Universidade de Aveiro Departamento de Química
Ano 2018

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica com especialização em Bioquímica Clinica, realizada sob a orientação científica da Professora Doutora Margarida Sâncio da Cruz Fardilha, Professora Auxiliar com Agregação do Departamento de Ciências Médicas, Universidade de Aveiro e da Professora Doutora Rita Maria Pinho Ferreira, Professora Auxiliar do Departamento de Química, Universidade de Aveiro

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Dedico este trabalho à minha mãe.

o júri

presidente

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palavras-chave

Cancro da próstata, exercício físico, função testicular, testosterona, espermatogénese, morfologia testicular, saúde vascular

resumo

O cancro da próstata (CaP) é um dos tipos de cancro mais comuns em homens e, para além de afetar a função da próstata, também afeta outros órgãos, como os testículos, causando disfunção testicular. O exercício físico tem sido associado a efeitos benéficos em pacientes com CaP, quer na prevenção, quer no resultado da doença. Em indivíduos saudáveis, o exercício físico pode ter um efeito benéfico ou prejudicial na função testicular, dependendo do tipo e intensidade. O exercício físico parece ainda ter um efeito benéfico em prevenir ou contrariar o efeito negativo na função testicular causado por várias condições, como o envelhecimento e a obesidade. Assim, o objetivo deste trabalho foi avaliar o efeito preventivo do exercício físico na disfunção testicular associada ao CaP.

Cinquenta ratos Wistar Unilever foram aleatoriamente divididos em quatro grupos: Sedentários, Sedentários+CaP, Exercitados e Exercitados+CaP. Os animais foram sacrificados cinquenta semanas após o primeiro treino e uma amostra de espermatozoides e de soro, e os testículos foram recolhidos. O peso dos animais e dos testículos, a concentração e morfologia dos espermatozoides, os níveis séricos de testosterona e a histologia do testículo foram analisados e comparados entre grupos. O CaP afetou negativamente o testículo, levando a uma redução do seu peso, acompanhada por uma diminuição da concentração e morfologia dos espermatozoides. Os animais com CaP exibiram ainda uma maior espessura arterial no testículo que, juntamente com o efeito de *feedback* negativo causado pelo aumento dos níveis de testosterona, pode ter contribuído para o efeito prejudicial na função testicular. O exercício físico, apesar de ter exacerbado o aumento dos níveis de testosterona causado pelo CaP, melhorou ligeiramente a concentração e morfologia dos espermatozoides dos ratos CaP. Nesses animais, o exercício físico pareceu ainda contrariar o aumento da espessura arterial do testículo e consequente diminuição do lúmen arterial. Pelo contrário, nos ratos controlo, o exercício físico pareceu diminuir a concentração e morfologia dos espermatozoides, demonstrando um efeito negativo no testículo. Nem o CaP nem o exercício físico foram associados a fibrose testicular.

Concluindo, o CaP afetou negativamente a saúde vascular do testículo e, consequentemente, a função testicular. O exercício físico pareceu melhorar a saúde vascular do testículo dos animais com CaP, o que se refletiu numa ligeira melhoria dos parâmetros de função testicular. Por outro lado, nos animais controlo, o exercício físico pareceu ter um efeito prejudicial no testículo.

Keywords

Prostate cancer, physical activity, testicular function, testosterone, spermatogenesis, testis morphology, vascular health

abstract

Prostate cancer (PCa) is one of the most common types of cancer in men and, in addition to impair prostate function, also affects other organs, like testis, causing testicular dysfunction. Exercise training has been associated with a beneficial effect for PCa patients, both in prevention and outcomes of the disease. In healthy subjects, exercise training can have either a beneficial or deleterious effect on testicular function, depending on the type and intensity. In addition, exercise training seems to have a benefic effect in preventing or counteracting the impairment of testis function caused by several conditions, like aging or obesity. Thus, the aim of this work was to explore the preventive effect of exercise training in PCa-induced testicular dysfunction.

Fifty Wistar Unilever male rats were randomly divided into four groups: Sedentary, Sedentary+PCa, Exercised and Exercised+PCa. Fifty weeks after the first training, the animals were sacrificed and sperm, testes and serum were collected. Animals and testes weight, sperm concentration and morphology, testosterone serum levels and testis histology were analyzed and compared between groups. PCa negatively affected testis tissue causing a reduction of testis weight accompanied by decreased sperm count and morphology. PCa-induced animals also exhibited higher testis arterial thickness which, along with a negative feedback effect caused by increased serum testosterone levels may contributed to the PCa-induced impairment of testis function. Despite exercise training seemed to exacerbate PCa-induced increase testosterone serum levels, it slightly improved sperm concentration and morphology of PCa rats. Moreover, in these animals, exercise training seemed to counteract the increased in arterial thickness and consequent decreased arterial lumen Contrarily, in control rats, exercise training seemed to decrease sperm count and morphology, demonstrating a negative impact in testis. Both PCa and exercise training were not associated to testis fibrosis.

In summary, PCa negatively affected testis vascular health and consequently testis function. Exercise training seemed to improve vascular health of testis tissue of tumor-bearing rats, which was reflected in a slightly improvement of testis function parameters. On the other hand, in control animals, exercise training seems to have a deleterious effect in testis tissue.

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Abbreviations

3β-HSD	3β hydroxysteroid dehydrogenase
17β-HSD	17β hydroxysteroid dehydrogenase
ADAM9	disintegrin and metalloproteinase domain-containing protein 9
APS	ammonium persulphate solution
ASC	caspase recruitment domain
BCA	bicinchoninic acid assay
Bcl-2	B-cell lymphoma 2
BN	bent-neck
BPH	benign prostatic hyperplasia
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CAT	catalase
COX	cyclooxygenase
CREB	cAMP response element
CRP	C-reactive protein
CRPCa	castration-resistant prostate cancer
DH	decapitated head
DHT	dihydrotestosterone
FH	flattened-head
FSH	follicle-stimulating hormone
FSHR	follicle-stimulating hormone receptor
G6PDH	glucose-6 phosphate dehydrogenase
GnRH	gonadotropin-releasing hormone
GPCR	G-protein coupled receptor
GPx	glutathione peroxidase
GSH	reduced glutathione
GST	glutathione S-transferase
HCR	high intrinsic capacity runners
HPG axis	hypothalamic-pituitary-gonadal axis
HSP70	heat shock protein 70
IGF-1	insulin growth factor 1
IGFBP-1	insulin growth factor binding protein 1
IL-1β	interleukin 1β
IL-6	interleukin 6

JAK2	Janus kinase 2
LCR	low intrinsic capacity runners
LEP	leptin
LH	luteinizing hormone
LHR	luteinizing hormone receptor
MDA	malondialdehyde
MDM-2	mouse double minute 2 homolog
MET	metabolic equivalent value
MNU	N-methyl-nitrosourea
NF-kB	nuclear factor kappa B
Nrf-2	nuclear factor erythroid 2p45-related factor 2
Ocn	osteocalcin
ODF-1	outer dense fiber protein 1
PCa	prostate cancer
PH	pin-head
PIN	prostatic intraepithelial neoplasia
PO_{2m}	microvascular oxygen partial pressure
RIPA	radioimmunoprecipitation assay buffer
RT	room temperature
SAMP8	senescence-accelerated prone mouse model
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
SHBG	sex hormone-binding protein
SOD	superoxide dismutase
StAR	steroidogenic acute regulatory protein
STAT3	signal transducer and activator of transcription 3
TBARS	thiobarbituric acid reactive substance
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline with 0.1% of Tween 20
TD	tail defect
TEMED	tetramethylethylenediamine
TGF-α	transforming growth factor α
TNF-α	tumor necrosis factor α
TURP	transurethral resection of the prostate
VEGF	vascular endothelial growth factor
VIP	vasoactive intestinal peptide

1. Introduction

1.1. Male reproductive system

The male reproductive system consists in internal (genital ducts and accessory glands – prostate, seminal vesicle and bulbourethral glands) and external (penis, testes and scrotum) structures, that function is to produce, support, transport and deliver male gametes (spermatozoa) into the female reproductive tract[1].

1.1.1. Testis

The testes are the most important organs of the male reproductive system, where the sperm cells are produced[2]. They are oval structures covered by a muscular structure called scrotum and are constituted by a highly convoluted and packed system of seminiferous tubules (Figure 1)[2]. Each testis contains approximately 370 seminiferous lobules with about 180 µm of diameter each. Seminiferous tubules are essentially constituted by Sertoli and germinative cells at different developmental stages, and enclosed by connective tissue, which include blood vessels, lymphatics, nerves and Leydig cells. The Sertoli and Leydig cells are involved in two main functions of testis: spermatogenesis and steroidogenesis[3].

Spermatogenesis is a tightly regulated process that starts at puberty and during which a diploid precursor spermatogonial stem cell undergo strictly orchestrated cellular processes including mitotic and meiotic divisions and spermiogenesis[4]. Spermatogonial stem cell undergo mitotic divisions and some of the daughter cells remain as spermatogonial stem cells, to maintain the lineage throughout the male adult life (undifferentiated type A spermatogonia), while others suffer a differentiation process, forming type B spermatogonia, that further divides into primary spermatocytes[5]. In meiotic divisions, four haploid cells (spermatids) arise from a diploid primary spermatocyte[4]. Finally, in spermiogenesis, extensive morphological transformations and remodeling of cellular components occur, which include nucleus elongation; chromatin condensation; formation of the acrosome, which contains the hydrolytic enzymes required for sperm-egg interaction and fertilization; and formation of a long tail lined with mitochondria. Along spermatogenic process, several developmental germ cell subtypes can be identified based on microscopic appearance

(Figure 1): spermatogonia, primary spermatocyte, secondary spermatocyte, round spermatid, elongating spermatid and mature spermatozoon, which develop from the basal lamina to the lumen of the seminiferous tubule[6]. Sertoli cells are considered the supporting cells and, besides its role in testis development, they are in tight communication with germ cells through multiple sites, playing a vital role in maintenance of spermatogenesis. In addition to provide physical support to germ cells, Sertoli cells also provide critical factors necessary for the successful of this process, mainly biochemical stimulation factors in the form of growth factors and nutrients[3,7]. In rat, spermatogenesis is very similar to the human's. The main difference is in the organization of different cell types within the seminiferous tubules, which is associated to differences in spermatogenic cycle stages[8]. In human, spermatogenic cycle is divided in six stages and each stage is present in one quadrant of the tubule, which leads to a disorganized appearance. On the other hand, rat spermatogenic cycle is constituted by 14 stages and each stage occupy a different longitudinal space[2,8].

Steroidogenesis is the process of biosynthesis of testosterone from cholesterol, which occur since embryonic phase, in Leydig cells. The Leydig cells are located near the blood vessels, where they secrete the synthesized testosterone. The testosterone biosynthesis involves the orchestrated action of several proteins: steroidogenic acute regulatory protein (StAR), cytochrome P450 heme-containing enzymes (Cyp11a and Cyp17) and hydroxysteroid dehydrogenases (3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β-hydroxysteroid dehydrogenase (17β-HSD))[9]. Testosterone plays a vital role in stimulate spermatogenesis by binding to androgen receptor in Sertoli cells; in the differentiation, development and maturation of men reproductive organs; and in regulation of accessory glands functions[3]. Besides these reproductive functions, testosterone also exerts functions in other parts of the body. In bones, this hormone is essential in maintain bone mineral density and architecture by increasing the bone formation and decreasing bone resorption. In addition, testosterone improves body composition, by increasing lean body mass and decreasing body fat mass. The wide-ranging benefits of testosterone also includes the improvement of cardiovascular health with reduction of cardiovascular diseases risk and reduction of blood glucose[10].

The testis functions are controlled by the hypothalamic-pituitary axis, through the secretion of several hormones. Therefore, a pulsatile secretion of gonadotropin releasing hormone (GnRH) by hypothalamus stimulate the anterior pituitary to synthesize and secrete two types

of gonadotropins: luteinizing hormone (LH) and follicle-stimulating hormone (FSH). LH binds to LH receptor (LHR) in Leydig cells stimulating the testosterone biosynthesis. In turn, FSH acts in FSH receptor (FSHR) located in Sertoli cells, playing an important role in the determination of testicular Sertoli cells number and in induction and maintenance of spermatogenesis[11]. The testosterone, produced in Leydig cells exerts a negative feedback effect on hypothalamic-pituitary axis, decreasing the secretion of both GnRH by hypothalamus and LH and FSH by pituitary. This negative feedback effect is essential for maintaining the testosterone serum levels in a controlled range[11,12].

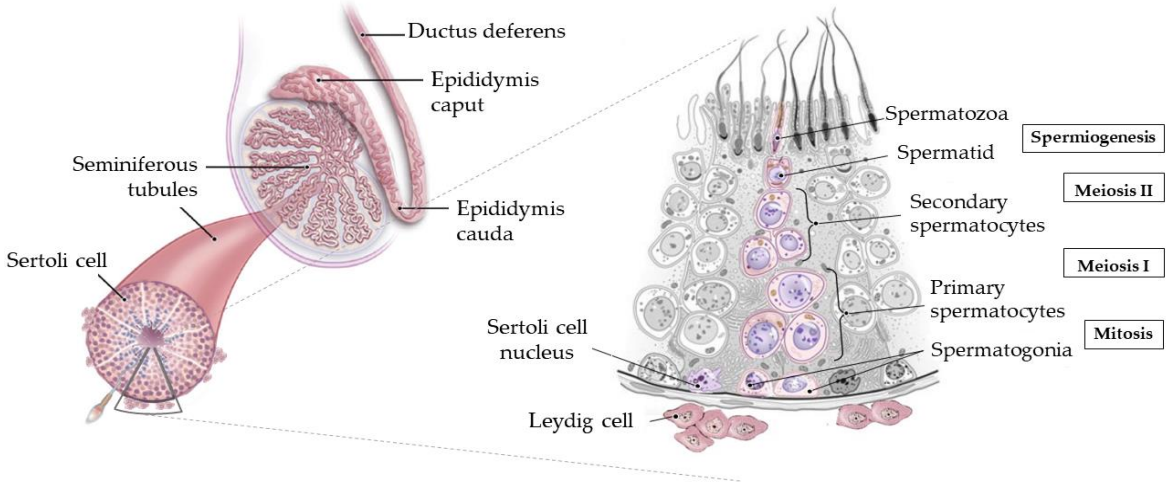


Figure 1: Anatomy and cellular organization of human testis. Testis is composed by seminiferous tubules and each seminiferous tubule is constituted by Sertoli cells and several types of germinative cells corresponding to each step of spermatogenesis; and surrounded by Leydig cells.
Figure was adapted from Samplaski *et al*. 2010[13].

1.1.2. Prostate gland

The prostate is a fibromuscular and glandular organ situated at the base of the bladder, which forms multiple lobes around the urethra. The prostate gland is divided in three histologically distinct glandular zones: the central zone, the peripheral zone and the transition zone; and a nonglandular region: the anterior fibromuscular stroma[14].

This accessory gland is extremely important for the male reproductive system, since it stores and secretes an alkaline fluid essentially composed by citrate, cholesterol and prostaglandin. The fluid constitutes approximately one-third of the volume of semen, and it has a key role in both viability and motility of the sperm cells, especially in the acidic environment of female reproductive tract[3,15].

1.2. Prostate cancer

Prostate cancer (PCa) is a common cancer in men, that often originates in prostatic epithelial cells (adenocarcinoma) and which results in a malignant enlargement of the prostate gland, frequently of the peripheral glandular zone[16].

1.2.1. Epidemiology

Nowadays, cancer is a major public health problem and, in men, PCa is one of the most worrying types of cancer, being considered the 2nd most frequent type of cancer and the 5th leading cause of death by cancer worldwide. A total of 1.10 million new cases of PCa were reported in 2012, 307 000 of whom died from this disease[17,18]. Although PCa incidence has increased in previous years in most countries, the mortality rates have been decreasing over the years, which indicates a good prognosis for many of the PCa patients[19].

One of the factors that contributes to the increased incidence and decreased mortality of PCa is the improvement of diagnosis, with the emergence of new biomarkers that allow earlier detection. The discovery of new biomarkers, along with patients' follow-up and multiple treatments available, causes PCa to be associated with significant healthcare costs[20,21]. Thus, this high economic burden of PCa associated with its high incidence reinforces the need of develop preventive measures for this disease.

1.2.2. Risk Factors

Given PCa high and increasing incidence, it has become important to understand what causes it. Although the exact etiology is difficult to know, there are some described risk factors. The major risk factor recognized for PCa is the patients' age, and it is known that older men are at higher risk to develop PCa, especially over the age of 50[22]. Family history[22], genetic alterations[23] and high serum levels of insulin-like growth factor 1 (IGF-1) also increase PCa risk[24]. Environmental factors were also suggested as strong determinants of PCa risk[25]. Indeed, high consumption of legumes, nuts and fish, and an active lifestyle were associated to decrease incidence of PCa[26,27]. Regarding physical activity, despite the apparent benefits, this is a subject that actually generates some controversy, which will be discussed later.

1.2.3. Pathology

The biological behavior of PCa exhibit high variability[28]. Nevertheless, tumors scored in the same stage, frequently have a similar outlook and are treated in the same way. Hence, staging PCa is important and the AJCC (American Joint Committee on Cancer) TNM (tumor-node-metastasis) system is widely accepted to stage this disease. This system based on the evaluation of primary tumor extent and spread to other parts of the body; prostate-specific antigen (PSA) levels; and Gleason score, to assign a stage to PCa. The Gleason score is a widely used measure based on microscopic histologic appearance of the carcinoma cells[29].

The staging of PCa largely affects the treatment selection. Despite in initial stages radical prostatectomy and radiotherapy are the preferred choices, the advanced stages, not eligible for surgery, are frequently treated with chemical or surgical castration, since PCa development is highly dependent of androgens[30]. Nevertheless, despite the initial sensibility of PCa to androgen blockage, PCa may evolves into an irreversible stage designated castration-resistant (CRPCa), considered a lethal form of the disease[31].

1.2.4. Outcomes

The PCa is a type of cancer that usually progresses slowly and are frequently asymptomatic at the time of diagnosis[32]. Nevertheless, there are several negative outcomes that decrease the patients' quality of life. The prostate tumor growth is associated with problems in the urinary tract since, as they proliferate, the PCa cells can reach the urinary tract and cause obstruction of the bladder outlet or urethra affecting the urine flow. In more severe cases, PCa patients may suffer from renal failure[33]. Sexual problems are also common in PCa patients, like erectile dysfunction which is characterized by the inability to get and maintain an erection during sexual activity[34]. Testis function may also be affected and this topic is discussed in more detail in next section. Finally, in a significant fraction of PCa patients tumor spreads to other parts of the body, giving rise to tumor metastasis. The most common site of metastasis described for PCa is the bones and is associated to bone pain and weakening, leaving the patients more susceptible to bone fractures. Other common sites of PCa metastasis include lung, liver, pleura and adrenal glands[35].

1.3. Prostate cancer-induced testicular dysfunction

PCa has been associated to testicular dysfunction and, although the underlying mechanisms have been underexplored, thus far, there are two principal mechanisms proposed, which are summarized in Figure 2. PCa patients, after radical prostatectomy, presented elevated serum levels of free and total testosterone, LH and FSH, which led to the hypothesis that prostate gland or PCa cells can secrete one or more substances that affect the hypothalamic-pituitary gonadal (HPG) axis[36].

Normal male rats, after prostate removal exhibit an increase in testosterone biosynthesis, with higher activity of 3β-HSD and 17β-HSD and in FSH serum levels. However, alterations in LH serum levels was not verified. Together, these results raise the question of whether PCa itself exerts a negative feedback in HPG axis or normal prostate gland alone is responsible for all the observed alterations[37]. To clarify this question, the testosterone levels in moderate- (Gleason score 5-7) and high-grade (Gleason score>7) PCa were compared. High-grade PCa exhibit lower testosterone serum levels, compared with healthy men and moderate-grade PCa patients, and this effect is not age-related[38]. In agreement, the changes in hormone levels after prostatectomy are more pronounced in high-grade PCa. Additionally, patients treated with transurethral resection of the prostate (TURP), which consists in a partial removal of the prostate gland commonly used in benign prostatic hyperplasia (BPH) treatment, did not present changes in hypothalamic-pituitary hormones serum levels[39,40]. Thus, it was proposed that PCa cells may have the ability to secrete one or more inhibitory factors that exert a negative feedback effect in HPG axis, but their identity is not known. One proposed candidate is inhibin, since its expression was more intense in prostate tumors than in normal prostate gland and it exert a negative feedback action on the secretion of FSH [41,42]. Nevertheless, a more recent study did not find differences in inhibin serum levels between patients with and without PCa and between patients with low- and high-grade PCa[43]. Thus, despite its potential, inhibin does not appear to be responsible for the impairment of testis function observed in PCa patients.

On the other hand, a polymorphism in GPRC6A receptor (rs2274911 - Pro91Ser) was also associated to PCa patients and proposed as contributor for PCa-induced testicular dysfunction[44]. The GPRC6A is a G protein-coupled receptor activated by several ligands

and with numerous functions in different organs[45–47]. In testis, this receptor is highly expressed in Leydig cells and the most important ligand is osteocalcin (Ocn), which induces cyclic adenosine monophosphate (cAMP) production that mediates its effects[48,49]. GPRC6A- and Ocn-deficient male mice exhibit lower testosterone serum levels, size and weight of testis and sperm count, compared to wild type[48,49]. Accordingly, Ocn increased the expression of testosterone biosynthesis enzymes: StAR, Cyp11a, Cyp17 and 3β-HSD, by the binding of cAMP response element binding protein (CREB) to enzyme genes promoters[48]. The action of Ocn in GPRC6A in Leydig cells were considered a second endocrine axis that controls testosterone biosynthesis in testis[50]. GPRC6A is also expressed in Sertoli cells, spermetogonia and spermatids, but in lower amounts and its functions in these cells are still unknown[49]. The Pro91Ser polymorphism is associated with subfunction of the receptor and patients carrying these polymorphism exhibit low sperm count and a significant risk of spermatogenic impairment[44,51]. In addition, GPRC6A and Ocn are also highly expressed in PCa cells. In these cells, GPRC6A polymorphism is associated to decreased PCa cells proliferation, with consequent delay of PCa progression and improvement of survival rate[52–54].

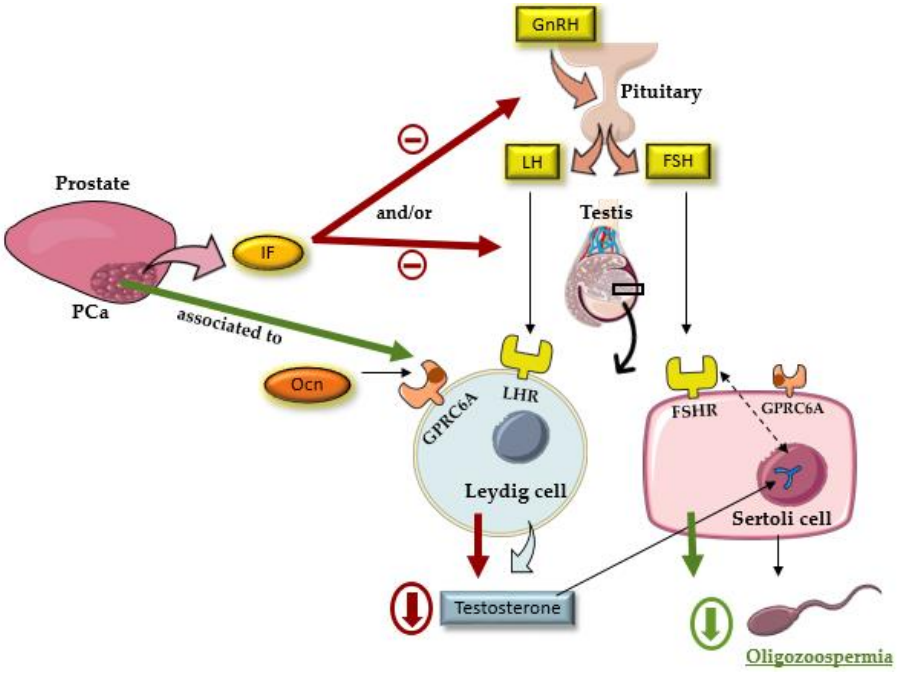


Figure 2:Overview of the effects of prostate cancer (PCa) in the hypothalamic-pituitary-testicular axis.
Abbreviations: **GnRH:** gonadotropin-releasing hormone; **LH**: luteinizing hormone; **FSH**: follicle-stimulating hormone**; PCa:** prostate cancer; **IF**: inhibitory factor; **Ocn**: osteocalcin; **LHR**: LH receptor; **FSHR**: FSH receptor; ● :polymorphism.
Figure was produced using Servier Medical Art.

1.4. Effect of physical activity on prostate cancer

1.4.1. Therapeutic approach

The benefits of physical activity were confirmed for the treatment of several diseases, including musculoskeletal, cardiovascular, pulmonary and neurological diseases[55], but its success as a non-pharmacological therapy for PCa is still a topic under investigation. To date, five large prospective cohort studies reported a negative relationship between exercise training after diagnosis and progression and/or mortality of PCa[56–60]. Some mechanisms, summarized in Figure 3, were proposed to explain these findings.

Several *in vitro* studies verified that PCa cells incubated with serum from moderate-to-vigorous exercised men exhibit decreased growth rate and increased apoptosis, compared to PCa cells incubated with serum from sedentary participants. Decreased serum insulin levels in exercised subjects seems to be responsible for this effect[61–63]. Insulin inhibits sex hormone-binding globulin (SHBG) production. Therefore the decrease in its concentration after exercise training increase SHBG levels[64]. Increased SHBG leads to decreased free serum testosterone levels, and consequently decreased PCa cells growth rate[64,65]. Reduction in insulin serum levels was also associated to increased insulin-like growth factor binding protein-1 (IGFBP-1) and consequent decreased IGF-1 levels in serum of exercised participants[61–63,66]. IGF-1 is known to contribute to degradation of p53 by mouse double minute 2 homolog (MDM-2)-induced degradation, so its decreased levels increased p53, contributing to PCa cells apoptosis[62,63,67]. Wistar male rats submitted to swimming training exhibited increased levels of serum testosterone and dihydrotestosterone (DHT), compared to sedentary ones, which suggest a contribution to PCa cells growth[68]. However, the increased testosterone may be caused by increased LH levels to compensate the low free testosterone levels after exercise training[64]. Decreased androgen receptors expression in prostate of exercised rats also suggest a lower PCa cell growth in this subjects[68].

PCa has long been associated with a low-grade chronic inflammatory state, responsible in part for maintaining the survival and contributes to proliferation, metastasis and angiogenesis of tumor prostate cells, through the activation of several signaling pathways by pro-inflammatory mediators[69]. Exercise training has been associated with decreased inflammatory mediators, by increasing the methylation of apoptosis-associated speck-like

protein containing a caspase recruitment domain (ASC) gene[70]. The methylation of ASC gene causes down-regulation of its expression and consequently decrease the release of pro-inflammatory mediators, like interleukin-1beta (IL-1β)[71]. Interleukin-6 (IL-6) can stimulate PCa cells proliferation through the activation of the androgen receptor and exercise training, in addition to decrease androgen receptor expression, was associated to downregulation of IL-6[68,72,73]. In adult men, exercise training was associated with a lower chance to have high levels of C-reactive protein (CRP), a marker of inflammation[74]. Prostaglandins, produced by cyclooxygenases (COX) are important for PCa progression, since a COX-2 inhibitor caused PCa cells apoptosis by blocking Akt phosphorylation[75,76]. In patients with a disease characterized by an inflammatory state, decreased levels of COX-2 mRNA and protein after exercise training were observed. An association between decreased COX-2 expression and a decrease in pro-inflammatory cytokines, was also suggested[73].

Prostate tissue, both normal and malignant, express vasoactive intestinal peptide (VIP) and its two receptors, $VPAC_1$ and $VPAC_2$[77]. However, PCa tissue present higher levels of VIP and overexpress $VPCA_1$. VIP exhibited a mitogenic effect in PCa cells, since it promotes PCa cells proliferation by an androgens-independent mechanism[78]. In humans, it was reported that circulating levels of VIP are under tight control by the production of natural anti-VIP antibodies, which causes VIP specific hydrolysis through cleavage of seven peptide bonds, resulting in fragments with reduced biological activity[79]. In serum of PCa patients significantly lower levels of antibodies anti-VIP were observed, which seems to explain the high VIP levels observed in these patients[80]. Contrariwise, exercised subjects had increased levels of these type of antibodies, indicating that exercise training seems to work as a stimulus for the production of natural anti-VIP antibodies, and then to decrease levels of circulating VIP. These selected subjects were athletes and participated in several types of sports like swimming, karate, rowing, among others, for a period of eleven years[80].

Prostate cells can generate reactive oxygen species (ROS), and this generation increased in PCa cells when compared to normal prostate cells[81,82]. Moreover, antioxidant defenses, like glutathione S-transferase (GST), glutathione peroxidase (GPx) and superoxide dismutase (SOD), were decreased in PCa, since nuclear factor erythroid 2p45-related factor 2 (Nrf2) was downregulated[83,84]. Thus, increased ROS production, together with decreased antioxidant defenses in PCa, leads to increased oxidative stress, which increased levels of

lipid peroxidation, which is verified by the high levels of thiobarbituric acid reactive substances (TBARS)[84]. Oxidative stress contributes to PCa cells survival by increasing the expression of disintegrin and metalloproteinase domain-containing protein 9 (ADAM9)[85]. Exercise training, not leading to exhaustion, was associated to an adaptive response characterized by increased circulating antioxidant defenses, to counteract the increase in ROS production by exercise[86]. After three types of exercise training (endurance, resistance and concurrent training), both higher levels of erythrocytes SOD activity and reduced levels of malondialdehyde (MDA) were observed, indicating decreased levels of lipid peroxidation. Endurance and concurrent training groups also presented higher erythrocytes GPx activity and increased total antioxidant capacity of plasma. Accordingly, other authors also verified an increased activity of SOD and catalase (CAT), and decreased levels of hydrogen peroxide and MDA, after exercise training[73]. Despite all the proposed mechanisms of exercise training, there is no defined threshold of exercise intensity and frequency to give rise to each of the mentioned beneficial alterations. The high heterogeneity of the studies regarding the duration, intensity and frequency of exercise training makes it difficult to establish a range in which exercise training is benefic for therapeutic of PCa patients.

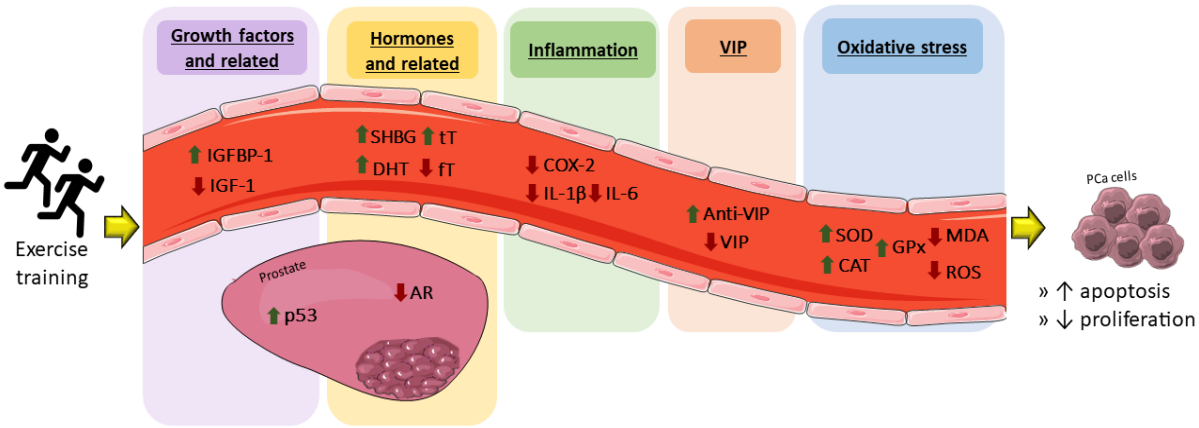


Figure 3: Overview of altered factors in circulation and prostate tissue that are responsible for the beneficial effect of exercise training of moderate intensity, for prostate cancer (PCa) patients.
Abbreviations: **IGF-1:** insulin-like growth factor-1; **IGFBP-1**: IGF binding protein-1 **SHBG**: sex hormone-binding globulin**; fT**: free testosterone; **tT**: total testosterone; **DHT**: dihydrotestosterone**; IL-1β**: interleukin-1β; **IL-6**: interleukin-6; **COX-2**: cyclooxygenase-2; **VIP**: vasoactive intestinal peptide; **SOD**: superoxide dismutase; **MDA**: malondialdehyde; **ROS**: reactive oxygen species; **GPx**: glutathione peroxidase.
Figure was produced using Servier Medical Art.

1.4.2. Preventive approach

In addition to treatment, prevention of diseases is also a critical issue and physical activity has also been associated with benefic effects in this regard. Its benefits in primary prevention has already been demonstrated for some diseases[87–89], including some types of cancer, like breast[90], colon[91], renal[92], and bladder[93] cancers. For others, like PCa, the impact of physical activity in prevention of disease is still a controversial issue.

Several population-based studies aimed to evaluate the association between exercise training and PCa incidence, reporting an inverse association (Table 1)[27,94–100]. Additionally to a global reduced risk of developing PCa, exercise training was associated to a lower risk of high-grade PCa at diagnosis, which is associated with an aggressive clinical course and poor outcomes of disease[94–98]. Until now, only one relevant study evaluated the effect of exercise training on PCa incidence risk using animal models. In this work, Esser *et al*[101] used transgenic mice with predisposition to develop PCa caused by a C3(1)tag that leads to a direct expression of SV40 on prostate tissue. Among group 1, corresponding to mice who ran more than five kilometers *per* day (ten weeks), 83% had normal dorsolateral prostates, comparing with 43% in mice who ran less than five kilometers *per* day (group 2). The same effect was observed for ventral prostates, but this was less pronounced. Thus, both these results indicated a reduced risk of prostate alterations in mice who ran more kilometers *per* day. These authors also found a lower frequency of prostatic intraepithelial neoplasia (PIN) pathology, classified as a pre-cancerous condition, in mice from group 1. Additionally, and according to other population-based studies, group 1 was also associated to a reduced chance to had advanced disease at diagnosis[101]. Moreover, some studies (Table 1) did not agree with the previously mentioned and reported a null[102,103] or negative[100,104,105] association between exercise training and the risk of PCa.

Table 1:Summary of the studies that evaluated the association between several types and intensities of physical activity and the risk of developing prostate cancer (PCa).

Type of study and population	Subjects characteristics	Type of physical activity	Physical activity cut-off	Effect on total PCa risk	Reference
Prospective	307 participants Median age: 64	Recreational	≥ 9 MET-h/week	White men ↓ Black men ↔	Singh *et al.*[94]
Prospective	286 participants Median age: 68	Household Recreational Occupational	–	↓	De Nunzio *et al.* [95]
Prospective	163677 participants Mean age: 62	Recreational (mainly.19-29 age)	≥ 4h/week	White men ↔ Black men ↓	Moore *et al.*[96]
Prospective (Europe)	127923 participants Median age: 54	Occupational	Manual	↓	Johnsen *et al.*[97]
Prospective (Sweden)	100303 participants Mean age: 60	Recreational Occupational	> 60 min/day Manual	↓	Orsini *et al.*[27]
Prospective (United States)	51529 participants Mean age: 54	Recreational	> 29 MET-h/week	↓	Giovannucci *et al.*[98]
Case-control	2365 participants Mean age: 67	Strenuous recreational (early 50s)	–	↓	Darlington *et al.*[99]
Prospective (China)	592 participants Mean age: 71	Recreational	≥ 20 min regularly	↔	Tai *et al.*[102]
Prospective (United States)	86404 participants Mean age: 64	Recreational	≥ 35 MET-h/week	↔	Patel *et al.*[103]
Case-control (Canada)	1014 participants Mean age: 67	Household Recreational Occupational	≥ 26.8 MET-h/wk/y ≥ 25.1 MET-h/wk/y ≥ 161.9 MET-h/wk/y	↑ ↓ ↓	Friedenrich *et al.*[100]
Prospective	34757 participants Mean age: 57	Recreational	≥ 11 MET-h/week	Normal men ↓ Obese men ↑	Littman *et al.*[104]
Case-control (Sweden)	2617 participants Mean age: 67	Household Recreational	≥ 4.6 MET-h/day ≥ 13.5 MET-h/day	↑	Wiklund *et al.*[105]

PCa: prostate cancer; **MET**: metabolic equivalent value; ↑: increase; ↓: decrease; ↔: no effect

Despite some controversy, it was suggested a benefic effect of exercise training in reducing PCa incidence. Thus far, the molecular mechanisms that explain this association are not fully elucidated. However, at least some of the alterations summarized in Figure 3, may contribute

to this association. The different effect of exercise training between black and white men, and between obese and normal weight men (Table 1) suggest a role of androgens in the effect of exercise training, since these two groups share differences in hormonal profile, particularly in androgens levels[106,107]. Oxidative stress was also proposed as a stimulator of prostate carcinogenesis mainly by causing damage in DNA and by increasing oncogene expression[108,109]. Likewise, chronic inflammation was suggested as a contributor to prostate tumor development[69], and VIP was also considered a stimulator of PCa development since it contributes to the malignant transformation of a specific human normal prostate epithelial cell line[110]. Thus, both hormonal alterations and reduced chronic inflammation, oxidative stress and VIP serum levels after exercise training, seems to, at least in part, contribute to decreased risk of developing PCa. Until now, there is a lack of robust evidences on this subject, since most studies reported in literature have limitations that difficult the establishment of a definitive conclusion. Main limitations are studies' heterogeneity with regard to intensity, duration and frequency of exercise training programs, and the way the information about participants' physical activity was gathered that was assessed based on validated self-reported questionnaires but that can lead to misclassifications of the participants.

1.5. Impact of physical activity on testicular function

To date, there are no available human studies on testis that evaluate the effect of exercise training in testis function; however, there are some studies that assess the effect of exercise training in sperm concentration and characteristics, and in hormonal profile, which are indirect measures of testis function. Several population based-studies reported a positive association between moderate-intensity exercise training and several parameters of testis function[111–114]. Increased levels of total and free testosterone, LH, FSH and sperm count, and improvement of sperm motility and morphology was observed in trained groups. However, not all the alterations were consensual between all studies. For example, Gaskin *et al*[113], contrary to other authors, did not found a significant association between exercise training and sperm motility and morphology. The mechanisms responsible for these benefic effects were not yet clarified.

Contrary, some studies reported a negative impact of moderate-intensity exercise training in testicular function[115–118]. Lower levels of free and total testosterone, LH and FSH, and higher level of SHBG were observed in moderate-intensity trained mem. It was also observed decreased semen parameters in moderate-intensity group but this alterations did not reach statistical significance[115]. Thus far, based on animal model studies, some candidates had been proposed as responsible for these negative effects of exercise training in testis functions. Moderate-intensity exercise training caused vascular dysfunction with weakened oxygen supply to testis tissue, since it was observed a decreased microvascular oxygen partial pressure (PO_{2m}) value in testis of trained group[117]. An impaired spermatogenesis was also suggested, since it was observed decreased levels of outer dense fiber protein 1 (ODF-1) in exercised rats, which is involved in the correct formation of sperm, being essential to a rigid head-tail junction[116,119]. Finally, contrary to other authors. Wise *et al*[118] did not verify an association between exercise training of diverse types, including running, bicycling and weightlifting, at different hours *per* week, and semen parameters. An exception was observed for men who bicycled as a primary form of exercise. In this case, it was demonstrated a decreased sperm count and motility for men who bicycled for five or more hours *per* week, comparing with those who don't exercised regularly.

For high-intensity exercise training there is an apparent consensus in a harmful impact in testis functions, both for a shorter[120–122] or longer period[101,109]. In a human randomized controlled study, it was verified lower levels of total and free testosterone, LH and FSH higher levels of SHBG, and decreased semen parameters. All these alterations were more pronounced than for moderate-intensity exercised participants. In addition, it was also demonstrated a positive relation between the significance of the observed differences and the weeks of exercise training, suggesting that more prolonged exercise training programs are associated with more pronounced alterations in the hormonal and semen parameters[115]. Several studies with animal models agree with a negative impact of intensive exercise training in hormonal and seminal parameters, and the decreased in testosterone levels was corroborated by decreased testis activity of some steroidogenic enzymes (3β-HSD and 17β-HSD)[120–123]. But the involvement of hypothalamic-pituitary axis in these alterations is not consensual, since some studies did not found significant alterations in FSH serum levels[106,107] and others verified increased levels of LH[122]. Lower levels of heat shock protein 70 (HSP70) in spermatogenic cells after high-intensity exercise training constitute an

additional evidence of impaired spermatogenesis, since it is associated with maturation arrest in the germinative cell populations, which may causes low sperm count and may be associated with male infertility[124,125]. Oxidative stress was proposed as one of the responsible for these prejudicial effects. Decreased testis activity of enzymatic antioxidant defenses like SOD, CAT, GPx and GST, reduced content of non-enzymatic defenses like reduced glutathione (GSH) and ascorbic acid, and increased levels of lipid peroxidation markers (MDA and conjugated dienes) were observed in intensive exercised group[120–123]. Ribose deficit seems to be also involved in testis dysfunction of intensive exercised men. A decreased in ribose levels was confirmed by reduced levels of glucose-6-phosphate dehydrogenase (G6PDH), enzyme that catalyze the first step of pentose phosphate pathway. Administration of D-ribose restored MDA, GSH and ascorbic acid for near their basal levels, and increased testosterone and LH levels, indicating a key role of ribose deficit in oxidative stress and consequently in testis dysfunction[122].

Lifelong or later in life exercise training of different types (swimming and running), frequencies and durations was associated with benefic effects at testis level by the protection/reversion of some age-associated impairments of testicular function[126–128]. Using a senescence-accelerated prone mouse model (SAMP8), lifelong moderate-intensity exercised group presented higher levels of Nrf-2 in testis, which leads to an increased expression of antioxidant enzymes, confirmed by the increased mRNA levels of SOD and GPx[126]. Decreased oxidative damage in testis of exercised groups was also observed, with reduced levels of protein carbonyls, 8-isoprostanes, MDA and 8-hydroxy-20-deoxyguanosine. Reduction of testis inflammation after exercise training was also observed by the decreased levels of nuclear factor kappa B (NF-kB), a mediator of inflammatory molecules. Reduced levels of pro-inflammatory mediators (IL-1β and tumor necrosis factor alpha (TNF-α)) and COX-2, and increased levels of anti-inflammatory mediators (IL-10 and transforming growth factor alpha (TGF-α)) confirmed an anti-inflammatory effect of moderate-intensity exercise training in testis[126]. In agreement with the reduction of oxidative stress and inflammation in testis by lifelong exercise training, it was verified an improvement in testicular function. Increased levels of serum and testis testosterone, confirmed by the higher levels of steroidogenic enzymes StAR and Cyp11a and increased number of Leydig cells and spermatogonia in seminiferous tubules were observed after lifelong exercise training[112,113]. Exercised older rats was also associated with attenuated age-related testicular

atrophy and reduced levels of DNA damage, measured by levels of phosphorylated histone H2AX[128]. The effect of exercise training o seems to have a higher protection in lifelong or earlier initiation of exercise training program, compared to exercise training later in life[126].

Low intrinsic capacity runners (LCR) rat models were also used to evaluate the effect of exercise training in reversion of impaired testicular function[116]. This animal model is characterized by lower sirtuin-1 levels on testis, and consequently attenuation of spermatogenesis and higher frequency of abnormal sperm and DNA damage[129]. After twelve weeks of moderate-intensity exercise training, decreased levels of ROS and increased ODF-1 were observed, suggesting improvement in spermatogenesis[116]. Increased lactate dehydrogenase C levels was also observed in testis, which is essential in germ cells energy metabolism and, consequently, in sperm function[130]. However, trained LCR rats presented a higher acetylation of p53, especially comparing to higher intrinsic running capacity (HCR) rats, which suggest an increased genetic instability in these rats, more pronounced with exercise training. Moreover, all the aforementioned benefic alterations were not verified in rats with HCR, suggesting additional evidences of a specific benefic effect of exercise training in testis, in the presence of a condition that affects its function[116].

Doxorubicin treatment also induced impairment of testis function, mainly by decreased testis antioxidant defenses and consequent causing oxidative damage[131,132]. Exercise did not significantly affect carbonylated and nitrated proteins and MDA levels, so did not appear to have a positive effect in preventing oxidative damage in testis and consequently in preventing impairment of sperm motility. However, it was suggested a benefic effect of exercise training by negatively modulation of testis proteome susceptibility to oxidative modification[132].

Obesity was associated to decreased spermatogenesis and testosterone levels[106,133]. Obese male mice were characterized by increased levels of serum leptin (LEP), which suggested leptin resistance, confirmed by the reduced testis expression of leptin receptors and of two precursors of leptin signal transduction: Janus kinase 2 (JAK2) and signal transducer and activator of transcription 3 (STAT3), in this group of mice. Exercise training for eight weeks both high- and moderate-volume restored the LEP-JAK-STAT signaling pathway in testis. However, only for moderate-volume was verified an improvement of testicular function, with increased expression of steroidogenic factor-1 that is a regulator of transcription of

some steroidogenic enzymes like StAR and Cyp11a, which were also elevated in this group of mice. Accordingly, it was also reported an increased level of testosterone and sperm count and an improvement in sperm motility in this group[133].

Thus, the impact of exercise training in testicular function is still a very controversial topic mainly due to the high heterogeneity between studies. Despite for moderate-intensity exercise training there are inconsistent published studies in literature, for intensive exercise training there seems to be a consensual association with impairment in spermatogenesis and hormonal profile. In the presence of a condition that causes impairment of testicular function, like aging or obesity, there are several evidences that exercise training preventing and/or counteracting these alterations.

2. Aims

In order to add new insights on the impact of physical activity in testicular function of PCa subjects, we used control and PCa-induced animal models, in which half of the animals were submitted to an exercise training program. Thus, we intended to characterize the morphological adaptations of testis tissue induced by PCa and/or exercise training, and relate them with the effect of these conditions in testicular function parameters. For this purpose, we intended:

i) To characterize the effect of PCa and exercise training in body and testis weight;

ii) To assess the effect of PCa and exercise training in steroidogenesis and spermatogenesis;

iii) To study the effect of PCa and exercise training in testis morphology.

3. Material and Methods

3.1. Animals

A total of 50 Wistar Unilever male rats aged four weeks old were acquired to Charles River Laboratories company (France), placed in quarantine for two weeks and then randomly distributed in cages (five per cage). The animals were randomly divided in four experimental groups: Sedentary (SED), Sedentary+PCa (SED+PCa), Exercised (EX) and Exercised+PCa (EX+PCa); and maintained in bioterium of University of Trás-os-Montes and Alto Douro (UTAD) in controlled conditions, including a temperature of 18±2ºC, a 12 hours light/dark cycle and a relative humidity of 55±5%. The animal protocol was approved by the animal well-being responsible organ of UTAD and by Direção Geral de Alimentação e Veterinária-DGAV (license nº 021326).

In order to induce prostate lesions to PCa groups (31 rats), it was administered flutamide subcutaneously (20mg/Kg prepared in 10% propylene glycol and 5% ethanol) during consecutive 21 days. After two days, testosterone propionate (100mg/Kg dissolved in corn oil (Sigma)) was injected subcutaneously. N-methyl-nitrosourea (MNU, 30mg/Kg prepared in citrate buffer 0.1 M pH 4.8) was administered intraperitoneally two days later, and after 15 days subcutaneous implants of crystallin testosterone (Sigma) were inserted in interscapular region through a small incision followed by suture. This procedure was carried out under anesthesia (75mg/Kg of ketamine and 10mg/Kg of xylazine) and the implants (Dow Corning) were prepared in medical silicone tubes (4cm) filled with testosterone and with the extremities sealed with medical glue (G.E. RTV-108).

Forty-one weeks after MNU administration, the animals were euthanized through overdose of anesthetics (association of ketamine and xylazine), administered intraperitoneally, followed by exsanguination through cardiac puncture. The animals weight was measured and serum, testes and epidydimal sperm from epididymis cauda were collected. The testes were weighted and stored at -80ºC for further analysis.

3.2. Endurance training protocol

The animals of exercised groups (Exercised and Exercised+PCa) were exercised since six weeks old in a level treadmill (Treadmill Control LE 8710, Harvard Apparatus, USA) for 50 weeks. The exercise program included five days per week, 30 minutes per day during the first week (habituation period) and then 60 minutes per day until the end. The speed of treadmill was set for 70% of the maximal speed capacity of the animals with PCa and every fifteen days the speed capacity was re-evaluated to correct the exercise intensity. In order to subject the animals to a similar stress, the animals from the sedentary groups (Sedentary and Sedentary+PCa) were regularly placed on a turned off treadmill for few minutes.

3.3. Testosterone serum levels

Testosterone levels were determined on serum aliquots, using testosterone ELISA kit – Caymann Chemical (Ann Arbor, MI. USA). This kit is a competitive assay based on the competition between testosterone and a testosterone-acetylcholinesterase conjugate, with a concentration range from 3.9 to 500pg/mL and a sensitivity of approximately 6pg/mL. Based on the kit instructions, it was prepared eight testosterone standards with decreasing testosterone concentrations, the absorbance at 412nm was determined using a microplate reader (Tecan® Infinite M200) and a standard curve generated. The serum samples were diluted and based on the standard curve the testosterone concentration was determined in pg/mL.

3.4. Sperm concentration

An undiluted preparation of sperm was examined on a glass slide to set the appropriate dilution, based on Table 2. The adequate volume of 1× phosphate-buffered saline (PBS) were added to the samples and the counting was performed in a Neubauer chamber. The total number of spermatozoa counted was divided by the volume in which the counting was made, and then, based on the respective applied dilution, the sperm concentration was determined. The results were expressed as number of spermatozoa $\times 10^6$ *per* milliliter.

Table 2: Appropriated dilution, with the respective volume of semen and phosphate-buffered saline (PBS), according to the number of spermatozoa counted using 40× microscopic objective.

Spermatozoa per 40× field	Dilution	Semen (µL)	PBS (µL)
"Swim-up"	1:2	100	400
<15	1:5	100	400
15-40	1:10	50	450
40-200	1:20	50	950
>200	1:50	50	2450

3.5. Sperm morphology

Diff-Quik rapid staining method was used to prepare the glass slides to analyze sperm morphology[134]. In this method, a sperm smear was fixated and stained. To fixate the air-dried sperm smears, they were immersed in triarylmethane fixative solution (dissolved in methanol) for 15 seconds. The staining was performed by immersing the fixed sperm smears sequentially in staining solution 1 (eosinophilic xanthene) - 10 seconds, staining solution 2 (basophilic thiazine) - 15 seconds, and running tap water (10 to 15 times). All these solutions were provided by the Diff-Quik rapid staining kit. The sperm morphology was analyzed using a Zeiss optical Primo Star microscope (Carl Zeiss AG, Germany) and the following defects were identified: decapitated-head (DH), flattened-head (FH), pin-head (PH), bent neck (BN) and tail defects (TD). *Per* sample, it was counted a total of 333 spermatozoa (when possible) and for each spermatozoon only one defect was considered. Head-defects was considered more relevant than tail defects. The percentage of each defect were calculated.

3.6. Sperm motility

A preliminary analysis of sperm motility was also performed yet most spermatozoa were immotile. This may be due to the anesthetics administered to the animals at sacrifice since ketamine was already associated to decreased sperm motility[135]. Nevertheless, this constrain did not significantly affects this work since sperm motility mainly reflects epididymal function, instead of testicular function.

3.7. Histology of testes

The right testis tissues were embedded in paraffin forming paraffin-blocks. The paraffin blocks were sectioned (5µm) with a manual microtome. For each sample, it was performed two glass slides with three or more cuts *per* slide. One glass slide *per* sample was deparaffinized in xylol, dehydrated with alcohol in decreasing concentrations (100%, 95% and 75%) and stained with haematoxylin and eosin (H&E). These specimens were then examined in a bright-field optical microscopy and digital images were captured, using ZEN Microscopy software. These images were further analyzed using ImageJ software. The mean area and number of seminiferous tubules in three random microscopic fields were measured. Moreover, the thickness of arterial wall and the diameter of arterial lumen were also measured and the ratio between them were calculated. To the other glass slide, deparaffinization and dehydration was also applied using the same procedure and then Sirius red staining was applied. The analysis of these glass slides was performed using the same procedure previously described. The basal lamina thickness was measured in three random microscopic fields.

3.8. Biochemical markers

The left testes (six animals *per* experimental group) were macerated with liquid nitrogen until get a powder. Then, the testis samples were lysate with the adequate volume of ice-cold 1× radioimmunoprecipitation assay buffer (RIPA) lysis buffer with addition of proteases and phosphatases inhibitors (1 mL of buffer *per* 250 mg of testis tissue, approximately) and incubated for 30 minutes at 4ºC with agitation. The samples were centrifuged at 4ºC, 20 min at 16000g and the soluble fraction were collected. The protein concentration of the soluble fractions was determined using the bicinchoninic acid (BCA) protein quantification assay (Fisher Scientific, Loures, Portugal). In a 96-well plate, 1 µL of each sample were added to 24 µL of 1× RIPA lysis buffer. The standard protein concentrations were prepared as described in Supplementary table 1. Then, 200µL of working reagent (WR) were added to each well and the plate was incubated 30 min at 37ºC. The WR were prepared by mixing BCA reagent A with BCA reagent B (50:1). Once the 96 well-plate cooled to RT, the absorvance was read at 562 nm using an Infinite® 200 Pro (Tecan, Switzerland). A standard curve of absorvance (562nm) vs bovine serum albumin

(BSA) concentration was obtained and used to determine the total protein concentration of the samples.

The testis lysates (soluble fractions) were resolved by 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and proteins were electrotransferred onto nitrocellulose membranes. The gel was run at 200 V and then electrotransferred at 200 mA for two hours. The efficiency of the protein transference to the membrane was confirmed using Ponceau Staining that was also used as the loading control (Supplementary figure 1). Non-specific protein-binding sites on the membrane were blocked with 5% BSA or 5% non-fat milk in 1× Tris-buffered saline (TBS 25mM Tris-HCl, pH 7.4, 150mM NaCl) containing 0.1% Tween 20 (TBS-T) for one hour. The blots were then washed with 1×TBS-T and incubated with primary antibodies (rabbit anti-VEGFA, mouse anti-Boule -Table 3) overnight and two hours (respectively) at 4ºC. After the incubation, the blots were washed three times for ten minutes with 1× TBS-T and then incubated with the appropriate secondary antibody for one hour at RT. Blots were washed three times for ten minutes with 1× TBS-T and once with 1× Tris-buffered saline (TBS 25mM Tris-HCl, pH 7.4, 150mM NaCl) (TBS) and immunodetected using the Odyssey infrared Imaging System (Li-COR® Biosciences, US). The results were analyzed using QuantityOne 4.6.6 (Basic)® software - Biorad.

All the solutions used in this protocol were prepared as described in Supplementary table 2.

Table 3: Summary of the primary antibodies used and their main characteristics (host, dilution, molecular weight (MW) of the predicted band, reference and supplier).

Name	Host	Dilution	MW (KDa)	Reference	Supplier
Anti-VEGFA	Rabbit	1:1000 (5% BSA)	27 KDa	ab46154	abcam
Anti-Boule	Mouse	1:500 (5% non-fat milk)	31 KDa	sc-166660	Santa Cruz

3.9. Statistical analysis

The results are expressed as mean ± standard error of the mean (SEM). The statistical significance of the differences between the four experimental groups were tested using Kruskal-Wallis test followed by multiple comparison post-hoc Dunn's test. The statistical significance between groups were measured based on *P*-value and differences with a *P*-value

< 0.05 were considered significant. Statistical analysis was performed using GraphPad Prism software (version 7.0).

3.10. Summary of the experimental design

A brief scheme resuming the experimental design used in this study is shown in Figure 4 .

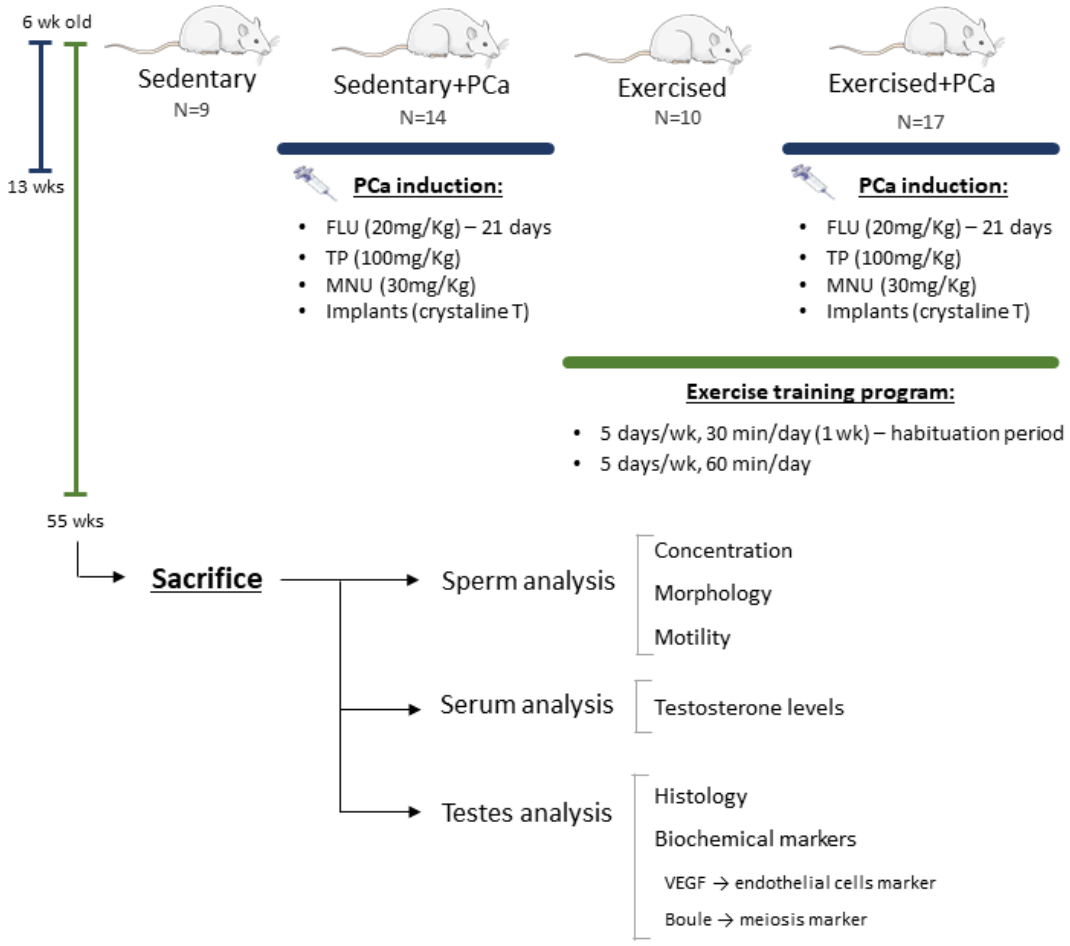


Figure 4: Summary of the experimental setup. At the top different treatment applied to each group and at the bottom the different analysis performed.
Abbreviations: **PCa**: prostate cancer; **FLU**: flutamide; **TP**: testosterone propionate; **MNU**: N-methyl-nitrosourea; **T**: testosterone.

4. Results

4.1. Characterization of the animal model

As expected, animals from PCa groups exhibited prostate lesions such as hyperplasia and microinvasive carcinoma. The animals body weight, left and right testes weight and the respective body-testes weight ratios of the experimental groups are described in Table 4. PCa-induced animals both sedentary and exercised presented a reduction in body weight when compared with respective controls, but these differences did not reach statistical significance. A significant decrease in body weight were observed in the Exercised and Exercised+PCa animals, when compared with respective sedentary groups (P=0.0015 – EX vs SED; P=0.0004 – EX+PCa vs SED+PCa). PCa-induced rats both sedentary and exercised also exhibit a significant decrease in both right (P=0.0011 – SED+PCa vs SED; P=0.0077 – EX+PCa vs EX) and left (P=0.0003 – SED+PCa vs SED; P=0.0121 – EX+PCa vs EX) testis weight, and, accordingly, in right (P=0.0002 – SED+PCa vs SED; P=0.0144 – EX+PCa vs EX) and left (P<0.0001 – SED+PCa vs SED; P=0.0066 – EX+PCa vs EX) testis-body weight ratios, compared to respective sedentary and exercised controls. Exercise training exhibited a tendency to decrease testes weight of control and PCa-induced rats. Regarding testes-body weight ratios, there was a decrease in exercised control group compared with sedentary but without statistical significance, and a slight increase in exercised PCa-induced rats compared with respective sedentary but also without significance.

Table 4: Effect of prostate cancer (PCa) and exercise training on animals' body weight, left and right testis weight and testes/body weight ratios. The results were expressed as mean ± SEM and the statistical significant were represented based on *P*-value. **P*-value<0.05; ***P*-value<0.01; ****P*-value<0.001; *****P*-value<0.0001.

Mean Weight	Sedentary	Sedentary+PCa	Exercised	Exercised+PCa
Body (g)	541.05±13.42	494.59±9.12	434.81±8.91 **	421.64±7.40 *** (b)
Right Testis(g)	2.148±0.085	1.058±0.084 **	1.621±0.158	0.955±0.036 ** (a)
Right testis/ Body (mg/g)	3.986±0.172	2.138±0.156 ***	3.701±0.335	2.278±0.095 * (a)
Left testis (g)	1.591±0.031	0.657±0.069 ***	1.133±0.142	0.553±0.011 * (a)
Left testis/ Body (mg/g)	2.831±0.075	1.316±0.117 ****	2.601±0.0331	1.320±0.039 ** (a)
		vs. Sedentary	vs. Sedentary	(a) vs. Exercised (b) vs. Sedentary+ PCa

4.2. Testicular function parameters analysis

To determine the effect of PCa and exercise training in testis function, testosterone serum levels and sperm concentration and morphology were analyzed. The testosterone serum levels of different groups are presented in Figure 5. PCa-induced rats both sedentary and exercised exhibited significant higher levels of testosterone serum levels comparing with respective controls (*P*=0.0005 – SED+PCa vs SED; *P*=0.0003 – EX+PCa vs EX). Exercise training showed a tendency to increase testosterone serum levels in controls and PCa rats.

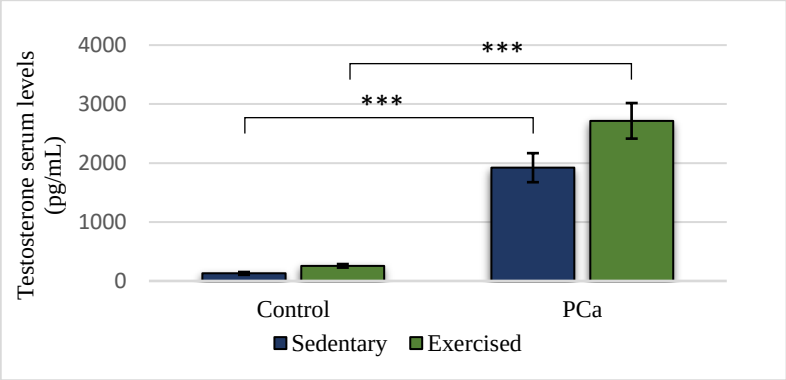


Figure 5: Effect of prostate cancer (PCa) and exercise training on testosterone serum levels. The results were expressed as mean ± SEM and the statistical significance were represented based on *P*-value. ***P-value<0.001.

The sperm concentration of the four experimental groups are represented in Figure 6. PCa-induced rats presented abruptly decreased sperm concentration, compared with respective controls (*P*<0.0001 – SED+PCa vs SED; *P*=0.0184 – EX+PCa vs EX). In control animals, exercise training caused a slightly decrease in sperm concentration, but this alteration did not reach statistical significance. Contrary, in PCa-induced animals, exercise training tended to increase the sperm concentration, but also without statistical significance.

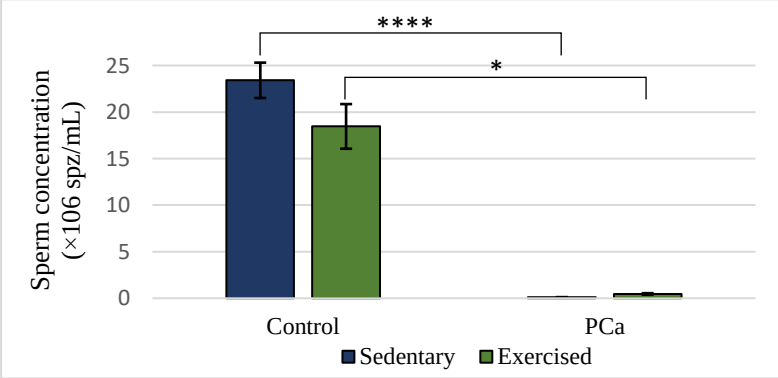


Figure 6: Effect of prostate cancer (PCa) and exercise training on sperm concentration. The results were expressed as mean ± SEM and the statistical significance were represented based on *P*-value. *P-value<0.05; ****P-value<0.0001.

The sperm morphology of the different groups is described in Table 5, based on the percentage of different morphologic forms. PCa-induced rats, both sedentary and exercised, exhibited a highly decreased percentage of normal sperm forms (P<0.0001 – SED+PCa vs SED; P=0.0215 – EX+PCa vs EX). Despite exercise training tended to decrease the percentage of normal forms in control rats, in PCa-induced rats it was observed a non-significant slightly increase. PCa also caused an increase in the percentage of decapitated-head (DH) (P=0.0078 – SED+PCa vs SED; P<0.0001 – EX+PCa vs EX) and flattened-head (FH) (P=0.0009 – SED+PCa vs SED; P=0.0002 – EX+PCa vs EX) forms, both in sedentary and exercised animals. Pin-head (PH) forms also increased in PCa-induced rats, but only significantly in sedentarys (P=0.0006). Bent-neck (BN) and tail defect (TD) decreased in PCa-induced rats, but only significantly in exercised rats (P=0.0068 – BN; P<0.0001 – TD). Exercise training decrease PH forms in PCa-induced rats (P=0.0021) and seemed to increase TD sperm forms in control rats and increased DH in PCa-induced and control rats.

Table 5: Effect of prostate cancer (PCa) and exercise training on percentage of sperm morphologic forms. The results were expressed as mean ± SEM and the statistical significance were represented based on P-value. $^{*}P$-value<0.05; $^{**}P$-value<0.01; $^{***}P$-value<0.001; $^{****}P$-value<0.0001.
N: normal; DH: decapitated-head; FH: flattened-head; PH: pin-head; BN: bent-neck; TD: tail defects.

Sperm morphology	Sedentary	Sedentary+PCa	Exercised	Exercised+PCa
N (%)	72.480±2.360	0.460±0.211 ****	43.650±3.780	1.180±0.210 * (a)
DH (%)	6.700±0.571	69.130±4.550 **	15.550±4.520	83.240±2.550 **** (a)
FH (%)	1.616±0.233	17.800±2.920 ***	0.926±0.166	11.690±2.100 *** (a)
PH (%)	0.067±0.041	3.465±0.964 ***	0.030±0.028	0.319±0.135 ** (b)
BN (%)	8.542±1.930	4.158±0.847	6.845±1.739	1.305±0.1950 ** (a)
TD (%)	10.630±1.380	4.800±1.729	33.630±3.690	1.968±0.444 **** (a)
		vs. Sedentary	vs. Sedentary	(a) vs. Exercised (b) vs. Sedentary+ PCa

4.3. Testis morphology analysis

To determine the histological alterations in testicular tissue caused by PCa and exercise training, microscopic illustrations were captured and analyzed. Representative microscopic illustrations (10× amplification – H&E staining) are represented in Figure 7. The number of seminiferous tubules per microscopic field and the area of seminiferous tubules (μm^2) of experimental groups are present in Figure 8. PCa-induced animals exhibited high number of seminiferous tubules per microscopic field with a reduced area, compared with control animals, both sedentary (P<0.0001 – number and area of seminiferous tubules) and exercised

(*P*=0.0386 – number of seminiferous tubules). Despite exercise training seemed to increase the number of seminiferous tubules *per* microscopic field and reduced their area in control animals, in PCa-induced animals, exercise training showed a tendency to decrease the number and increase the area of seminiferous tubules.

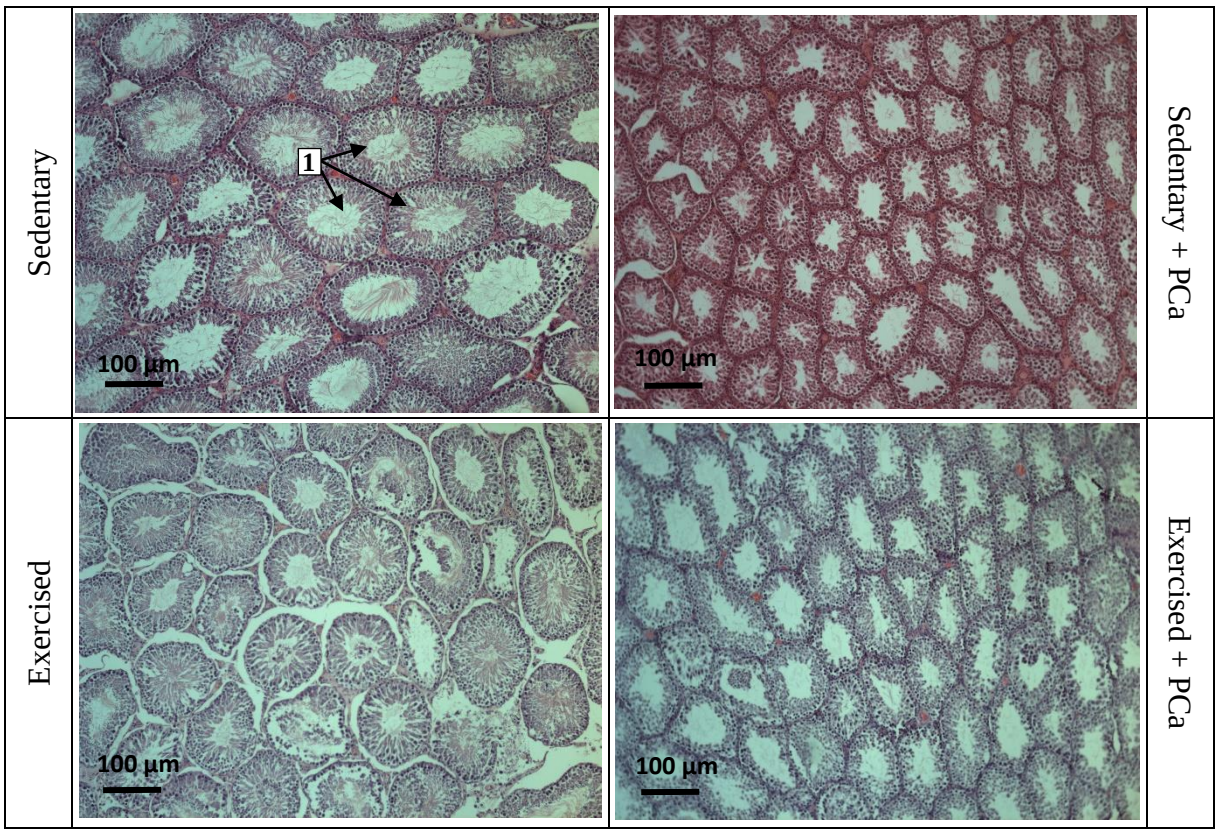


Figure 7: Representative illustrations of histological morphology of rat testes of four experimental groups. The testes cross sections were stained with haematoxylin and eosin. (10× amplification).
Legend: 1 – Seminiferous tubules.

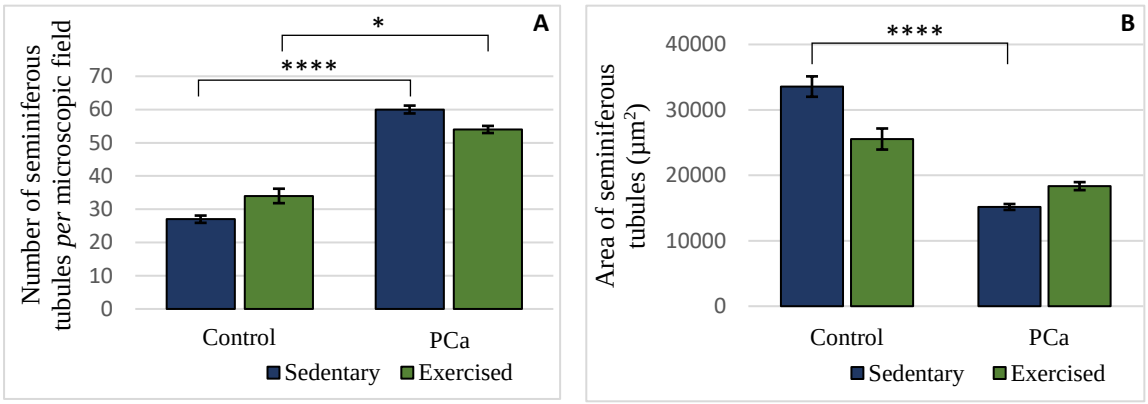
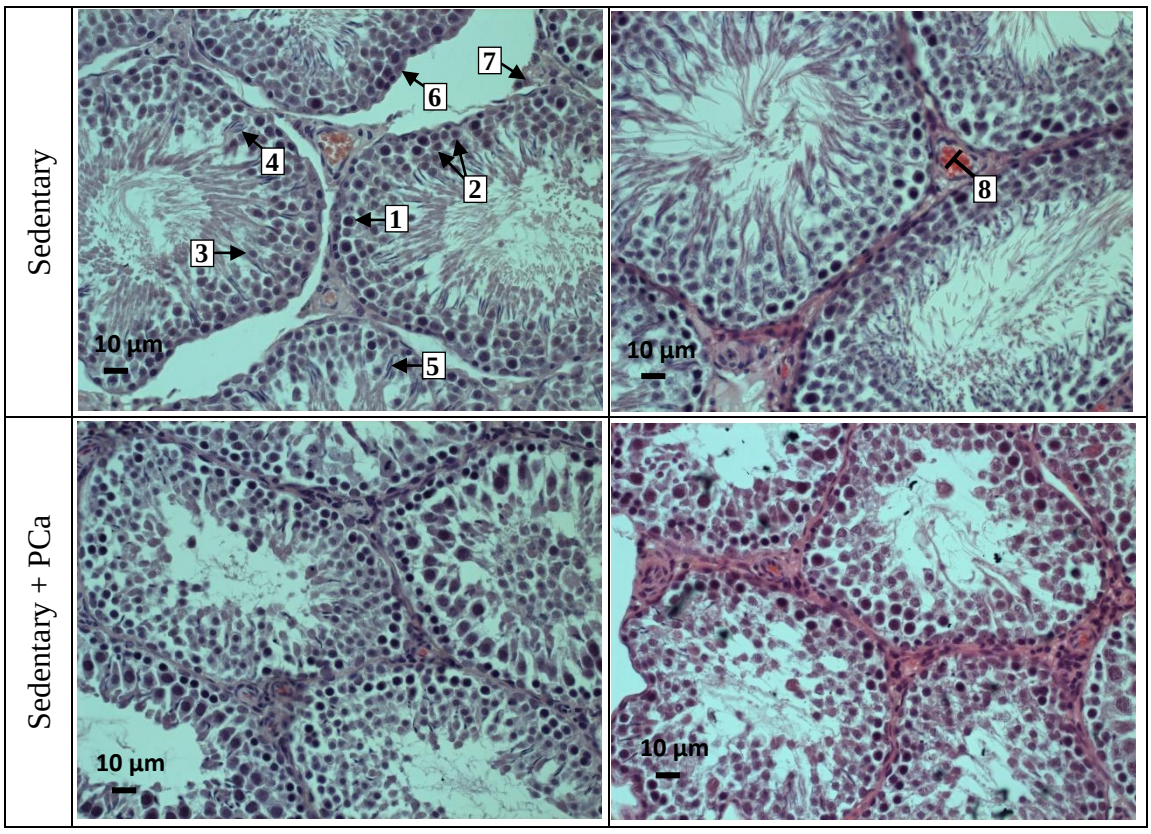


Figure 8: Effect of prostate cancer (PCa) and exercise training on (A) the number of seminiferous tubules per microscopic field and (B) the area of seminiferous tubules. The results were expressed as mean ± SEM and the statistical significance were represented based on *P*-value. * *P*-value<0.05; **** *P*-value<0.0001.

Representative microscopic illustrations (40× amplification – H&E staining) of different groups are represented in Figure 9. The ratio between lumen diameter and arterial thickness of experimental groups are exhibit in Figure 10. PCa-induced rats presented higher arterial thickness in testis, which resulted in a reduced lumen diameter, or even in closing of some arterial lumen, both in sedentary and exercised animals (P<0.0001 – SED+PCa vs SED; P=0.0091 – EX+PCa vs EX). PCa-induced rats also exhibited a decreased cellular density with very few spermatids and virtually none mature spermatozoa in the center of seminiferous tubules, compared with respective controls. Instead, some centers of seminiferous tubules were filled with immature germ cells, like primary or secondary spermatocytes, or presented a laced aspect. Exercise training exhibited a tendency to increase the lumen diameter/arterial thickness of testis tissue of PCa rats (P=0.0677 – EX+PCa vs SED+PCa) and seems to improve the maturation of sperm cells, since it was observed more spermatids and also some sperm heads in the center of seminiferous tubules. In control rats, exercise training also exhibited a tendency to slightly increase the arterial lumen of testis tissue and did not noticeably affected the maturation of sperm cells, since it was observed sperm cells at the different maturation stages, including mature spermatozoa.



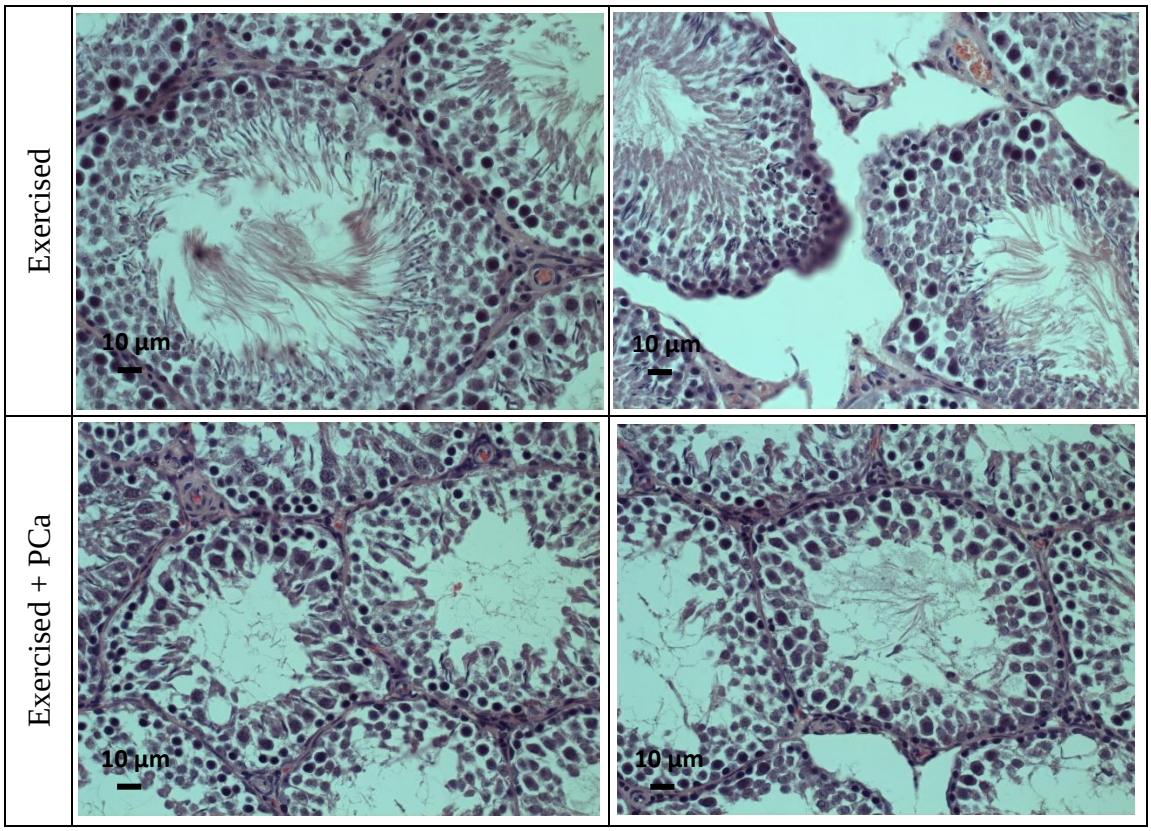


Figure 9: Representative illustrations of histological morphology of rat testes of four experimental groups. The testes cross sections were stained with haematoxylin and eosin. (40× amplification).
Legend: 1 – Spermatogonium; 2 – Spermatocytes; 3 – Round spermatid; 4 – Elongating spermatid; 5 – Mature spermatozoon; 6 – Sertoli cell nucleus; 7 – Leydig cell; 8 – Arterial lumen.

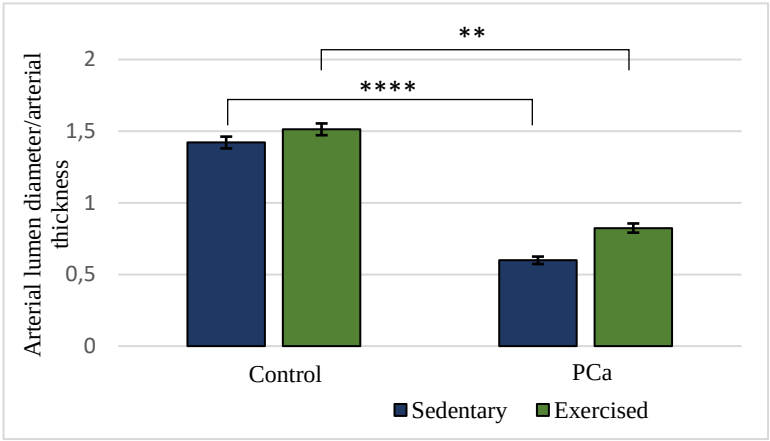
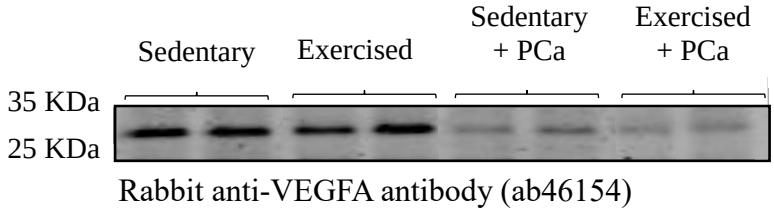


Figure 10: Effect of prostate cancer (PCa) and exercise training on the ratio between the arterial lumen diameter and the arterial thickness. The results were expressed as mean ± SEM and the statistical significance were represented based on *P*-value. ** *P*-value<0.01; **** *P*-value<0.0001.

To confirm some of the observed morphological alterations in testis tissue, the Boule (a marker of meiosis progression) and vascular endothelial growth factor (VEGF) (a marker of endothelial dysfunction) testis levels were measured. The levels of Boule in testis tissue of the four experimental groups are represented in Figure 11. Both PCa and exercise training did not significantly affect Boule testis level.

Figure 11: Effect of prostate cancer (PCa) and exercise training on the Boule testis levels. The results were expressed as mean ± SEM. A representative immunoblot is shown above the graph.

The levels of VEGF in testis tissue of the four experimental groups are represented in Figure 12. PCa-induced rats presented decreased testis levels of VEGF, compared with respective controls both sedentary and exercised, but these differences did not reach statistical significance. Exercise training showed a tendency to decrease VEGF levels, that is more pronounced in control animals, but no significant differences were reached.



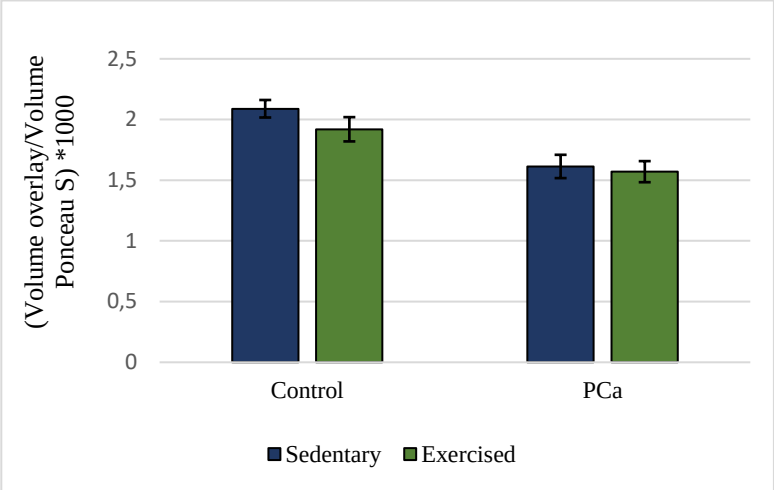
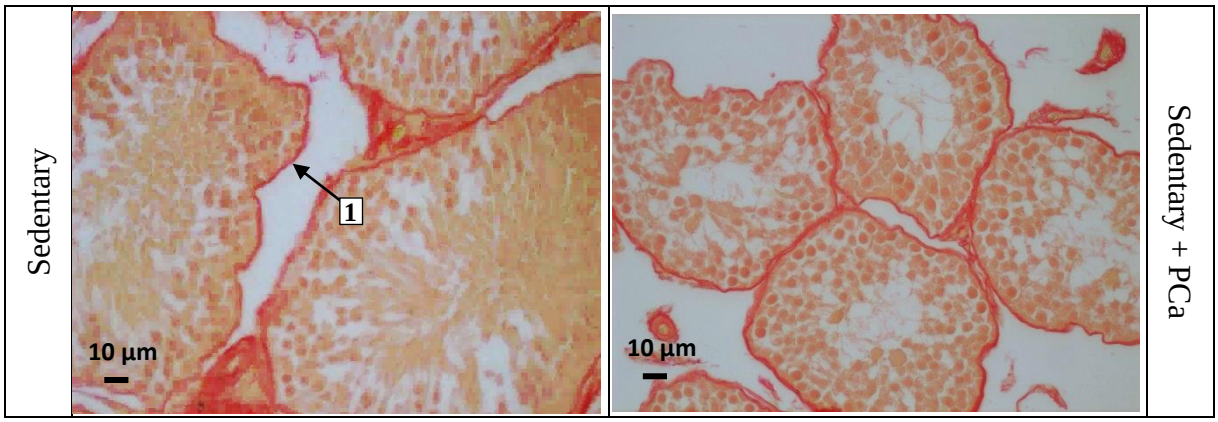


Figure 12: Effect of prostate cancer (PCa) and exercise training on the VEGF testis levels. The results were expressed as mean ± SEM. A representative immunoblot is shown above the graph.

Representative microscopic illustrations (40× amplification – sirius red staining) of the four experimental groups are presented in Figure 13. The basal lamina thickness (µm) of different groups are represented in Figure 14. PCa and exercise training did not alter the pattern of red staining of testis cross sections. Quantitatively, the basal lamina thickness was similar between groups. Nevertheless, PCa demonstrated a tendency to increase the basal lamina thickness, both in sedentary and exercised animals. Exercise training also seems to slightly increase the basal lamina thickness of both control and PCa-induced animals.



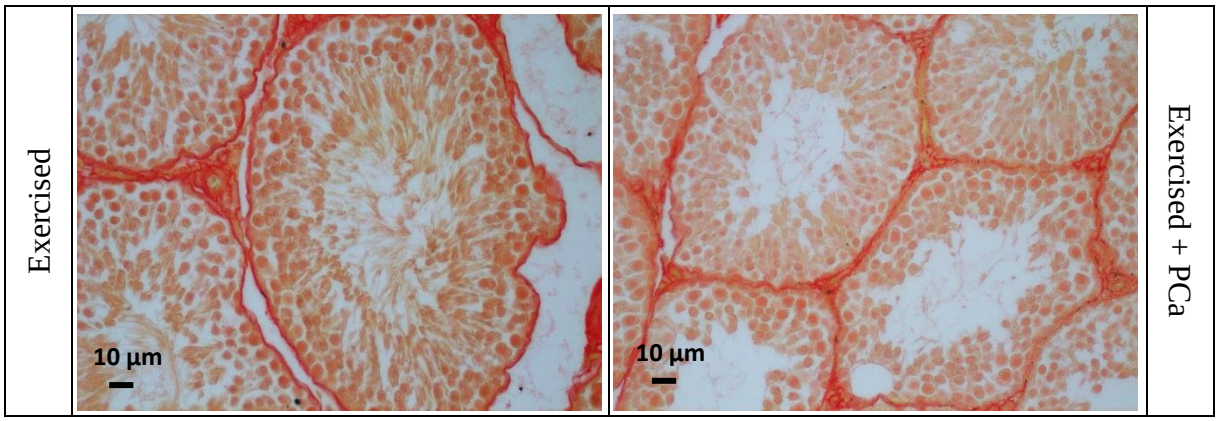


Figure 13: Representative illustrations of histological morphology of rat testes of four experimental groups. The testes cross sections were stained with sirius red. (40× amplification).
Legend: 1 – Basal lamina.

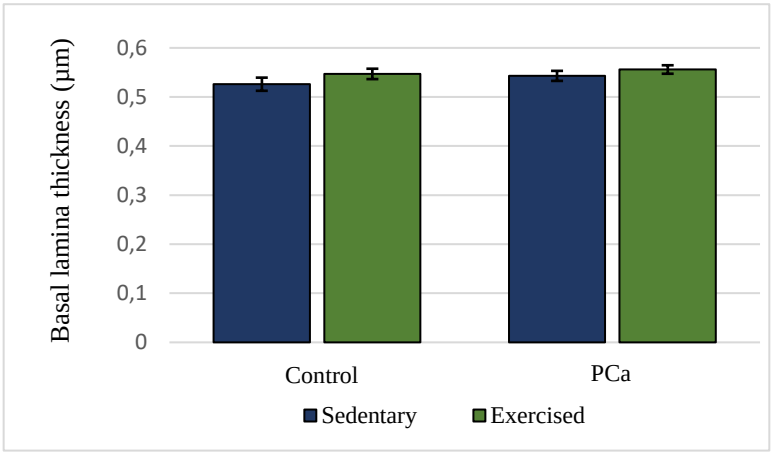


Figure 14: Effect of prostate cancer (PCa) and exercise training on the testis basal lamina thickness. The results were expressed as mean ± SEM and there isn't statistical significance between groups.

5. Discussion

During the last decades, PCa has emerged as one of the most worrying causes of death in men worldwide, making the treatment and prevention of this disease a major challenge for the scientific community[18]. Physical activity has been associated to numerous health-related benefits, being considered by many authors as a real polypill against diseases[136]. Indeed, it has become clear that physical activity plays a vital role in treatment and prevention of several diseases, including cancer[136–138]. There are growing evidences that some of the psychological and physiological challenges faced by cancer survivors can be prevented, attenuated or even treated with exercise training, which makes it associated with improved health and quality of life of cancer survivors[139]. Despite these apparent benefits, it becomes important research on the safety of this approach, including the analysis of the effect of exercise training in other tissues beyond the tissue where the cancer develops.

For the aforementioned reasons, it seems important to study the effect of exercise training in testicular tissue of PCa subjects, since testis is one of the most affected organs by this disease. Moreover, it seems also pertinent to add new insights on the effect of PCa in testicular tissue, since it is an underexplored topic. To the best of our knowledge, only a case-control study has focused on the effect of lifelong exercise training in PCa[100] and none have focused on the effect in testicular tissue of these subjects. We evaluated for the first time the impact of exercise training in testis of PCa-induced subjects. To do so, we used rat model because of the limitations inherent to the evaluation of exercise training behavior in human over the period necessary for prostate cancer development and to the ethical constrains[140]. Wistar Unilever rats were chosen for this study as these animals have the ability to produce adenocarcinomas that originates from a prostate lobe for which there is a human homologue[141]. A combination of hormonal stimulation – testosterone - with a carcinogen – MNU - was used to induce PCa. This protocol exhibits high rate of accessory sex glands tumor development and in Wistar Unilever rats the vast majority of cancers induced by this regimen arise in prostate gland, making this animal model suitable for the proposed study[141,142]. The type of exercise training is also a critical issue. In this study, treadmill training was chosen, because it allows easy control of exercise training intensity.

The exercise training protocol intensity was accurately defined based on maximal speed capacity, defined as 70%, which is considered moderate-intensity exercise training[143].

The results of animal's body weight were as expected, i.e. both PCa and exercise training were associated to weight loss (Table 4). Weight loss is a non-specific symptom that can be associated with several types of cancer. In PCa patients, decreased body weight is a common feature, mainly in advanced stages[144]. The presented results, although no significant, agree with this theory and with other authors that observed a decrease in animals body weight after MNU + testosterone PCa induction protocol[141,145]. In exercised rats, the decrease in body weight by PCa was less pronounced compared with sedentary ones, which may suggest that the disease did not progress to such advanced stages in these subjects. Exercise training also led to reduced body weight, which agree with other authors[132,143,146,147], and indicates that the treadmill training protocol applied was enough to cause an adaptive response in rat's body.

PCa caused decreased testes weight and testis/body weight ratios in both sedentary and exercised rats (Table 4), suggesting a negative effect of PCa in this organ[2]. Exercise training seemed to counteract this negative effect in PCa-induced rats, since, despite a tendency to slightly decrease testis weight, it exhibited a tendency to increase testis-body weight ratios. Instead, in control animals, exercise training was associated to a negative effect in testis, since it caused decrease testis weight and testis-body weight ratio (Table 4)[2].

In order to verify if the alterations in testis weight by PCa and exercise training are reflected in changes of testicular function, some testis function parameters were analyzed. Several authors have associated PCa to reduced testosterone levels, especially in more advanced stages[38,40,148]. Contrarily, in the present work, PCa subjects exhibited higher testosterone serum levels (Figure 5). Probably this increase did not reflect an increase in testosterone testicular production, but results of the testosterone injection and continuous release of testosterone by the implants, used to induce PCa, that masked a possible reduction of testosterone production[142]. Exercise training has been associated to alterations in reproductive hormonal profile in men, but the direction of these alterations is controversial. Some authors reported increase testosterone serum levels, along with increased LH and FSH levels, while others described reduced levels of both total testosterone, LH and FSH after moderate-intensity exercise training[111,112,114,115]. The present study seems to agree with an

improvement of testosterone production by exercise training, since a tendency to increase total testosterone serum levels after exercise was observed in both control and PCa-induced animals (Figure 5).

Low sperm count is a key factor of the male infertility and may be associated to several conditions, including aging, obesity and drug treatments[128,131,133]. PCa was also associated with decreased sperm concentration[44], which agree with the results of the present study, since a strong decrease of sperm concentration was observed in PCa-induced rats (Figure 6). Similar to what happens with testosterone serum levels, there is no consensus in literature regarding the effect of exercise training in sperm concentration. Indeed, while some authors observed increase sperm count after exercise training, others reported a decrese[113–115]. Nevertheless, it seems consensual that, in subjects with a condition that causes impairment of testicular function, exercise training may prevent or ameliorate these negative effects[126,132,133]. The effect of exercise training in sperm count of PCa subjects has never been evaluated and this work, contrarily to the expected did not observed a significant effect, despite the slightly tendency to increase sperm count. On the other hand, exercise training showed a trend to negatively affect spermatogenesis in control rats, since a decrease of sperm count was observed, but this alteration did not reach significance (Figure 6).

The number of abnormal sperm produced spontaneously, varies dramatically with species. In normal fertile human the number of normal sperm is around 4-12%, while in rats 95% or more normal sperm are common[134,149]. Probably this difference is associated to differences in lifestyle. Contrarily, in the present study, sedentary control rats exhibited 72% of normal sperm (Table 5). According to this result, a very recent work from Horst *et al*[150] established that, in Wistar rat strain, the normal sperm varied between 67 and 74%, by contrast with previous findings. The present study verified that PCa highly decrease the percentage of normal sperm, confirming a negative effect of this disease in spermatogenesis. In addition, PCa also increase the percentage of some abnormal sperm forms (DH, FH and PH), while decrease others (BN and TD) (Table 5). In most studies, sperm morphology is simply analyzed based on normal sperm percentage. While some authors reported either an increase or decrease, others did not observe any effect of exercise training in normal sperm percentage[112–115]. In the present work, while in control animals exercise training showed a trend to decrease normal sperm percentage, in PCa-induced animals, it seems to improve

sperm morphology, counteracting the decreased sperm count caused by PCa (Table 5). Regarding the effect of exercise training in sperm abnormal forms, it was observed a tendency to increase DH sperm (Table 5). Torma *et al*[116] associated exercise training to decreased levels of ODF-1. Since ODF-1 is important in the tight linkage of sperm head to tail[119], it is expected that its decreased levels increase the chance of sperm head-tail linkage dissociation, which corroborates the observed increase in DH sperm.

Given the changes observed in sperm concentration and morphology, it has become clear that PCa and probably exercise training affects spermatogenesis. To clarify how this process was affected the morphology of testis tissue was evaluated. The histology of the rat testis is similar to the human with the development of sperm cell precursors from the basal lamina to the lumen of seminiferous tubules. All the precursors are microscopically identic to the humans'. An exception is the spermatozoon, whose head in rat is sickle-shaped instead of its characteristic round head of human spermatozoon[2]. The histological results demonstrated that PCa decrease the size of seminiferous tubules (Figures 7 and 8). Reduction of seminiferous tubules dimensions may be responsible for the observed decrease in testis weight observed in PCa subjects, reflecting lumen contraction, that may be caused by loss of elongating spermatids or decreased production of testosterone[2]. Indeed, the results of the present work showed a post-meiotic disruption of spermatogenesis in PCa rats, with very few elongating spermatids and virtually none mature spermatozoa (Figure 9). Corroborating the post-meiotic arrest of spermatogenesis, PCa was no associated with alterations in Boule testis levels (Figure 11). Boule is essential for meiosis progression and reduced levels of Boule were associated to meiotic arrest[151–153]. Thus, the similar levels observed between control and PCa-induced rats suggested that meiosis steps occurred normally in these animals. Clinically, a post-meiotic disruption of spermatogenesis is considered less severe than pre-meiotic or meiotic disruptions, since some authors have already demonstrated a successful fertilization and embryo development after round spermatid injection[154,155]. The laced aspect of the center of seminiferous tubules observed in PCa rats (Figure 9), may be attributed to cell necrosis. In fact, other authors associated these appearance to necrotic cells' debris, which may indicate premature death of germ cells, specially elongating spermatids[156]. Exercise training exhibited a contradictory effect in control and PCa-induced rats. In PCa-induced rats, it demonstrated a tendency to counteract the negative effect of PCa in testis, increasing seminiferous tubules dimensions, which was accompanied by an

apparent improvement of sperm cells maturations, with more elongating spermatids and some sperm heads Contrarily, in control rats, exercise training seemed to decrease seminiferous tubules size but there is no an apparent effect in sperm cells maturation (Figures 7,8 and 9).

Histological results of this study also revealed a vascular effect of PCa in testicular tissue. PCa led to an increase in arterial thickness of testis accompanied by a decreased arterial lumen diameter (Figures 9 and 10), which may be responsible, at least in part, for the compromised testicular function observed in these subjects. Decreased arterial lumen diameter reduced testis blood flow and consequently oxygen supply, causing impairment of testis function[117]. Dominguez et al[117] associated exercise training to decreased health and function of testis and attributed this effect to the reduction of testicular PO_{2m}, and consequent vascular dysfunction. In contrast, in this study it was verified a tendency to increase arterial lumen diameter of testicular tissue after exercise training in both control and PCa-induced rats, that was more pronounced in PCa rats, indicating an improvement of testis vascular health by exercise training (Figure 10). To better understand what caused these vascular alterations in testis tissue, the VEGF testis levels were determined (Figure 12). PCa decreased VEGF, which suggested that the increase in arterial thickness of testis tissue were not caused by the proliferation of endothelial cells[157]. Alternatively, a possible explanation for the increased arterial thickness may be the proliferation of vascular smooth muscle cells and collagen deposition. The observed decrease levels of testis VEGF in PCa-induced animals agrees with the literature, since loss of VEGF in testis was associated to reduced, including reduced sperm count, as observed in the present study[158].

According to the literature, the rat testis collagen is a major constituent of the extracellular matrix, which composed the basal lamina ,that surround the seminiferous tubules[159]. Sirius red staining allows to determine the collagen content, by staining it in red[160]. Accordingly, in this work, it was observed a pattern of red staining surrounding the seminiferous tubules (Figure 13). Thus, it was possible to determine the basal lamina thickness and there were no differences in this parameter between groups (Figure 14). Fibrosis results of the excess deposition of connective tissue, typically caused by a chronic inflammatory response, and may be associated to organ failure[161]. In the present work, the pattern of red staining of testis tissue remains unchangeable between groups, indicating that neither PCa nor exercise

training were associated to testis fibrosis (Figure 13). This results also indicated that collagen deposition does not seem to contribute for the observed increase testis arterial thickness in PCa-induced animals.

The exogenous administration of testosterone used to induce PCa led to a great increase of testosterone serum levels in PCa rats, that may also contribute to the negative effect of PCa in testicular tissue observed in these subjects. The high levels of testosterone are responsible for a negative feedback effect in hypothalamus-pituitary axis and thus, by decreasing LH and FSH levels, contributes to decrease testis function[11]. These alterations constitute a strong limitation of this work, since, main of the alterations observed in testis of PCa subjects may be caused by the negative feedback mechanisms of testosterone, instead of by PCa itself. So, this confounding factor makes difficult the establishment of definitive conclusions.

6. Conclusions and future prospects

The main goal of this study was to evaluate the effect of PCa and exercise training in testis of Wistar Unilever male rats. To reach this goal, we successfully analyzed the morphology of testis tissue and associated it with some functional parameters. The integrated analysis of the results obtained in the present work led us to the following conclusions:

i) PCa significantly decreased testis weight, with reduction of seminiferous tubules dimensions. These alterations were reflected in reduced testis function with lower sperm count and percentage of normal sperm. The negative feedback in hypothalamus-pituitary axis caused by increased levels of serum testosterone by PCa induction protocol, along with the vascular alterations in testis tissue seems to contribute to these observed negative alterations in testis.

ii) Lifelong moderate-intensity exercise training did not significantly affect testicular tissue of PCa subjects. Nevertheless, it exhibited a tendency to improve PCa-induced impairment of testis, since it seems to slightly increase sperm count and normal sperm percentage. Despite exercise training seems to increase testosterone serum levels, it demonstrated a tendency to decrease the arterial thickness of testis tissue, which may, at least in part, explain the observed effect.

iii) Accordingly, lifelong moderate-intensity exercise training also did not significantly affect testicular tissue of control subjects. But, contrarily to its effect in PCa subjects, exercise training demonstrated a tendency to impair testis function with decreased sperm count and normal sperm percentage.

When analyzing the conclusions regarding exercise training, care should be taken since the type, intensity and duration of exercise training program greatly affects the results. Thus, it is not possible to generalize the conclusions of this study, and we only address the effect of treadmill training of the applied duration and intensity.

The opposite effect of exercise training in testis of control and PCa bearing animals suggested that other mechanisms may also contribute to the observed effect. Thus, future studies should, in addition to confirm the benefic effect of exercise training in testis of PCa subjects, elucidate the involved mechanisms. Particularly, the involvement of oxidative

stress and inflammation should be addressed since they are the main proposed candidates. Moreover, it will be also important characterize the signaling pathways modulated in testis by PCa.

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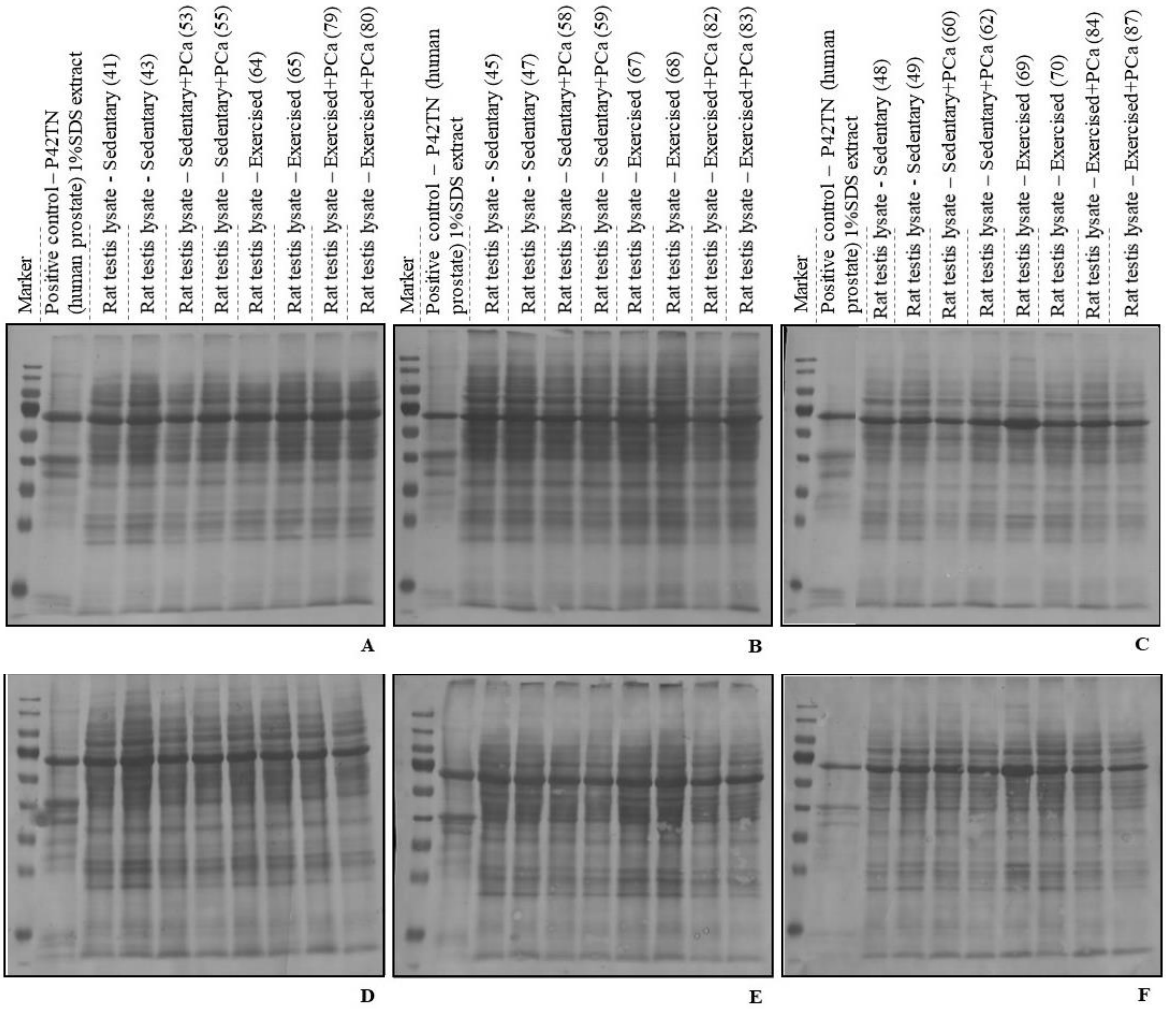
Supplementary data

Supplementary table 1 - Standards for BCA assay

Standards	BSA (µl)	1% SDS (µl)	Protein (µg)
P_0	-	25.0	0
P_1	0.5	24.5	1
P_2	1.0	24.0	2
P_3	2.5	22.5	5
P_4	5.0	20.0	10
P_5	10.0	15.0	20
P_6	20.0	5.0	40



Supplementary figure 1 – Ponceau staining used as the loading control in blot overlay. A, B and C are biological replicates. D, E and F are technical replicates of A, B and C, respectively.

Supplementary table 2 - Solutions for Western blot

Running gel 12% (2 gels, 1.5 mm thickness)	ddH_2O	6.360 mL
	Tris 1.5 M pH8.8	5.000 mL
	Acrylamide 40%	5.880 mL
	Bisacrylamide 2%	2.360 mL
	SDS 10%	0.200 mL
	APS 10%	0.100 mL
	TEMED	0.020 mL
Stacking gel 4% (2 gels, 1.5 mm thickness)	ddH_2O	4.736 mL
	Tris 0.5 M pH6.8	2.000 mL
	Acrylamide 40%	0.784 mL
	Bisacrylamide 2%	0.320 mL
	SDS 10%	0.080 mL
	APS 10%	0.040 mL
	TEMED	0.008 mL
Tris-HCl 1.5 M pH 8.8 buffer	For 1 L dissolve 181.5 g Tris in 800 mL deionized water. Adjust pH at 8.8 with HCl and make up to 1 L with deionized water.	
Tris-HCl 0.5 M pH 6.8 buffer	For 1 L dissolve 60 g Tris in 800 mL deionized water. Adjust pH at 6.8 with HCl and make up to 1 L with deionized water.	
10% APS (ammonium persulfate)	For 10 mL of deionized water add 1 g of APS.	
10% SDS (sodium dodecilsulfate)	For 500 mL of deionized water dissolve 50 g of SDS.	
4X Loading gel buffer	For 10 mL add 44 mL glycerol, 250 µL Tris-HCl 0.5 M pH 6.8 buffer, 0.8 g SDS, 0.2 mL β-mercaptoethanol and 3.3 mL deionized water. Add bromophenol blue (a small amount). Keep it at RT for short periods or at 4ºC for longer periods.	
Tris-Gly 10X Stock	For 1 L dissolve 30.30 g Tris (250 mM) and 144.10 g Gly (1.92 M) in 1 L of deionized water.	
Running buffer 1X	For 1 L add 800 mL deionized water, 100 mL Tris-Gly 10X and 10 mL 10%SDS. Make up to 1 L with deionized water.	
Transfer buffer 1X	For 1 L add 100 mL Tris-Gly 10X to 700 mL of deionized water and 200 mL methanol.	

10X TBS Stock (Tris buffered saline)	For 0.5 L dissolve 6.055g Tris in deionized water and adjust pH at 8.0. Add 43.8325 g NaCl and make up to 500 mL with deionized water.
1X TBST (TBS + Tween 20)	For 1 L add 100 mL TBS 10X and 500 µL Tween-20 to 900 mL of deionized water.
5% BSA in TBST 1X	For 100 mL of solution dissolve 5 g of BSA in TBST 1X.
5% non-fat milk in TBST 1X	For 100 mL of solution dissolve 5 g of non-fat milk in TBST 1X.