cldn4, and ZO1 have been described in human, rat and mouse epididymis (Stevenson et al., 1986; Gregory et al., 2001; Gregory and Cyr, 2006; Shum et al., 2008; Dubé et al., 2010; Ruan et al., 2014; Kim and Breton, 2016). However, the specific role of each one of these proteins, their participation in the formation of TJs, and their probable interaction in the epididymal epithelium remains to be established (Furuse et al. 1999; Gregory and Cyr, 2006; Findley and Koval, 2009; Van Itallie and Anderson, 2013; Kim and Breton, 2016).

ZO1 showed a regular expression pattern along the epididymis of common vampire bats, which was present only in the TJs in the initial segment of mice (Kim and Breton, 2016). Its expression, however, was different between seasons. The high expression of ZO1 in the corpus region at the dry season may indicates the importance of ZO1 for the establishing of TJs with cytosolic proteins during this period, when compared to the rainy season, in common vampire bat's epididymis. It is well known that ZO1 interacts directly with transmembrane proteins, as claudins, mediating the connection of TJs to the actin cytoskeleton and other cytosolic proteins in other mammals (Stevenson et al., 1986; Ruan et al., 2014; Lingaraju et al., 2015; Kim and Breton, 2016).

In this study, we observed expression of Cldn1, Cldn3 and Cldn4 in the TJs, and in the basolateral membrane of epididymis epithelial cells in the rainy and dry seasons. Previous studies showed that Cldn1, Cldn3 and Cldn4 are not restricted to the TJs, but they can be also expressed in the basolateral membrane in rats and in mice (Gregory et al., 2001; Shum et al. 2008; Kim and Breton, 2016). The significance of this location outside of TJs remains unknown. Due the differences observed in their distribution along the epididymis, and between the seasons, we might conclude that Cldn1, Cldn3 and Cldn4 in the common vampire bat shows a certain level of plasticity, especially when we analyze the influence of the seasonality in the reproduction.

The expression distribution between Cldn1, Cldn3 and Cldn4 was different in the proximal regions of the epididymis when compared both seasons. The factors that regulate the blood epididymis barriercomponents and their distribution are unknown. However, a previous study with orchidectomized rats demonstrated a change in the Cldn1 labeling pattern in the epididymis initial segment (Gregory et al., 2001), which might indicates that androgens produced by the testis could regulate Cldn1 expression in epididymis, especially in the proximal region. The fact that common vampire bat can breed along the year (Greenhall et al., 1983; Alencar et al., 1994; Gomes and Uieda, 2004), might indicates a regular androgens levels, even though, the androgens levels may change in different individuals. The fact of Cldn3 expression in the caput region was different between rainy and dry season, might indicates that Cldn3 may be is more affected by variations in the androgen levels than Cldn1 and Cldn4. However, more studies are necessary to understand the relation between androgens and the claudins distribution in the epididymis of the common vampire bat.

In this study, we observed Cldn1, Cldn3 and Cldn4 labeling in basal cells. It is well known that basal cells are located at the base of the epithelium and present a huge plasticity (Shum et al., 2008; Kim et al. 2015; Roy et al. 2016). It is believed that the presence of Cldn1 in the basal cells of rats epididymis provides a ‘‘molecular ladder” that would support basal cell elongation (Shum et al., 2008), and Cldn4 in mice epididymis could be an additional potential mediator of basal cell elongation (Kim and Breton, 2016). Although we did not observed basal cells elongation in the common vampire bat epididymis (unpublished data), we believe that Cldn1, Cldn3 and Cldn4 are involved in the cell adhesion, since we observed their expression along the basal cell membrane, and in the paracellular transport of solute across basal and adjacent cell types.

In summary, in this study, we identified Cldn1, Cldn3, Cldn4 and ZO1 expression in the epididymal epithelial in a Neotropical bat species in two different seasons. The epithelial cells from the epididymal duct of common vampire bat are connected together by TJs proteins, and shown the expression of Cldn1, Cldn3, Cldn4 and ZO1 along the entire epididymis, with differences in their location, and season. ZO1 expression was observed just in the TJs, in all regions and in both seasons. Cldn1, Cldn3 and Cldn4 expression was observed in TJs and in the basolateral membrane, and varied according to the region and season. In addition, we observed Cldn1, Cldn3 and Cldn4 expression in the basal portion of the epithelium in the basal cells along the epididymis in both seasons.

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CONCLUSÕES GERAIS

- O ducto epididimário de *Desmodus rotundus* pode ser dividido em quatro regiões, segmento inicial, cabeça, corpo e cauda, e é formado por um epitélio pseudoestratificado composto por células principais, basais e claras.
- A região da cabeça apresentou maior altura do epitélio epididimário e o menor diâmetro luminal.
- A região da cauda apresentou maior diâmetro de ducto.
- O compartimento de ducto foi composto por epitélio pseudoestratificado e lúmen repleto de espermatozoide em todas as regiões, enquanto no compartimento de interducto foram observados fibroblastos, músculo liso e vasos sanguíneos.
- A porcentagem do epitélio diminuiu a partir da região proximal para a região distal do epidídimo, enquanto o percentual luminal aumentou.
- Componentes do interducto, tecido conjuntivo e vasos sanguíneos, apresentaram maior percentual na região da cauda.
- As células principais foram as mais frequentes no epitélio epididimário, seguida pelas basais e claras. Cada tipo celular apresentou características ultraestruturais semelhantes em cada uma das regiões do epidídimo analisadas.
- Células principais apresentaram formato colunar, com grânulos PAS-positivos no citoplasma, marcação PAS-positiva no estereocílio, e características ultraestruturais relacionadas com funções de absorção e secreção. Além disso, também apresentaram expressão da proteína aquaporina 9 no esteriocílio ao longo de todo o epidídimo.
- Células basais foram observadas na porção basal do epitélio, adjacente à membrana basal, foram positivamente marcadas para citoqueratina 5, e formaram uma rede de comunicação entre elas através da emissão de prolongamentos citoplasmáticos laterais na base do epitélio. Não foi observada nenhuma extensão citoplasmática da célula basal em direção ao lúmen.
- Células claras apresentaram formato caliciforme, diferentemente do que foi descrito em ratos e camundongos, com núcleo na região apical da célula ao longo de todo o epitélio. Apresentaram baixa frequência na região da cauda, marcação positiva para H^+ V-ATPase. Não foi observado aparato endocítico.
- Foram observados quatro diferentes componentes de *tight junction* no epidídimo do morcego vampiro comum, claudina 1, claudina 3, claudina 4 e ZO1.

- ZO1 foi observada estritamente na região da *tight junction*, independente da região ou estação analisada.
- Todas as claudinas estudadas apresentaram marcação positiva na região da *tight junction* e nas membranas basolaterais de todos os tipos celulares. Entretanto, foi observada variação entre as regiões na mesma estação, e entre as estações.
- Porção basal do epitélio epididimário, células basais, foi marcada para todas as claudinas, com diferença entre as regiões, mas independente das estações.