Muscular atrophy, impaired epithelial autophagy and UCHL1 increase in androgen-deficient cauda epididymis

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Abstract

In epididymis, cimetidine induces androgenic failure due to reduced sex hormone-binding globulin stromal levels and blockade of androgen receptor (AR) nuclear import. UCHL1, a hydrolase of ubiquitin-proteasome system (UPS), seems to play a role in autophagy and apoptotic pathway. However, the role of UPS and autophagy in epididymis has not been clarified. We evaluated UCHL1 and autophagy in epididymal cauda epithelium under androgenic deficiency induced by cimetidine, focusing on the interplay among these processes and apoptosis. The integrity of epididymal muscular layer was also evaluated. Male rats received cimetidine (CMTG) or saline (CG). Seminal vesicles were weighed, the expression of androgen-responsive genes *Crisp1* and connexin 43 (Cx43) in cauda epididymis was evaluated, and cauda fragments were processed for light and transmission electron microscopy. The epithelium height and muscular thickness were measured. TUNEL, immunohistochemistry for caspase-3 and Cx43, and immunofluorescence for AR, Bcl-2, UCHL1, MAP LC3A, and p62/SQSTM1 (autophagic markers) were performed. Bcl-2, UCHL1, and Cx43 were detected by Western blot. In CMTG, the reduction in seminal vesicles weight accompanied by downregulation of *Crisp1* and Cx43 confirmed epididymal androgenic failure. These results were associated with muscular atrophy, apoptosis and weak Cx43 and AR immunoexpression, supporting the androgenic dependence of muscular integrity. The high UCHL1 levels and reduction in Bcl-2 reinforce UCHL1 role in epithelial cells death. The intense immunoexpression of LC3A and p62/SQSTM1 indicates autophagic disturb, which in association with high UCHL1 levels, points to a role of UPS and autophagy in the regulation of epididymal epithelial cells viability under androgenic control.

Reproduction (2020) **159** 693–705

Introduction

Spermatozoa storage in cauda epididymis depends on epithelial activity, which creates a microenvironment responsible for the maintenance of sperm quiescence (Cooper 1986, Cornwall 2009, Robaire & Hinton 2015). The epithelial cells are regulated by dihydrotestosterone (DHT) (Wong & Yeung 1978, Wong *et al.* 1978, Moore & Bedford 1979), a biologically active androgen in epididymis that binds to androgen receptor (AR) and regulates the expression of genes involved in cell survival (Ezer & Robaire 2003, Robaire & Hamzeh 2011). Thus, androgen withdrawal induces apoptosis in all epididymal regions (Fan & Robaire 1998, Kempinas & Klinefelter 2014) and impairs sperm reproductive competence due to alterations in sperm storage in cauda epididymis (Orgebin-Crist *et al.* 1975, Foldes & Bedford 1982, Robaire & Hinton 2002). Besides androgens, several processes maintain sperm quality in epididymis and studies have suggested that extracellular

ubiquitination may be involved in the elimination of defective spermatozoa (Sutovsky *et al.* 2001, Sutovsky 2003, Baska *et al.* 2008, Eskandari-Shahraki *et al.* 2013). Ubiquitination is a process in which the covalent attachment of a ubiquitin molecule (Kleiger & Mayor 2014) generates a signal that directs the cellular components for destruction in cellular proteolytic machinery: the ubiquitin-proteasome system (UPS) or in the autophagic pathway (Shaid *et al.* 2013, Ji & Kwon 2017). This process is regulated by deubiquitinating enzymes (DUBs), which are responsible for disassembling ubiquitin-substrate covalent link in order to edit or remove ubiquitin signal (Komander *et al.* 2009). Thus, DUBs are crucial regulators of almost all cellular process, such as transcription, cell cycle, and apoptosis (Amerik & Hochstrasser 2004). The ubiquitin carboxyl-terminal hydrolase L1 (UCHL1), a protein gene product 9.5, is a DUB involved in both deubiquitination and ubiquitination processes (Liu *et al.* 2002). Although UCHL1 has been demonstrated in testes (Kwon *et al.*

2004a) and in epithelial cells of caput and cauda epididymis (Santamaria *et al.* 1993, Kwon *et al.* 2006), the role of this hydrolase in these organs has not yet been clarified. However, studies have confirmed the role of UCHL1 in the molecular regulation of cell death, since different tumors present low levels of this enzyme (Wang *et al.* 2008, Li *et al.* 2010, Ummanni *et al.* 2011). The efferent duct ligation in mice lacking UCHL1 failed to induce apoptosis in caput epididymis epithelium (Kwon *et al.* 2006). Moreover, in cryptorchid mice lacking (Kwon *et al.* 2004b) or overexpressing (Wang *et al.* 2006) UCHL1, the testis shows resistance to apoptosis or high germ cell death index, respectively. A directly proportional correlation between UCHL1 and germ cell death has also been demonstrated in testes of rats treated with the antidepressant fluoxetine (Câmara *et al.* 2019). Besides apoptosis, UCHL1 is involved in the regulation of autophagic flux, since pharmacological inhibition of this enzyme results in activation of the autophagic pathway in oligodendroglial cells (Cartier *et al.* 2012, Pukaß & Richter-Landsberg 2015). This finding is reinforced by the fact that, either in HeLa cells (Yan *et al.* 2018) or in cardiac fibroblasts (Zhang *et al.* 2014), the overexpression of this enzyme suppresses autophagy.

During autophagy, bulks of cytoplasmic components are sequestered into double membrane vesicles, called autophagosomes, and delivered to the lysosomes for degradation or recycling (Kaur & Debnath 2015). The microtubule-associated protein 1A/1B-light chain (MAP LC3A) is essential for autophagosome formation due to the conjugation with phosphatidylethanolamine, forming LC3-II (Klionsky *et al.* 2016). LC3-II is inserted in the autophagosomal membrane, being an autophagic receptor that interact with adaptor proteins (Shaïd *et al.* 2013). These adaptor proteins, including p62/SQSTM1, can bind to ubiquitinated cargos, which are delivered to the autophagosome (Kaur & Debnath 2015). Since p62/SQSTM1 is also degraded, this specific substrate of the autophagosome has been used to determinate the autophagic flux (Klionsky *et al.* 2016). Cell homeostasis and survival are maintained by autophagy (Doherty & Baehrecke 2018). In this case, the cytoprotective effect of autophagic process is associated with the degradation of pro-apoptotic factors. When a stimulus overcomes this cytoprotective effect, cell death machinery is activated. Therefore, autophagy and apoptosis are interplayed processes, in which autophagy inhibits apoptosis and vice versa (Mariño *et al.* 2014).

Cimetidine is an H₂ receptor antagonist used to treat gastric ulcer (Brogden *et al.* 1978) and different types of cancer as adjuvant therapy (Kubecova *et al.* 2011). Male patients treated with cimetidine have demonstrated alterations in serum testosterone levels (Wang *et al.* 1982), gynecomastia (Spence & Celestin 1979), and reduction in sperm concentration (Van Thiel *et al.*

1987). Studies have indicated that cimetidine binds to AR, antagonizing the androgen function in kidney cells, prostate and pituitary (Funder & Mercer 1979, Winters *et al.* 1979, Knigge *et al.* 1983). Moreover, rats treated with cimetidine have demonstrated reduced serum testosterone levels (Beltrame *et al.* 2015, 2019, de Santi *et al.* 2018) associated to changes in the seminiferous epithelium (Sasso-Cerri & Miraglia 2002, Sasso-Cerri & Cerri 2008, Beltrame *et al.* 2011, 2019, Beltrame & Sasso-Cerri 2016) and sperm parameters (Beltrame *et al.* 2019). In epididymis, cimetidine reduces sex hormone-binding globulin (SHBG) levels and impairs the AR nuclear import (de Santi *et al.* 2018), leading to epithelial cells apoptosis and confirming that this drug is an androgen disrupter in the male reproductive system. This drug also induces apoptosis in peritubular myoid cells (França *et al.* 2000, Sasso-Cerri & Miraglia 2002), testicular vascular smooth muscle cells (Beltrame *et al.* 2012), and vas deferens muscular layer (Koshimizu *et al.* 2013), pointing to a sensibility of smooth muscle to androgen deficiency. It is known that the contractility of the smooth muscle layer that surrounds epididymal epithelium contributes to the movement of spermatozoa along the epididymis (Robaire & Hinton 2015, Elfgen *et al.* 2018). Therefore, the regulation of the epididymal smooth muscle contraction by paracrine and endocrine factors (Elfgen *et al.* 2018), such as androgens (Sujarit & Pholpramool 1985), is essential for sperm maturation and storage (Elfgen *et al.* 2018). Here, we investigated the integrity of cauda epididymis muscular layer under androgenic deficiency induced by cimetidine. Since the role of ubiquitination and autophagy in epididymis as well as the hormonal control of these processes have been poorly addressed, the immunoexpression of UCHL1 and autophagic markers was evaluated in the androgen-deficient epididymal epithelium, focusing on the interplay among apoptosis, ubiquitination and autophagy.

Materials and methods

Animal procedures

The protocol regarding animal use and experimental procedures was approved by the Ethical Committee for Animal Research of São Paulo State University (UNESP/FOAr; CEUA 28/2014).

Ten adult *Holtzman* male rats 100 days old were maintained in polypropylene cages under standard light conditions (12 h light:12 h darkness cycle) at controlled temperature ($23 \pm 2^\circ\text{C}$) and humidity (55 ± 10), with water and food *ad libitum*. The animals from cimetidine group (CMTG; $n=5$) received daily intraperitoneal injections of cimetidine (Hycimet, Hypofarma, Ribeirão das Neves, Brazil) 100 mg/kg of body weight (Beltrame *et al.* 2011, 2012, 2015, 2019, Koshimizu *et al.* 2013, de Santi *et al.* 2018), while the animals from control group (CG=5) received saline by the same route. The animals

were treated for 52 consecutive days, period that induces testicular changes (França *et al.* 2000, Sasso-Cerri & Miraglia 2002, Sasso-Cerri & Cerri 2008, Beltrame *et al.* 2011, 2012) as well as androgenic failure in cauda epididymis (de Santi *et al.* 2018). After treatment, the animals were anesthetized with 80 mg/kg bw of ketamine hydrochloride (Francotar, Virbac do Brasil Ind. Com. Ltda, Jurubatuba, Brazil) and 8 mg/kg BW of xylazine hydrochloride (Virbaxyl; Virbac do Brasil Ind. Com. Ltda Jurubatuba, Brazil). In order to obtain identical halves, each cauda epididymis was sectioned in the sagittal plane. The halves of right cauda epididymis were fixed for histological analyses and embedded in glycol methacrylate (historesin) or paraffin. One half of left cauda epididymis was collected and stored at -80°C for Western blot analysis. Fragments of the other half were collected for Transmission Electron Microscopy (TEM) or immediately immersed in RNA Keeper stabilizing reagent (LGC Biotecnologia; Cotia, Brazil; 14-0002-01) for real time PCR. The seminal vesicles were removed and weighed.

Histological procedures for light microscopy

Cauda epididymis of CG and CMTG were fixed for 48 h in 4% formaldehyde buffered at pH 7.4 with 0.1 M sodium phosphate, dehydrated in ethanol, and embedded in glycol methacrylate (historesin) or paraffin. Morphological analyses were performed in 5- μm -thick historesin sections stained with H.E (Cerri & Sasso-Cerri 2003). The paraffin sections were submitted to TUNEL (TdT-mediated dUTP-biotin Nick End Labeling) method and immunofluorescence reactions.

In an attempt to maintain a standardized analysis, according to our previous study (de Santi *et al.* 2018), only the proximal region of cauda epididymis (Reid & Cleland 1957, Robaire & Hermo 1988), corresponding to segments 15 and 16 (Domeniconi *et al.* 2016), was analyzed in this study.

Transmission Electron Microscopy (TEM)

Fragments of cauda epididymis were fixed for 16 h in a mixture of freshly prepared 4% formaldehyde and 5% glutaraldehyde buffered at pH 7.2 with 0.1 M sodium cacodylate. After washing in 0.1 M sodium cacodylate at pH 7.2, the tissue fragments were transferred to sodium cacodylate-buffered 1% osmium tetroxide at pH 7.2 for 1 h, immersed in 2% aqueous uranyl acetate for 1.5 h, dehydrated in graded concentrations of ethanol, treated with propylene oxide, and then embedded in Araldite (Beltrame *et al.* 2012, 2015). Semithin sections stained by 1% toluidine blue solution were used for morphological analysis and suitable regions were carefully selected for trimming of the blocks. Ultrathin sections were collected on grids, stained in alcoholic 2% uranyl acetate and lead citrate, and examined in a transmission electron microscope (Tecnai G2 Spirit, FEI Company).

Epithelium height and muscular layer thickness

In three non-serial sections stained with H.E or Masson's trichrome, ten sections of epididymal ducts were captured, totalling 30 sections per animal. The images were captured

using a DP-71 camera attached to Olympus BX-51 microscope. In each duct stained with H.E., the epithelial height was measured from the cell base (in contact with the basement membrane) to the apical surface, excluding the stereocilia. In the sections stained with Masson's trichrome, the thickness of the smooth muscle layer surrounding the epididymal epithelium was also measured. Two measurements of epithelium and muscular layer were performed in each duct section, totalling 60 measurements per animal, using the analysis system Image-Pro Express 6.0 (Olympus).

TUNEL method

TUNEL method was performed as previously described (Sasso-Cerri & Miraglia 2002, Beltrame *et al.* 2011, 2012) and according to the ApopTag Peroxidase In Situ Kit (Millipore) manufacturer's instructions.

The sections were treated with proteinase K (Sigma-Aldrich) and the endogenous peroxidase was inactivated. The sections were incubated with terminal deoxynucleotidyl transferase (TdT) for 1 h at 37°C . After incubation with anti-digoxigenin peroxidase, the reaction was revealed with 3,3'-diaminobenzidine (DAB; Sigma-Aldrich) and counterstained with Carazzi's haematoxylin. Sections used as negative controls were incubated in a TdT-free solution. In four non-serial sections per animal, the number of TUNEL-positive smooth muscle cells was quantified in a total standardized perimeter of 3000 μm and the number of positive cells per micrometre was obtained.

Immunofluorescence and immunohistochemistry reactions

PGP 9.5 (UCHL1), MAP LC3A, p62/SQSTM1 (markers of autophagy), Bcl-2, and AR were detected by immunofluorescence, whereas caspase-3 (marker of apoptosis) and connexin-43 were detected by immunohistochemistry. Non-serial paraffin sections were immersed in 0.001 M citrate buffer pH 6.0 and heated in a microwave oven at 90°C during 30 min for antigen recovery. For the immunohistochemistry reaction, the endogenous peroxidase was inactivated with hydrogen peroxide. The sections were incubated with BSA 2% for 30 min to block non-specific binding. After washes in PBS, the sections were incubated in a humidified chamber at 4°C overnight with the following primary antibodies: mouse anti-PGP9.5 (Protein Gene Product) MAB (1:800; Biocare Medical, Concord, USA; CM 329 AK), mouse anti-p62/SQSTM1 MAB (1:100; Santa Cruz; sc-28359), mouse anti-Bcl-2 MAB (1:200; Santa Cruz; sc-7382), rabbit anti-MAP LC3A polyclonal antibody (1:90; Santa Cruz; sc-134226), rabbit anti-AR polyclonal antibody (1:150; Millipore; 06-680 PG-21), and rabbit anti-active caspase-3 antibody (1:100; Abcam). The sections submitted to immunofluorescence reactions were washed in PBS and incubated at room temperature in darkness for 1 h with appropriate secondary antibodies. Detection of PGP9.5, p62/SQSTM1, and Bcl-2 was performed using Alexa Fluor 488 anti-mouse antibody (1:1000; Life Technologies), whereas MAP LC3A and AR were detected by Alexa Fluor 594

anti-rabbit antibody ReadyProbes® reagent (Life Technologies; R37117). After washes in PBS, nuclear staining was performed with DAPI (Life Technologies) for 5 min in darkness at room temperature and the slides were mounted with Fluoromount G mounting medium (EMS, Hatfield, USA). For detection of active caspase-3 by immunohistochemistry, the sections were washed in PBS and incubated with Labelled StreptAvidin-Biotin Kit (Universal Dako LSAB, Dako Inc.) at room temperature. The reaction was revealed with 3,3'-diaminobenzidine (DAB, Sigma-Aldrich) and the sections were counterstained with haematoxylin.

The protocol for immunolocalization of connexin-43 was performed according to [Hermo et al. \(2007\)](#) and [Oliveira et al. \(2016\)](#), with some modifications. The sections were treated with 0.3 M glycine for 5 min and washed in PBS. Antigen recovery was performed in a microwave oven at 90°C (3 × 15 min) using 0.1 M citrate buffer pH 6.0. After inactivation of endogenous peroxidase with hydrogen peroxide, the sections were washed in PBS for 10 min and incubated at 37°C for 2 h with the mouse anti connexin-43 MAB (1:30; Santa Cruz; sc-271837). The sections were washed in PBS, incubated for 1 h at room temperature with the Dako Envision Dual Link System-HRP (Dako Inc., Carpinteria; K4061). The reactions were revealed with DAB, and the sections were counterstained with haematoxylin.

For all markers evaluated, negative controls were performed by incubating the sections with non-immune serum in place of primary antibodies.

UCHL1, MAP LC3A, and p62/SQSTM1 immunofluorescent areas

Immunofluorescent areas were analyzed using DFC 550 Camera (Leica) attached to a BM4000 B LED microscope (Leica) and the Leica Application Suite software (LAS 4.3, Leica).

In four non-serial sections per animal, the immunofluorescent area of UCHL1, MAP LC3A, and p62/SQSTM1 was measured in proximal cauda epididymis. The immunolabeled areas were measured in a total standardized epithelial area of 50,000 µm² per animal and the immunofluorescent area per square micrometre was obtained.

All parameters of the software Leica Application Suite (LAS 4.3, Leica), including threshold adjustment and color range (hue, saturation, and intensity), were rigorously standardized in all sections for the analyzes of immunofluorescent areas.

Western blot

The protocol was performed according to [de Santi et al. \(2018\)](#). Frozen cauda epididymis fragments were homogenized in lysis buffer for protein extraction, and protein samples (30 µg for UCHL1 or Bcl-2 and 45 µg for Cx43) were separated in SDS-PAGE (15% for UCHL-1 or Bcl-2 and 10% for Cx43) and transferred to nitrocellulose membrane (GE Healthcare). The membranes were incubated overnight at 4°C with mouse anti-PGP9.5 (Protein Gene Product) MAB (1:1000; Biocare Medical, Concord, USA; CM 329 AK), mouse anti-Bcl-2 MAB

(1:200; Santa Cruz; sc-7382), and mouse anti-Cx43 MAB (1:500; Santa Cruz; sc-271837). After washes in PBS/T, the membranes were incubated for 1 h at room temperature with HRP-conjugated anti-mouse secondary antibody (1:2000; Sigma-Aldrich). The membranes were washed in PBS/T and the reactions were detected using ECL Plus Western Blotting Substrate (Boster Biological Technology, Pleasanton, USA; AR1196). For positive controls, the membranes were stripped and incubated with rabbit anti-β-tubulin MAB (1:1000; Abcam; ab108342) and HRP-conjugated anti-rabbit secondary antibody (1:7500; Sigma-Aldrich). The assays were reproduced in triplicate for CG and CMTG.

Real time polymerase chain reaction (qPCR)

The expression of the androgen-responsive genes *Crisp1* ([Haendler et al. 1997](#), [Roberts et al. 2001](#)) and *Connexin43* (Cx43) ([Lydka et al. 2011](#), [Firestone & Kapadia 2012](#)) was evaluated by qPCR. RNA was extracted from cauda epididymis samples using Aurum Total RNA Mini Kit (Bio-Rad Laboratories; 732-6820), and RT was performed using High-Capacity cDNA RT Kit (Applied Biosystems; 4368814). For qPCR, PowerUp SYBR Green Master Mix (Applied Biosystems; A25742) and the QuantStudio 3 Real-Time PCR instrument (Applied Biosystems) were used. Standard curves at each individual run were used for all genes analyzed, and gene expression was presented as ratio to the housekeeping control. All primers were designed using the murine sequence available at the University of California, Santa Cruz (UCSC), Genome Browser and the Primer3 program ([Koressaar & Remm 2007](#), [Untergasser et al. 2012](#)) (Table 1).

Statistical analysis

The data were checked for normal distribution by the D'Agostino-Pearson normality test and analyzed by Student's *t*-test at a significance level of *P* < 0.05 using GraphPad Prism 6.01 software.

Results

Cimetidine-induced androgenic failure in cauda epididymis

Either the relative weight of seminal vesicles or the expression of the androgen-responsive genes *Crisp1* and Cx43 reduced significantly in CMTG (Figs 1A, B and 5H).

Table 1 Sequence of primers used in qPCR reactions.

Gene	Sequence (5'–3')
<i>Crisp1</i>	CCTCATTGGCTGTGATGTTG CGTAATAAAGGCAGGGGTTG
<i>Cx43</i>	AGCAAGCTAGCGAGCAAAAC GAGTTCATGTCCAGCAGCAA
<i>β-Actin</i>	ACGGTCAGGTCATCACTATC TGCCACAGGATTCCATACC

Figure 1 The relative weight of seminal vesicles (A) and the expression of the androgen-responsive gene *Crisp1* (B) reduced significantly in CMTG (* $P=0.0117$ (A); * $P=0.0262$ (B)).

Androgenic failure on the muscular layer

In CG, an evident smooth muscular layer was observed surrounding the epithelium on one side (Fig. 2A and C) and interconnected with the connective tissue on the opposite side (Fig. 2A). However, in CMTG, either the muscular layer or the epithelium was thinner than in CG (Fig. 2A, B, C and D) and scarce portions of connective tissue interconnected with the muscular layer were observed (Fig. 2B). The morphometric analyses

Figure 2 Photomicrographs of proximal cauda epididymis region sections of animals from CG (A, C) and CMTG (B, D). In A and B, epididymal sections stained with Masson's trichrome show muscular layer (arrows) surrounding the epithelium (asterisks) and interconnected with the connective tissue (CT). In B, note the scarce connective tissue (CT); the muscular layer (arrows) and the epithelium (asterisks) are thinner than in A. In C and D, epididymal sections stained with toluidine blue show principal (thin arrows) and clear (thick arrows) cells in the epithelium. In D, note the thin muscular layer (M) and reduced epithelial height (Ep). Bars = 36 μm (A, B); 18 μm (C, D). The epithelial height (E) and the muscular thickness (F) of animals from CMTG are significantly reduced in comparison with CG (* $P=0.002$ (E); * $P=0.0089$ (F)).

confirmed the significant reduction in the epithelial height and in the muscular thickness of animals from CMTG (Fig. 2E and F). In CMTG, several smooth muscle cells positive to the TUNEL method or immunolabeled to active caspase-3 were found (Fig. 3B, C, E and F), whereas scarce labeled cells were seen in CG (Fig. 3A and D). In the sections used as negative controls, no immunolabeling or TUNEL positivity was observed (Fig. 3G and H). The number of TUNEL-positive smooth muscle cells increased significantly in CMTG (Fig. 3I).

Under TEM, superimposed layers of fusiform shaped-smooth muscle cells with elliptical and regularly outlined nuclei were normally in close contact to each other by adherens junctions in CG (Fig. 4A and C). Otherwise, in CMTG, the muscle cells showed convoluted surface and were detached from each other. The large spaces between these cells were filled with

Figure 3 Photomicrographs of proximal cauda epididymis region sections of animals from CG and CMTG subjected to caspase-3 immunohistochemistry (A, B, C) and TUNEL method (D, E, F). In A and D, scarce immunolabeled cells are observed in CG (arrows). In B and C, epithelial (thick arrows) and smooth muscle cells (thin arrows) are positive to caspase-3 immunohistochemistry. In E and F, TUNEL-positivity is observed in epithelial (thick arrows) and smooth muscle cells (thin arrows). G and H show negative controls of caspase-3 (G) and TUNEL (H). Bars = 18 μm (A, B, D, E, G, H); 7 μm (C, F). (I) In CMTG, the number of TUNEL-positive smooth muscle cells is significantly higher than in CG (* $P<0.0001$).

Figure 4 Electron micrographs of portions of smooth muscular layer surrounding the epididymal epithelium in CG (A and C) and CMTG (B, D, E). In A, superimposed layers of fusiform smooth muscle cells with elliptical and regularly outlined nuclei (arrows). In a portion of these cells under high magnification (C), note the close contact between the cells, which are attached by *adherens* junctions – dense plaques (arrowheads). In B, the smooth muscle cells surface are intensely convoluted and the round/ovoid nuclei irregularly outlined show electron dense chromatin in the periphery (arrows). Under high magnification (D), note that the cells are detached from each other and a large space filled with collagen fibrils (asterisks) are observed between them. In E, a damaged smooth muscle cell shows convoluted nucleus with masses of condensed chromatin in the periphery (arrows). Dense bodies (Db); Mitochondria (Mi).

collagen fibrils (Fig. 4B and D). Moreover, the round/ovoid or convoluted nuclei were irregularly outlined and showed masses of condensed chromatin in the nuclear periphery, typical of apoptosis (Fig. 4B and E).

In CG, a punctual and sparse Cx43 immunolabeling was detected in the smooth muscle cells of epididymal cauda (Fig. 5A and B). Moreover, either in CG or CMTG, Cx43 immunostaining in epithelial cells (probably basal cells) was often seen. However, a weak or no immunoexpression was noted in the thin muscular layer of CMTG (Fig. 5C, D and E). In epididymal cauda extracts of CMTG, low levels of Cx43 were detected by Western blot (Fig. 5G). The gene expression of Cx43 was also significantly lower in CMTG than in CG (Fig. 5H).

In the sections of cauda epididymis of animals from CG, an evident AR immunofluorescence was observed

Figure 5 Photomicrographs of proximal cauda epididymis region sections subjected to immunohistochemistry for detection of Cx43 and nuclear staining with haematoxylin. In A and B, a punctual and sparse immunostaining is observed in smooth muscle cells (arrows), whereas in C, D and E, no immunolabeling is noted in the thin muscular layer (asterisks). Either in CG (A) or CMTG (C, D, E), epithelial cells (probably basal cells) are also immunolabeled (arrowheads). (F) Negative control. Bars = 7 µm (A, C, D, E); 18 µm (B, F). (G) Western blot analysis of Cx43 in cauda extracts. A weak band at 43 kDa (Cx43 molecular weight) is observed in CMTG. Tubulin signal is observed in both groups. (H) Cx43 expression reduced significantly in CMTG * $P=0.0484$.

Figures 6 Photomicrographs of proximal cauda epididymis region sections subjected to immunofluorescence for detection of AR and nuclear staining with DAPI (overlay, B and D). Evident AR immunofluorescence is observed in the nuclei of epithelial cells (thin arrows), fibroblasts (thick arrows), and smooth muscle cells (arrowheads) in CG (A, B). In CMTG, a diffuse immunoreaction is observed in the epithelial cells cytoplasm (thin arrows) and a weak or no AR immunolabeling is observed in the nuclei of fibroblasts (thick arrow) and smooth muscle cells (asterisks) (C, D). E, negative control. Bars = 18 µm.

in the nuclei of epithelial cells, smooth muscle cells, and fibroblasts (Fig. 6A and B). However, in CMTG, the epithelial cells showed a diffuse AR immunorexpression in the cytoplasm, and the scarce fibroblasts and smooth muscle cells were weakly immunolabeled (Fig. 6C and D). In both reactions for detection of Cx43 or AR, no immunolabeling was detected in the negative controls (Figs 5F and 6E).

Immunorexpression of Bcl-2, UCHL1, and autophagy in the epithelial cells

The immunofluorescence for detection of PGP9.5 (UCHL1) in the epithelial cells showed a strong immunofluorescence in CMTG (Fig. 7C and D) when compared to CG (Fig. 7A and B). The quantitative analysis showed a significant increase in the PGP9.5 (UCHL1) immunofluorescent area per square micrometre of epididymal epithelium in CMTG (Fig. 7F). Moreover, these results were confirmed by the high levels of this protein detected by Western blot in CMTG (Fig. 7G).

A slight immunorexpression of the autophagic markers MAP LC3A (Fig. 8A and B) and p62/SQSTM1 (Fig. 8E and F) was observed in the epididymal epithelial cells of animals from CG, whereas an intense immunorexpression of these markers was detected in CMTG (Fig. 8C, D, G and H). The quantitative analyses confirmed the significant increase in the immunofluorescent area of both markers in CMTG (Fig. 8K and L).

A weak immunorexpression of Bcl-2 was detected in the epididymal epithelial cells of CMTG in contrast to a strong immunolabeling in CG (Fig. 9A, B, C and D). These results were in agreement with the low levels of this anti-apoptotic factor observed in the epididymal cauda extracts of CMTG in contrast to the high levels in CG (Fig. 9F).

In all sections used as negative controls for each antigen, no immunofluorescence was detected (Figs 7E, 8I, J and 9E).

Discussion

In cauda epididymis, cimetidine reduces sex hormone-binding globulin (SHBG) levels in the stromal tissue and impairs AR nuclear import in epithelial cells, leading to apoptosis in the epithelial cells and reduction in the epididymal duct diameter (de Santi *et al.* 2018). Thus, we investigated the integrity of proximal cauda epididymis muscular layer as well as the immunorexpression of UCHL1 (a component of UPS) and autophagic markers in the epithelium of the androgen-deficient epididymis induced by cimetidine. In the present study, besides a diffuse cytoplasmic AR immunolabeling in the epithelial cells, confirming failure in AR nuclear import, the reduction of seminal vesicle weight and downregulation of the androgen-responsive genes *Crisp1* and *Cx43*

Figures 7 Photomicrographs of proximal cauda epididymis region sections of animals from CG (A and B) and CMTG (C and D) showing immunofluorescence of PGP9.5 (UCHL1) and nuclear staining with DAPI. Note the enhanced UCHL1 immunolabeling (arrows) in CMTG when compared to CG. E, negative control. Bars = 18 μm . (F) The UCHL1 immunofluorescent area per square micrometre of epididymal epithelium increased significantly in CMTG (* $P=0.0006$). (G) Western blot analysis of PGP9.5 (UCHL1) levels in cauda extracts. A band at 27 kDa, corresponding to the PGP9.5 molecular weight, is stronger in CMTG than in CG. Tubulin signal is observed in both groups.

supported the androgenic dysfunction in CMTG. Moreover, the significant decrease in Cx43 was consonant with the presence of muscle cells apoptosis and reduction in the muscular layer thickness. These findings, in association with the weak immunorexpression of Cx43 and AR in the smooth muscle cells, confirm the importance of androgens for these cells. The presence of cell death in the epithelium, the low immunorexpression of Bcl-2, and reduction in the epithelium height were correlated with increased UCHL1 immunorexpression, indicating that this deubiquitinating enzyme (DUB) plays a role in the epididymal epithelial cells death under androgen deficiency. The intense immunorexpression of MAP LC3A and p62/SQSTM1 in CMTG indicates impaired autophagy and points to a correlation between UCHL1 and autophagy in the regulation of epididymal epithelial cells apoptosis under androgen regulation.

Androgenic failure on epididymal smooth muscle cells

We have demonstrated that cimetidine treatment reduces serum testosterone levels (Beltrame *et al.* 2015, 2019,

Figures 8 Photomicrographs of proximal cauda epididymis region sections showing MAP LC3A (A, B, C, D) and p62/SQSTM1 (E, F, G, H) immunofluorescence and nuclear staining with DAPI. Note the enhanced immunofluorescence for both markers in the epithelium of CMTG (arrows; C, D, G and H) in comparison to CG (A, B, E and F). (I and J) Negative controls. Bars = 18 μ m. (K and L) Either LC3A (K) or p62/SQSTM1 (L) immunofluorescence area per square micrometre of epididymal epithelium is significantly increased in CMTG. (* $P=0.0001$ (K); * $P<0.0001$ (L)).

de Santi *et al.* 2018) in association with changes in Leydig cells (Beltrame *et al.* 2015) and in the seminiferous epithelium (Sasso-Cerri & Miraglia 2002, Sasso-Cerri & Cerri 2008, Beltrame *et al.* 2011, 2019, Beltrame & Sasso-Cerri 2016). Here, the reduction in seminal vesicles weight in association with downregulation of the androgen-responsive genes *Crisp1* (Haendler *et al.* 1997, Roberts *et al.* 2001) and *Cx43* (Lydkja *et al.* 2011, Firestone & Kapadia 2012) confirms the androgen dysfunction induced by cimetidine in cauda epididymis, in which blockade of epithelial AR nuclear import and low SHBG stromal levels have been previously demonstrated (de Santi *et al.* 2018). In the present study, a weak AR immunofluorescence was also detected in the epididymal smooth muscle cells of CMTG. This result is in agreement with the low AR levels in the total

Figures 9 Photomicrographs of proximal cauda epididymis region sections showing Bcl-2 immunofluorescence and nuclear staining with DAPI in CG (A and B) and CMTG (C and D). In CG (A and B), an evident immunolabeling is noted in the basal portion of cells throughout the epithelium (arrowheads), whereas in CMTG (C and D), few cells show immunofluorescence (arrowheads). E: negative control. Bars = 18 μ m. (F) Western blot analysis of Bcl-2 levels in cauda extracts. A band at 26 kDa, corresponding to the Bcl-2 molecular weight, is stronger in CG than in CMTG. Tubulin signal is observed in both groups.

epididymal extract (de Santi *et al.* 2018) and is also consonant with the low expression of both *Cx43* and *Crisp1* demonstrated in the present study.

Cx43 is present in GAP junctions between smooth muscle cells, including in muscular layer of cauda epididymis (Cyr *et al.* 1996, Oliveira *et al.* 2016). Studies indicated that GAP junctions may transmit survival signals or inhibit death signals by limiting the flux of toxic molecules (Vinken *et al.* 2006, Du *et al.* 2017). Blockage of testicular connexins induces germ cell death (Lee *et al.* 2006), and the reduction in *Cx43* levels were associated with increased apoptosis in osteocytes (Davis *et al.* 2017), vascular smooth muscle cells (Tang & Fang 2017), and cardiomyocytes (Du *et al.* 2017). The animals from CMTG showed a high incidence of TUNEL-positive and caspase-3-immunolabeled muscle cells in cauda epididymis. Moreover, apoptotic ultrastructural features and loss of contact between muscle cells were also observed. These findings

associated with weak immunoexpression of Cx43 in the muscular layer confirm that these cells undergo apoptosis, at least in part, due to downregulation of Cx43 induced by androgenic dysfunction. Besides Cx43, apoptosis is also activated by excessive elevation of intracellular Ca^{+2} levels (Kondratskyi *et al.* 2015). Androgens can regulate intracellular Ca^{+2} levels due to the direct inhibition of type L Ca^{+2} channels (Scrugg *et al.* 2004, 2007). In epididymis, androgen deprivation may increase the levels of Ca^{+2} due to the inhibition of phosphodiesterase (Holtz *et al.* 1981). Therefore, the reduction in androgen bioavailability in CMTG may disturb Ca^{+2} homeostasis, inducing smooth muscle cells apoptosis, as also suggested in muscle cells of testicular blood vessels (Beltrame *et al.* 2012) and vas deferens (Koshimizu *et al.* 2013). Further studies are necessary to clarify the mechanism by which smooth muscle cells undergo apoptosis under androgenic deficiency in epididymis.

Our findings are in accordance with previous studies, which demonstrated the susceptibility of smooth muscle cells to cimetidine treatment (França *et al.* 2000, Sasso-Cerri & Miraglia 2002, Beltrame *et al.* 2012, Koshimizu *et al.* 2013). Since in cauda epididymis androgen supply is probably provided by the blood stream, the low immunoexpression of AR either in smooth muscle cells or in stromal fibroblasts may impair drastically the androgen supply to cauda epididymis.

UCHL1 and autophagy in the epididymal epithelial cells under androgenic dysfunction

The role of UCHL1 in the male reproductive system has not yet been fully understood. In fluoxetine-treated rats, this antidepressant reduces testosterone synthesis and increases the immunoexpression of UCHL1 in the seminiferous epithelium, indicating that androgens may regulate this hydrolase (Câmara *et al.* 2019). Here, the high levels of UCHL1 observed in the androgen-deficient epididymal epithelium support this hypothesis. The epididymal epithelium secretes UCHL1 (Baska *et al.* 2008) and other components of the ubiquitin-proteasome system (UPS) (Sutovsky *et al.* 2001, Baska *et al.* 2008), which were associated with the extracellular ubiquitination of defective spermatozoa in the luminal fluid (Sutovsky 2003, Eskandari-Shahraki *et al.* 2013). However, UCHL1 deficient mice caput epididymal epithelial cells, which normally undergo apoptosis under androgenic failure induced by efferent duct ligation, were resistant to cell death (Kwon *et al.* 2006). In rodent testes, UCHL1 probably controls the balance between pro-apoptotic and anti-apoptotic factors (Wang *et al.* 2006, Kwon 2007) and high levels of this enzyme have been directly proportional to the incidence of germ cells death (Kwon *et al.* 2004b, 2006, Câmara *et al.* 2019). Ectopically expressed UCHL1 in tumour cells reduces

the levels of anti-apoptotic proteins, such as Bcl-2 (Wang *et al.* 2008), and stabilizes the pro-apoptotic factor p53 due to its deubiquitination (Li *et al.* 2010). Moreover, mice lacking UCHL1 present high levels of the anti-apoptotic proteins Bcl-2 and Bcl-xL in the caput epididymis (Kwon *et al.* 2006). Therefore, the high incidence of apoptosis in the epididymal epithelial cells of cimetidine-treated rats (de Santi *et al.* 2018) may be associated with possible degradation of anti-apoptotic factors induced by increased UCHL1. The low Bcl-2 levels observed here support this idea and these results point to a role of this enzyme in the regulation of cell death in epididymis.

Besides apoptosis, UPS also seems to interact with the autophagic pathway (Ding *et al.* 2007), an essential process for the elimination of protein aggregates and damaged organelles, maintaining cell viability (Doherty & Baehrecke 2018). The autophagosome formation depends on MAP LC3A, and the levels of this protein have been used as autophagy marker (Klionsky *et al.* 2016). However, since autophagosome is a transient structure, LC3 levels alone do not reflect the autophagic flux (Klionsky *et al.* 2016). On the other hand, the autophagic receptor p62/SQSTM1 binds to ubiquitinated cargos, delivering them to the autophagosome (Kaur & Debnath 2015), being degraded during autophagy. Thus, when autophagy is disrupted, high levels of p62/SQSTM1 are found in cells (Klionsky *et al.* 2016). In the present study, the intense MAP LC3A and p62/SQSTM1 immunofluorescence indicates a reduction or blockage of the autophagic flux following cimetidine-induced androgenic failure. Inhibition of autophagy, evidenced by increase in these autophagic markers, has also been observed in Leydig cells of rats (Li *et al.* 2011) and humans (Gao *et al.* 2018) under reduced serum testosterone levels, as well as in osteocytes of ovariectomized rats (Florescio-Silva *et al.* 2018). Otherwise, androgens promote prostate cancer cell growth *in vitro*, at least in part, due to induction of autophagy (Shi *et al.* 2013, Blessing *et al.* 2017). Therefore, our findings reinforce the idea that autophagy is an androgen-dependent process.

When the intensity of a stressor overcomes the cytoprotective function of autophagy, the apoptotic pathway is activated (Mariño *et al.* 2014). During this process, proteins involved in autophagy are degraded, shutting down the autophagic process and amplifying the apoptotic response (Mariño *et al.* 2014, Ouyang *et al.* 2014). Inhibition of autophagy by siRNA, pharmacological inhibitors, or steroid deprivation results in increased apoptosis (He *et al.* 2012, Park *et al.* 2013, Florescio-Silva *et al.* 2018). Here, we demonstrated a correlation between epithelial cells apoptosis and increased UCHL1 in parallel to an interference in autophagy under androgen deprivation. Therefore, UCHL1 and autophagy are probably

involved in the maintenance of cauda epididymis epithelial cells viability. The pharmacological inhibition of UCHL1 enhances protein degradation by autophagic pathway (Cartier *et al.* 2012, Zhang *et al.* 2014, Pukaß & Richter-Landsberg 2015), whereas the overexpression of this enzyme suppresses autophagy (Zhang *et al.* 2014, Yan *et al.* 2018). This finding is confirmed by the fact that UCHL1 interacts with MAP LC3A to block autophagosome formation in HeLa cells (Yan *et al.* 2018). Therefore, the impairment in autophagy observed in the epididymal epithelial cells under androgenic failure may be associated with the increased UCHL1 immunoexpression. According to our knowledge, this is the first study suggesting an interplay among UCHL1, autophagy, and apoptosis in epididymal epithelial cells under androgenic control.

In conclusion, cimetidine-induced androgenic failure impairs the muscular layer of cauda epididymis, reducing Cx43, causing loss of adhesion between smooth muscle cells and inducing apoptosis. These data highlight the importance of stromal androgenic supply for the maintenance of smooth muscle cells viability in cauda epididymis. The reduction of epithelial high induced by androgenic failure may be associated with the reduction in Bcl-2 levels and epithelial cells apoptosis. This finding agrees with the increased levels of UCHL1 in the epithelium and points to a role of this deubiquitinating enzyme in the apoptotic pathway under androgenic control. Cimetidine-induced androgen deprivation also inhibited the autophagy, which in association with the increased UCHL1 levels reinforces a possible role of ubiquitin-proteasome system in the autophagic control. Therefore, our results suggest an interplay between UCHL1 and autophagy in the regulation of apoptosis in epididymal epithelium and that androgens seem to orchestrate these processes.

Declaration of interest

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

Funding

This research was supported by FAPESP (2012/23845-3; 2015/09341-0; 2015/12501-0; 2017/19829-6).

Author contribution statement

F de Santi, F L Beltrame, and E Sasso-Cerri conceptualized the research. F de Santi, P S Cerri, and E Sasso-Cerri performed data curation. P S Cerri performed formal analysis. E Sasso-Cerri acquired funding. F de Santi, I D Lunardi, F L Beltrame, P S Cerri, and E Sasso-Cerri performed laboratory investigations.

F de Santi, I D Lunardi, F L Beltrame, and P S Cerri devised the methodology. E Sasso-Cerri supervised the study. F L Beltrame, P S Cerri, and E Sasso-Cerri performed validity analysis. F de Santi visualized the study. F de Santi, I D Lunardi, F L Beltrame, and E Sasso-Cerri wrote the original draft. F de Santi, P S Cerri, and E Sasso-Cerri reviewed and edited the final version of the manuscript.

Acknowledgements

The authors thank Luis Antônio Potenza and Pedro Sérgio Simões for the histological technical assistance. The authors also thank Dr Flávia L Lopes for the primers design.

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Received 30 September 2019

First decision 29 October 2019

Revised manuscript received 13 March 2020

Accepted 19 March 2020