Title: GnRH immunization alters the expression and distribution of protein disulfide isomerases in the epididymis

Running Title: Expression of PDIA3 in the boar reproductive system

Authors and affiliations

Angela Maria Schorr-Lenz[1]

Jayse Alves[1]

Nicole Andrea Coberllini Henckes[1]

Pâmela Maria Seibel[1]

Adam Maurice Benham[2]

Ivan Cunha Bustamante-Filho[1]*

[1] Centro de Ciências Biológicas e da Saúde, Univates, Lajeado, RS, Brazil.

[2] School of Biological and Biomedical Sciences, Durham University, United Kingdom.

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* To whom correspondence should be addressed: Prof. Ivan Cunha Bustamante-Filho. Centro de Ciências Biológicas e da Saúde, Univates. Rua Avelino Tallini, 171, Lajeado, RS, Brazil, 95900-000. E-mail: ivanbustamante@univates.br

Abstract

Hypogonadism is defined as the inadequate gonadal production of testosterone. Low serum testosterone leads to infertility by impairing spermatogenesis and reducing sperm count, however the impact of hypogonadism in epididymal sperm maturation is poorly understood. From the testis, spermatozoa are transported into the epididymis where they find a specific microenvironment composed of a complex mixture of proteins that facilitate sperm storage and maturation. Inside the epididymal ductule, spermatozoa undergo several changes, resulting in their becoming capable of fertilizing eggs. Protein disulfide isomerases (PDIs) are known to participate in the folding and assembly of secreted proteins in the endoplasmic reticulum. However, little is known about the control and function of PDIs in the testis and epididymis, particularly during male development. The aim of this work was to compare the expression and distribution of PDI and PDIA3 (ERp57) in the testis and epididymis of healthy and GnRH-immunized boars. We detected higher amounts of PDIA3 and PDI in sperm preparations and fluid from the proximal regions of the epididymis of healthy boars. However, we observed an increase in PDIA3 expression in the testis and cauda epididymidis in the immunocastrated group. GnRH-immunized boars showed a marked increase of PDI content in cauda spermatozoa and fluid, indicating a possible endocrine dysregulation of PDI. The results of our study suggest that PDIs are associated with epididymal sperm maturation and may be attractive candidates for monitoring male fertility.

INTRODUCTION

Hypogonadism is defined as the inadequate gonadal production of testosterone, a common condition due to an intrinsic testicular failure (primary hypogonadism) or to a suboptimal stimulation by pituitary gonadotropins (secondary or central hypogonadism) (Corona, et al., 2015). In The USA 500,000 men per year are diagnosed with androgen deficiency, and prevalence is higher among those with comorbidities such as obesity (Chambers Anderson, 2015), diabetes mellitus (Tirabassi, et al., 2016), and HIV (Rochira Guaraldi, 2014). Also, androgen deprivation therapy and radiotherapy used for advanced prostate cancer treatment leads to hypogonadism (Vassilakopoulou, et al., 2016). The incidence and prevalence of male hypogonadism in the general population are unknown (Basaria, 2014), nonetheless it is estimated that 30% to 70% of men with male infertility have some degree of concurrent endocrine dysfunction (Hotaling Patel, 2014). Lower serum testosterone leads to infertility by impairing spermatogenesis and reducing sperm count, and is clinically observed as oligospermia and azoospermia (Mulhall Hsiao, 2014). However, the impact of hypogonadism in epididymal sperm maturation is poorly understood.

Post-testicular sperm maturation occurs as spermatozoa progress through the epididymis, and culminates in their ability of fertilizing eggs successfully (Cooper, 2007). The epididymis is composed of a single, long, convoluted tubule divided into three main anatomical regions (the caput, the corpus and the cauda). Segmented gene expression patterns throughout the tubule result in distinct protein secretion profiles in the intraluminal epididymal fluid (Thimon, et al., 2007). As a result, this tubule provides different microenvironments that are optimal for each step of sperm maturation

(Dacheux, et al., 2012).

In sperm physiology, several molecular chaperones play important roles guaranteeing proper protein folding and secretion, including the Protein Disulfide Isomerase (PDI) family. PDIs are generally involved in cycles of substrate reduction, oxidation, and direct isomerization. They also demonstrate molecular chaperone activity and can prevent the aggregation of proteins that do not contain Cys residues (McLaughlin Bulleid, 1998). There are at least 21 mammalian homologues of PDI, including PDIA3 (ERp57). Although the main function of PDIA3 is to mediate the folding and quality control of newly synthesized glycoproteins in the endoplasmic reticulum (Coe Michalak, 2010, Turano, et al., 2011), it has also been found in other subcellular locations, such as in the nucleus, cytoplasm, and at the cell-surface. Some studies have suggested that PDIA3 participates in transcriptional regulation (Coppari, et al., 2002), signal transduction (Coe, et al., 2010, Ramirez-Rangel, et al., 2011), membrane fusion (Schelhaas et al., 2007), hormone responses (Tunsophon Nemere, 2010) and sperm–egg fusion (Liu, et al., 2014).

PDIA3 was previously described in boar epididymal spermatozoa (Akama, et al., 2010) and is also associated with fertility potential in men. It was demonstrated recently that the amount of PDIA3 is reduced in the spermatozoa of male individuals with obesity-associated asthenozoospermia (Liu, et al., 2015). Interestingly, PDIA3 also binds to the hydroxylated, hormonal form of vitamin D3,1α,25-dihydroxycholecalciferol (1α,25-$(OH)_2$D3, calcitriol), and therefore may be involved in hormonal signalling processes (Nemere, et al., 2004, Turano, et al., 2011). This pathway activates numerous signal transduction cascades, such as protein kinase C (PKC),

phospholipases C (PLC) and A2 (PLA2), and extracellular response activated kinase (ERK), all of which have been shown to respond to the formation of the PDIA3-1α,25-(OH)2D3 complex (Boyan, et al., 2007, Chen, et al., 2010). These new functions suggest a broader role of PDIA3 in cell physiology. Thus, it may be that, in the epididymis, PDIA3 may not only act as a molecular chaperone, but might also modulate steroid biology.

The present work aimed to evaluate the presence of PDIA3 and PDI in the spermatozoa and epididymal fluid of boars, comparing gonadotrophin releasing hormone (GnRH)-immunized males with their healthy counterparts. Immunocastration is a routine procedure to prevent boar taint, an unpleasant odor caused by androstenone and skatole, both testosterone metabolites present in the adipose tissue and which become pronounced upon heating of pork meat (Brunius, et al., 2011). The immunological blocking of GnRH signaling decreases the production of FSH, LH and testicular steroids (including androstenone), resulting in reproductive organ atrophy, low sperm counts, and aggressive behaviour (Zamaratskaia, et al., 2008). Here we show that the depletion of serum testosterone induced by immunocastration alters the distribution of both PDIA3 and PDI in the boar epididymis. These results contribute to understanding the role of protein disulfide isomerases in epididymal physiology and provide a basis for using the GnRH-immunized boar as a model for studying the effects of hypogonadism on the epididymis and in sperm quality.

MATERIAL AND METHODS

Animals

Research procedures were conducted in accordance with the Brazilian federal laws for the use of animals for scientific purposes, and were validated by the Animal Experimentation Ethics Committee of Univates (no. 002/2015).

Large White and Duroc boars between 10 and 18 months old were used. For the control group (n = 9), testicles from healthy boars were generously donated by local producers after routine orchiectomy in the farm. For the GnRH-immunized group (Immunocastrated group, n = 9), testicles were obtained from a local slaughterhouse. Animals in this group were immunized with Vivax® (Pfizer) 2 months prior slaughtering, according to the manufacturer's instructions (two vaccinations within a one-month interval between each), with the slaughter being performed two months after the second dose when plasma testosterone levels were undetectable (Brunius, et al., 2011, Einarsson, et al., 2011, Zamaratskaia, et al., 2008).

RNA extraction and quantitative RT-PCR

Total RNA from testicular and epididymal tissues was extracted with the IllustraRNAspin Kit (GE Healthcare, USA), followed by treatment with DNase I (Thermo Fisher,USA). cDNA was synthetized with the SMART PCR cDNA Synthesis Kit (Clontech®; Mountain View, CA, USA), according to the manufacturer's instructions. First-strand cDNA synthesis was performed with oligodT and reverse transcriptase (M-MLV, Invitrogen®, Carlsbad, CA, USA) using 1.5 µg of RNA.

RT-qPCR was carried out in an Applied Biosystems StepOne Plus real-time cycler using the previously synthesized first strand cDNA samples as templates. PDIA3

sense and antisense primers were 5'-ATCCCAGAGAGCAATGATGG-3' and 5'-TGACCACACCAAGGAGCATA-3', respectively. Reaction settings were: 5 min at 94°C, followed by 40 cycles of 10 s at 94°C, 15 s at 60°C, 15 s at 72°C; samples were held for 2 min at 60°C for annealing of the amplified products and then heated from 60 to 99°C with a ramp of 0.3°C/s to provide the denaturing curve of the amplified products. Quantitative PCRs were performed in a final volume of 20 µL containing 10 µL of each reverse transcription sample diluted 100 times, 2 µL of 10X PCR buffer, 1.2 µL of 50 mM $MgCl_2$, 0.1 µL of 5 mM dNTPs, 0.4 µL of 10 µM primer pairs, 2.9 µL of water, 2.0 µL of SYBR green (1:10,000, Molecular Probe), and 1 µL of Platinum Taq DNA polymerase (5 U/µL, Invitrogen®, Carlsbad, CA, USA). Data were analyzed using the comparative Ct method (Livak Schmittgen, 2001). PCR efficiency from the exponential phase was calculated for each individual amplification plot with the LinRegPCR software (Ramakers, et al., 2003). In each plate, the average of PCR efficiency (Eff) for each amplicon was determined and used in future calculations. Ct values of *Actb* (β-actin) were used to normalize Ct values for *Pdia3* (Vadnais, et al., 2008). The equation Q_0 $_{Pdia3}$/$Q_{0\ Actb}$= [$(Eff_{Actb})^{CtActb}$ / $(Eff_{Pdilt})^{Ct\ Pdia3}$], where Q_0 corresponds to the initial amount of transcripts, was used for normalization (Livak Schmittgen, 2001, Ricachenevsky, et al., 2011). Each data point corresponds to three true biological replicate samples.

Collection of epididymal spermatozoa and fluid and sample preparation

Epididymal sections from regions 1, 5 and 9 (Dacheux, et al., 2005), corresponding to boar caput, corpus, and cauda, respectively, were dissected and the epididymal fluid (with spermatozoa) was flushed out, collected in tubes, and then

suspended in 250 µL of ice-cold phosphate-buffered saline (PBS). The suspensions were stirred and centrifuged (800 × g for 10 min), and the supernatant (epididymal fluid) was stored at -80°C after the addition of protease inhibitors. The sperm pellet was washed twice in 300 µL of PBS, gently stirred and centrifuged at 3,000 × g for 10 min. Finally, the sperm pellet was then lysed in RIPA buffer (25mM Tris-HCl (pH 7.6), 150mM NaCl, 1% NP-40, 1% (w/v) sodium deoxycholate, 0.1% (w/v) SDS) and centrifuged (12,000 × g for 1 h at 4°C). A protease inhibitor cocktail (Pierce) was added and the supernatant (sperm protein extract) was stored at -80°C until analysis. Protein extracts from the testis were obtained after centrifugation (12,000 ×g for 1 h at 4°C) of tissue homogenates in RIPA buffer. The supernatant was stored at -80°C until analysis.

SDS-PAGE and Western blotting

After protein determination (Lowry, et al., 1951), samples of epididymal fluid and sperm protein extract were resolved by 10% (w/v) SDS-PAGE (Sigma). After electrophoresis, proteins were transferred to nitrocellulose membranes (Hybond™ ECL™ nitrocellulose membrane, GE Healthcare) in a semi-dry transfer apparatus (Bio-Rad, Trans-Blot Turbo, Hercules, CA, USA). Membranes were incubated for 60 min at 4°C in blocking solution (Tris-buffered saline containing 5% (w/v) powdered milk and 0.1% (v/v) Tween-20, pH 7.4) and then incubated overnight at 4°C with antibodies for PDIA3 (rabbit) or PDI antibodies (rabbit) at a 1:2,000 dilution, or with monoclonal anti-actin (1:5,000). Membranes were then incubated with horseradish peroxidase conjugated anti-rabbit antibody (1:3,000; Invitrogen, Amersham Pharmacia Biotech).

Chemiluminescence (ECL, GE Healthcare) was detected on X-ray films (Kodak, Japan) and results were quantified by densitometry.

Statistical analysis

PDIA3 and PDI relative absorbance and PDIA3 mRNA relative expression were analyzed by means of two-way ANOVA followed by Tukey HSD or Student's *t* test ($P \leq$ 0.05) using the GraphPad Prism 6 Software (San Diego, USA).

RESULTS

PDIA3 mRNA expression in the boar testis and epididymis

The relative PDIA3 mRNA levels from the testis and epididymis of both experimental groups were compared (Figure 1). PDIA3 mRNA levels were increased in the testis of immunocastrated animals when compared to the control group ($P < 0.05$). Western blotting analysis revealed an increase ($P < 0.05$) of both PDIA3 and PDI protein in testicular homogenates obtained from the GnRH-immunized boars when compared to the control animals (Figure 2). Immunization also promoted an increase in mRNA levels in the caput and corpus of epididymidal homogenates ($P < 0.01$), but not in the cauda samples. The corpus epididymidis had a higher relative expression of PDIA3 mRNA than in the other epididymal regions of the GnRH-immunized group ($P < 0.05$).

PDIA3 and PDI protein distribution in epididymal sperm and fluid

In healthy boars, western blotting analyses showed higher protein expression of PDIA3 in cell extracts from the caput and corpus epididymidis, when compared to the cauda ($P < 0.05$). In contrast, spermatozoa from the cauda epididymidis of the GnRH-immunized animals demonstrated a higher expression of this chaperone (Figure 3A and 3C).

For a better understanding of PDIA3 distribution in the swine epididymis, we performed a relative quantification of this protein in epididymal fluid. PDIA3 immunocontent was higher in fluid obtained from the proximal epididymis in both healthy and immunocastrated animals (Figure 3B and 3D). Moreover, immunocastration reduced PDIA3 concentration in the fluid from the caput and corpus epididymidis ($P < 0.01$). On the other hand, treated boars showed an increase of this chaperone in cauda samples when compared to controls ($P < 0.0001$).

The same distribution pattern, as well as the GnRH-immunization effect, was also observed for PDI (Figure 4). The PDI chaperone was increased in both epididymal sperm samples (Figure 4A and C) ($P < 0.05$) and fluid (Figure 4B and D) ($P < 0.001$) from cauda samples of immunized boars.

DISCUSSION

Hypogonadism is a common condition during aging, but also occurs in young men, albeit less frequently. The significance of this disease relies on evidences that strongly support the premise that low testosterone is an important biomarker not only for fertility, but also for morbidity and mortality in men because of its association with other

comorbidities such as obesity, metabolic syndrome and diabetes mellitus (Zarotsky, et al., 2014). Other factors that contribute to alterations in testosterone levels may also be considered, such as the exposure to endocrine disruptors (EDs), such as bisphenol A (BPA) and polychlorinated biphenyls (PCBs). In most studies regarding the effects of endocrine disruptors on steroids, only urinary EDs were correlated with steroid plasma levels. However, recently (Vitku, et al., 2016) and colleagues described that elevated seminal levels of BPA were associated with a significant decrease in sperm count, sperm concentration and morphology.

Androgens are responsible for maintaining epididymal structure and function, and therefore fluctuations on testosterone levels may impair epididymal maturation of spermatozoa. Administration of antiandrogens, such as flutamide, results in an accelerated sperm transit time through the epididymis, loss of sperm motility, and decreased ability of the cauda epididymis to store sperm (Robaire Hinton, 2015). Therefore, understanding how androgen deprivation alters the epididymal expression of proteins associated to sperm quality is relevant.

In translational research, the swine a widely accepted animal model, presenting unique advantages because of the similar anatomic and physiological characteristics it shares with humans (Swindle, et al., 2012). The GnRH-immunized (immunocastrated) model for the study of androgen deprivation in swine reproductive tissues could contribute to a broader understanding of the role androgens may have in epididymal physiology

The goal of this current work was to evaluate the influence of GnRH immunization on PDIA3 and PDI expression in the swine epididymis. Immunized boars presented higher amounts of PDIA3 in epididymal fluid and in spermatozoa collected from the caput and corpus epididymidis when compared to control animals, suggesting a possible role for this chaperone in sperm maturation. This is consistent with the work of Ellerman and colleagues, who suggested that PDIA3 at the sperm membrane may facilitate sperm-egg fusion (Ellerman et al., 2006). Proteins in the epididymal milieu are key players in post-testicular sperm maturation. Several proteins present in the epididymal fluid have been characterized and most of these, such as hexoaminidase, gluthathione peroxidase, and RNAse A, are secreted in the anterior part of the organ (Dacheux, et al., 2005). In fact, it is estimated that the secretory activity of the anterior region is 6 to 8 times greater than in the posterior region. Therefore, the higher concentrations of molecular chaperones, such as PDIA3 and PDI, in the proximal epididymal tubule of control samples may reflect a need for post-translational disulfide bond rearrangements, or a requirement to prevent aggregation of epididymal or sperm surface proteins.

The decline in PDIA3 expression during epididymal progression in healthy boars contrasts with the findings of (Akama, et al., 2010), who found that, in boars, PDIA3 remained unchanged during epididymal maturation. This may reflect a level of species/strain or age-related diversity in chaperone expression control in epididymal tissues. The mechanism by which PDI family proteins bind to sperm cells is unknown, but it is possible that they are secreted by the epididymal epithelium and transferred to sperm cells as they transit through the epididymis. In human spermatozoa, PDIA3 is

localized in the acrosome, the equatorial segment and the flagellum (Zhang, et al., 2007). Interestingly, their sperm surface distribution changes after acrosome reaction, becoming mostly expressed on the equatorial segment and the flagellum. This PDIA3 distribution in human spermatozoa suggests an important role not only for spermatogenesis, but also for mature sperm function, especially during processes that are crucial for human fertilization, such as sperm capacitation and the acrosome reaction. In addition, fusion with oocytes is dependent on PDIA3, since incubation with anti-PDIA3 antibodies inhibits human sperm binding to hamster oocytes *in vitro* in a dose-dependent manner (Zhang, et al., 2007).

The physiological modifications of the epididymal milieu, such as protein secretion and hormone flux, could explain the differences in PDI distribution observed in the experiments presented herein. Changes in epididymal fluid composition may result in regionalized secretion associated with specific protein resorption throughout the epididymal tubule (Castella, et al., 2004). PDIA3 might be involved in one or more of the pathways activated during epididymal sperm maturation, such as morphological maturational changes in head orientation and development of progressive motility. These maturational changes are promoted by androgen-dependent factors from epididymal principal cells, the only cell type with striking cytological differences between regions (Moore, 1998, Moore, et al., 1998).

Based on the finding that PDIA3 expression and localization is altered in immunocastrated boars, we propose that PDIA3 behaves as an androgen-responsive factor. The increase in its immunocontent in cauda sperm and epididymal fluid of

GnRH-immunized boars suggests this chaperone may have a role in endocrine regulation. Since PDIA3 is associated with protein quality control, its increased amount in cauda sperm and fluid of immunocastrated boars might be a response to impaired spermatogenesis, triggered by defective sperm cells in this epididymal region.

A similar distribution pattern along the epididymis was observed for PDI. This chaperone can bind with low affinity to hormones, including thyroid hormone (T3) and estrogen. The binding capability might be associated with the significant similarity between the PDI segments 120-163 and 182-230 to the estrogen receptor segments 350-392 and 304-349, respectively (Tsibris, et al., 1989). PDI can function as a high-capacity intracellular 17β-estradiol (E_2) binding protein, increasing the concentration and accumulation of E_2 in live cells. Intracellular PDI-bound E_2 can be released from PDI upon a reduction in E_2 levels, and the released E_2 can increase estrogen receptor-mediated transcriptional activity and mitogenic activity in cultured cells (Fu, et al., 2008). Moreover, the binding of E_2 by PDI also reduces the availability of this hormone. PDI also modulates the estrogen receptor (ER), being an important regulator of the ERα/ERβ ratio by altering ERα and ERβ levels in opposite directions, a change which is expected to modify cellular responses to estrogens in different target tissues (Fu, et al., 2008). For instance, while ERα mediates the proliferative effect of estrogens in breast cancer cells, ERβ seems to be anti-proliferative, negatively regulating ERα transactivation (Zhao, et al., 2007). Several published data demonstrate the different ways that protein disulfide isomerases may influence steroid hormone regulation and responsiveness (Fu, et al., 2008, Schultz-Norton, et al., 2006), and further work is required to understand how hormones interact with PDIs in the epididymis.

In summary, our results show that PDI and PDIA3 expression and distribution in the boar testis and epididymis is altered in GnRH-immunized boars, suggesting that protein disulfide isomerases are influenced by endocrine factors (Figure 5). These insights point to potential physiological roles for PDIA3 and PDI in sperm maturation and fertility, and suggest that PDIs may be sentinels of hypogonadism or other androgenic disruptions.

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AUTHOR CONTRIBUTIONS

Bustamante-Filho designed the experiments, conducted part of the experiments and wrote the manuscript. Schorr-Lenz, Alves, Henckes and Seibel conducted some of the sample collection, qPCR and western blots. Benham was a scientific advisor and revised the manuscript.

DISCLOSURES

The authors have nothing to disclose.

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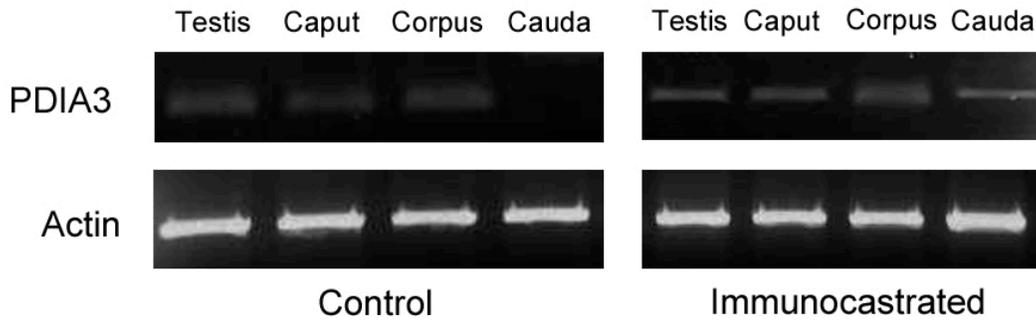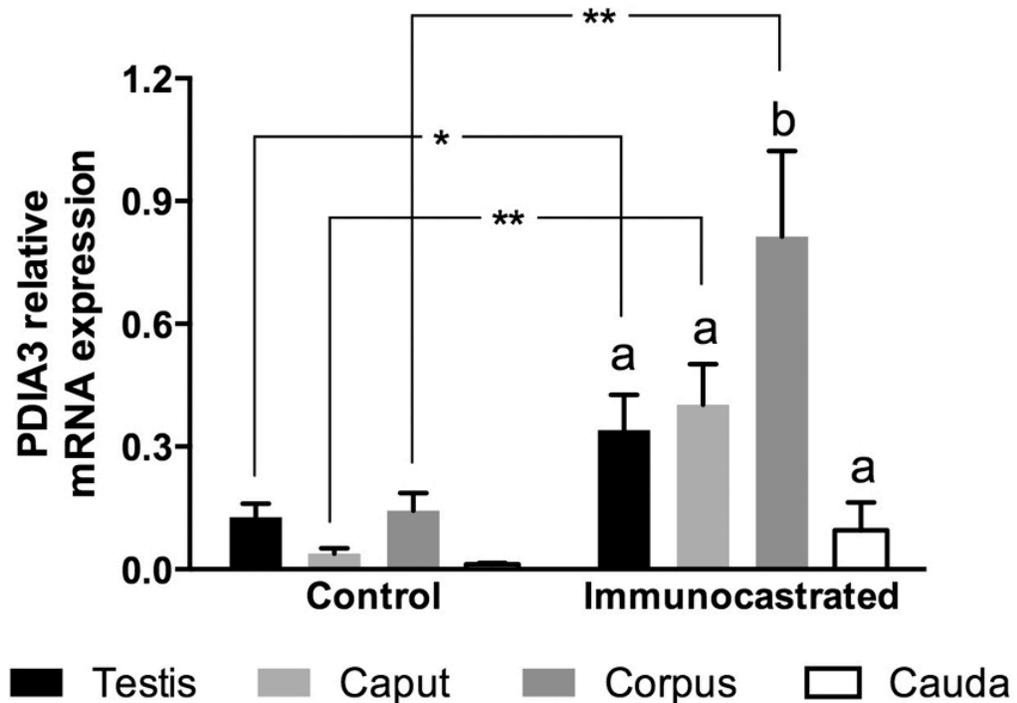
Figure 1 - PDIA3 mRNA levels in swine testis and epididymis (caput, corpus and cauda). (A) RT-PCR using specific primers for PDIA3 showing the specific 116 base pair amplicon. (B) qPCR showing an increase in PDIA3 mRNA expression in the GnRH-immunized group. Values are the averages of 9 samples for each group ± SD. Letters above bars denote differences between epididymal regions (P< 0.05). Asterisks denote statistically significant differences between groups (* P< 0.05; ** P< 0.01). 367x376mm (72 x 72 DPI)
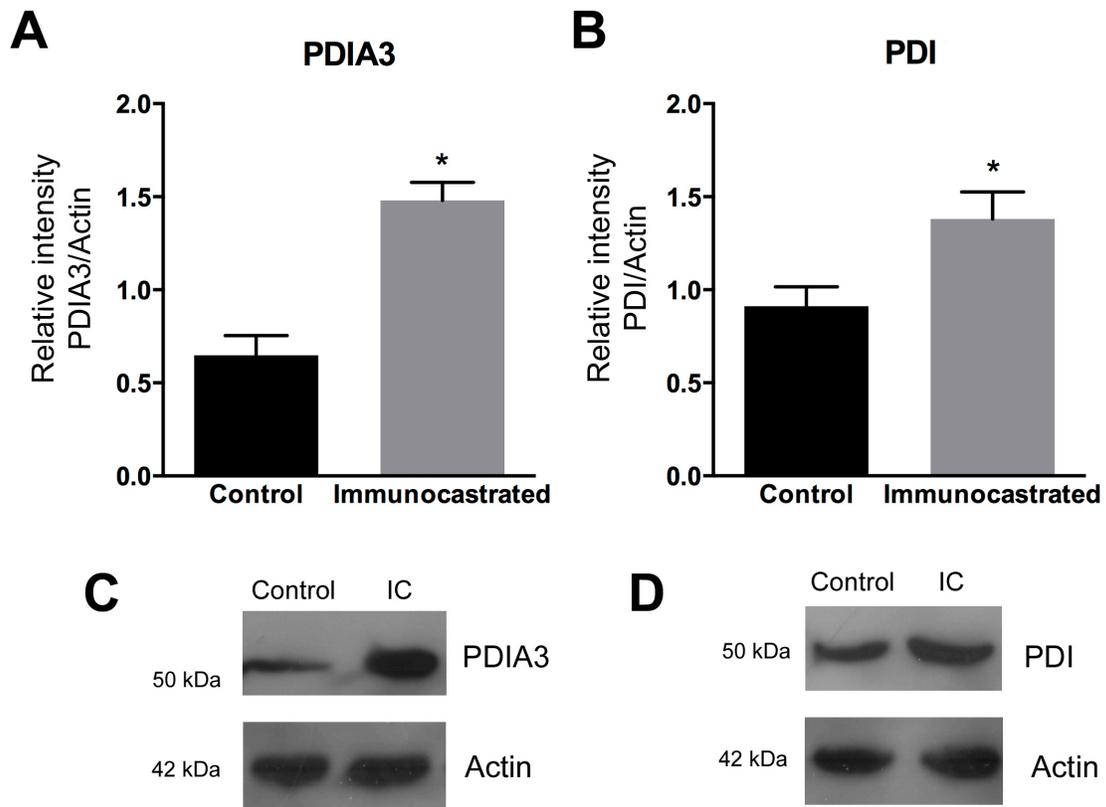
Figure 2 - PDIA3 (A) and PDI (B) immunocontent in the testis of healthy and immunocastrated (IC) boars. C and D show representative bands obtained by western blot. Asterisks indicates a statistically significant difference (* P< 0.05). 201x152mm (300 x 300 DPI)
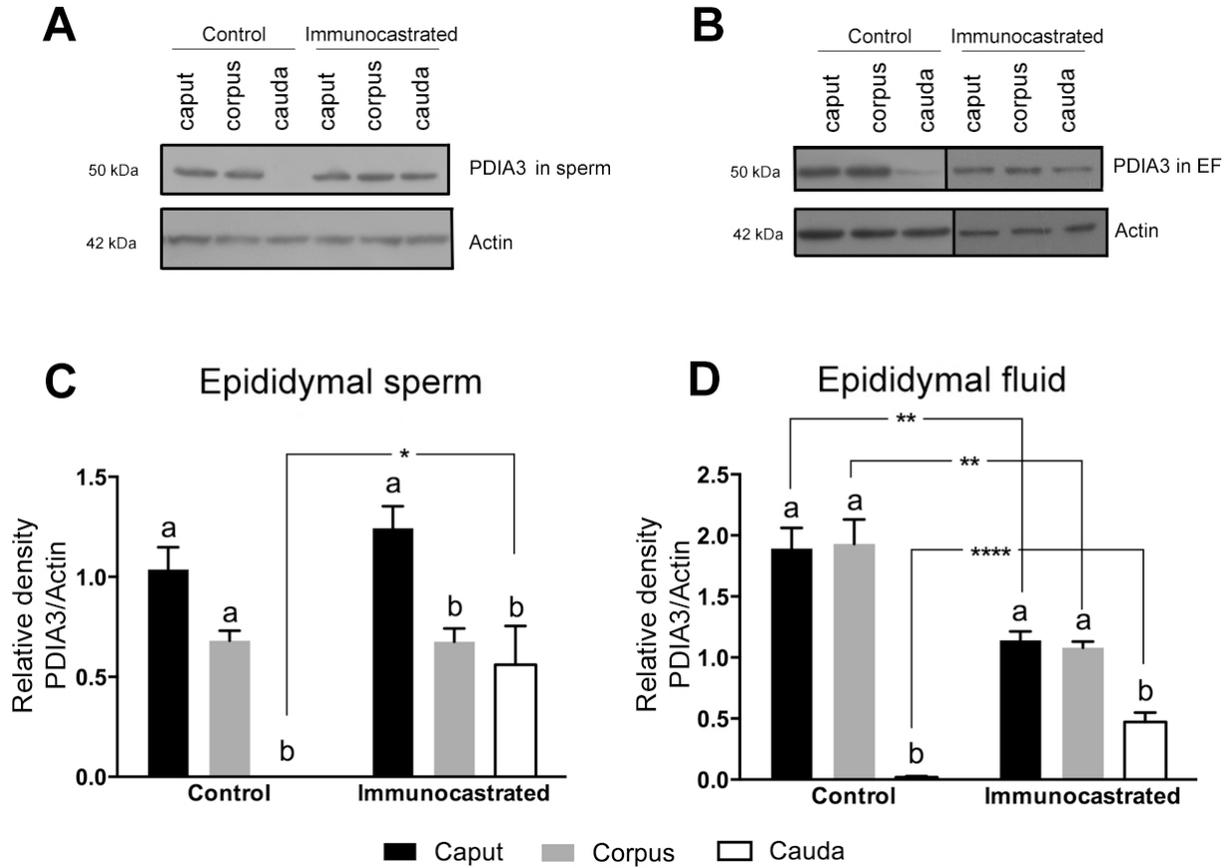
Figure 3 – PDIA3 expression in epididymal sperm (A and C) and epididymal fluid (EF) (B and D) collected from healthy and immunocastrated boars. (A) and (B) show the immunoblot of PDIA3 and actin (top panel). (C) and (D) show the quantification of PDIA3 in epididymal sperm and epididymal fluid, respectively. Letters above bars denote statistical differences between regions (P< 0.05). Asterisks indicate statistical differences between groups (* P< 0.05; ** P< 0.01; **** P< 0.0001). 220x159mm (144 x 144 DPI)
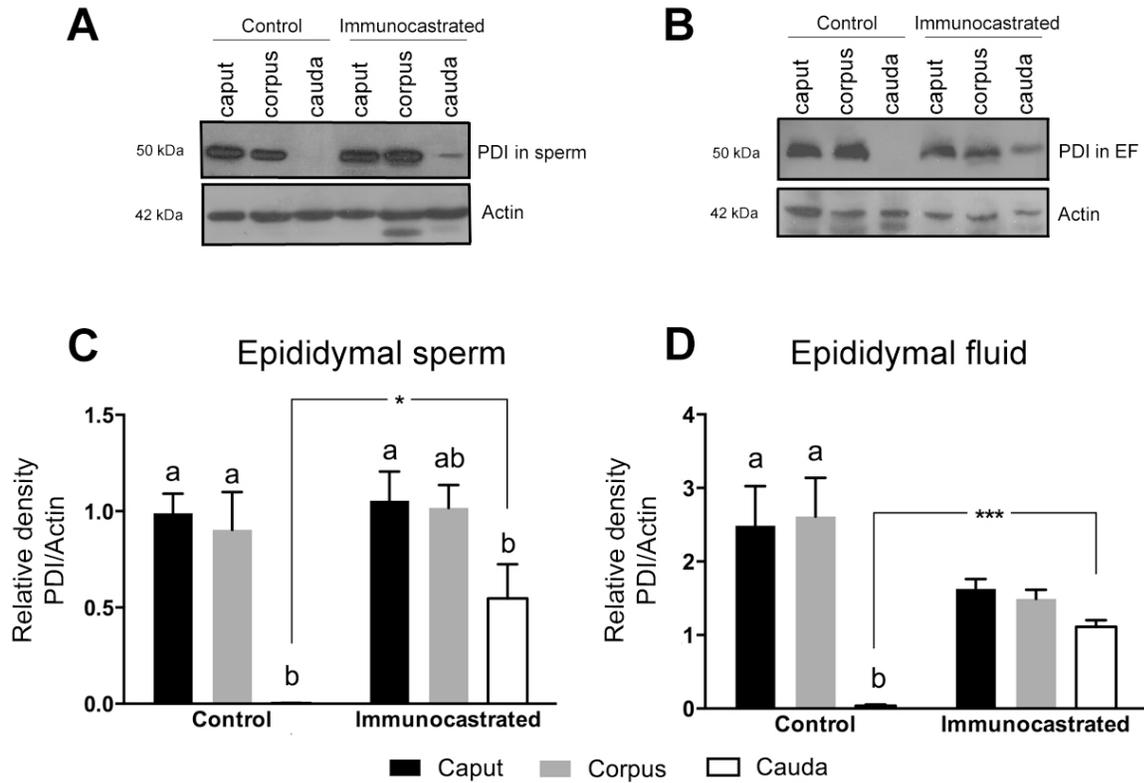
Figure 4 – PDI content in epididymal sperm (A and C) and epididymal fluid (EF) (B and D) collected from healthy and immunocastrated boars. (A) and (B) show the immunoblotting of PDI and actin (top panel). (C) and (D) show the quantification of PDI in epididymal sperm and epididymal fluid, respectively. Letters above bars denote statistical differences between regions (P< 0.05). Asterisks indicate statistically significant differences between groups (* P< 0.05; *** P< 0.001).
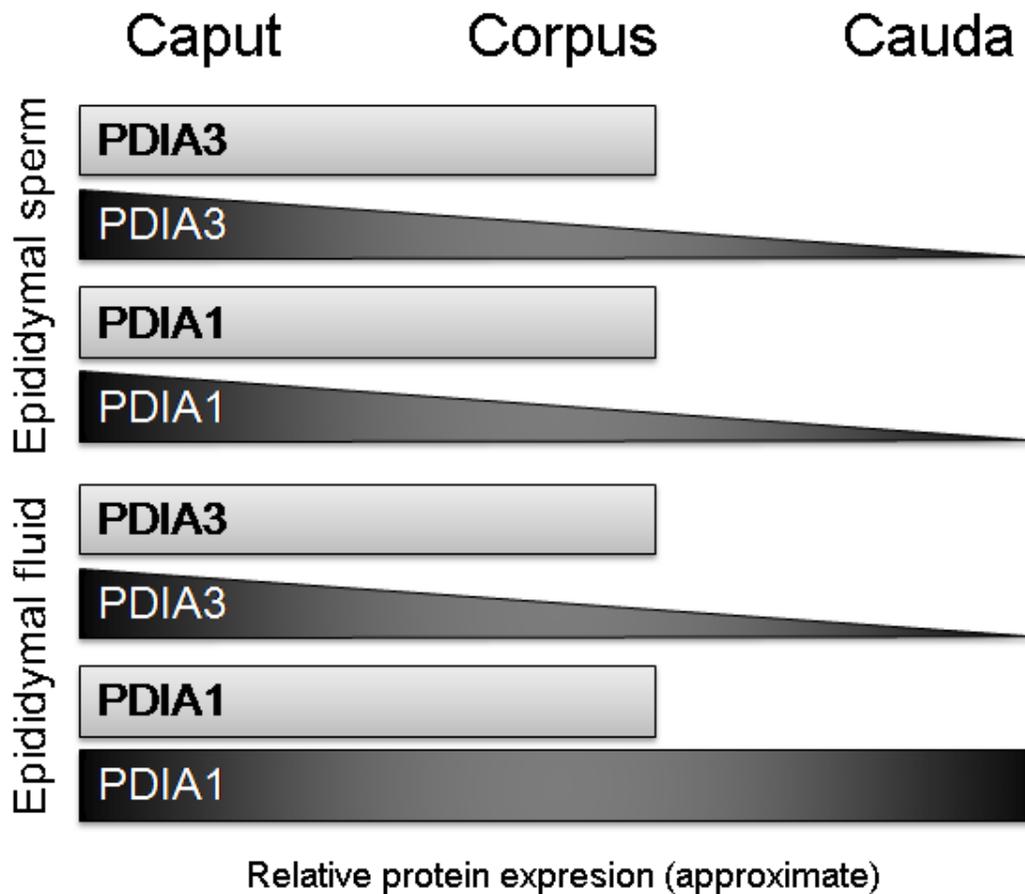
232x149mm (144 x 144 DPI)

Figure 5 – Schematic representation of the location (epididymal regions are approximate) of PDIA3 and PDI in epididymal fluid and sperm in boars. Light gray boxes indicate healthy animals and dark gray boxes indicate GnRH-immunized boars. 154x130mm (96 x 96 DPI)