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Validation of the Rat Model of Prostate Cancer: Correlating Seminal Vesicle Lesions With Dorsolateral Prostate Lesions

ELISABETE NASCIMENTO-GONÇALVES[1,2], FERNANDA SEIXAS[3,4], BRUNO MIGUEL MAIO OLIVEIRA[3], JOÃO EDUARDO TEIGA-TEIXEIRA[3], ANA I. FAUSTINO-ROCHA[1,5,6], BRUNO COLAÇO[3,7], RITA FERREIRA[2] and PAULA A. OLIVEIRA[1,4]

[1]*Centre for the Research and Technology of Agro-Environmental and Biological Sciences (CITAB), Inov4Agro, University of Trás-os-Montes and Alto Douro, Vila Real, Portugal;*
[2]*LAQV-REQUIMTE, Department of Chemistry, University of Aveiro, Aveiro, Portugal;*
[3]*Animal and Veterinary Research Centre (CECAV), Associate Laboratory for Animal and Veterinary Science – AL4AnimalS, University of Trás-os-Montes and Alto Douro, Vila Real, Portugal;*
[4]*Department of Veterinary Sciences, University of Trás-os-Montes and Alto Douro, Vila Real, Portugal;*
[5]*Department of Zootechnics, School of Sciences and Technology, University of Évora, Évora, Portugal;*
[6]*Comprehensive Health Research Centre, Évora, Portugal;*
[7]*Department of Zootechnics, University of Trás-os-Montes and Alto Douro, Vila Real, Portugal*

Abstract. *Background/Aim: Lesions in the seminal vesicle are described in the most used protocols for prostate cancer (PCa) induction. This study aimed to characterize the lesions of seminal vesicles associated with a protocol of PCa induction in rats to contribute to better characterization of this model. Materials and Methods: Forty-five male Wistar Unilever rats were randomly divided into two control groups: CONT1 (n=10) and CONT2 (n=10); and two PCa-induced groups: IND1 (n=10) and IND2 (n=15), sacrificed at 35 and 61 weeks, respectively. Animals from the induced groups were exposed to a multistep protocol for PCa induction. Animals, seminal vesicles and dorsolateral prostate were weighed. Seminal vesicles and dorsolateral prostate were submitted to histopathological and immunohistochemical analysis. Results: Animals in which PCa was induced had a lower mean body weight when compared with the control animals ($p<0.05$). The relative mean seminal vesicle weight was higher in groups with PCa when compared with control groups ($p<0.05$). Although the differences were not statistically significant, animals from the IND2 group developed more lesions than animals from the IND1 and CONT2 groups. It is worth noting that the animals from group IND2 developed papillary adenomas and carcinomas in situ, which were not observed in any other group. Similar to observations in seminal vesicles, animals from group IND2 developed more dorsolateral prostate lesions than animals from the IND1 group ($p<0.05$). Conclusion: We observed that the longer the exposure to testosterone was, the greater was the incidence of preneoplastic and neoplastic lesions in both the seminal vesicle and the prostate, suggesting that testosterone exposure affects the spectrum of developed lesions.*

Correspondence to: Paula A. Oliveira, CITAB, University of Trás-os-Montes and Alto Douro, Quinta dos Prados, 5001-801 Vila Real, Portugal. E-mail: pamo@utad.pt

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Seminal vesicles are accessory sex glands of the male reproductive system, responsible for the production of seminal fluid, which represents about 60-80% of the semen volume. In Man, the seminal vesicles are a pair of contorted tubes located in the pelvis, anterior to the rectum, inferior to the fundus of the urinary bladder and posterior to the prostate gland (1). The seminal vesicles are composed of an inner mucosal layer with pseudostratified columnar epithelium, a thin layer of loose connective tissue and a muscular layer with an inner circular and an outer longitudinal smooth muscle, whose contraction drives the secretion into the ejaculatory ducts. Externally, there is an adventitial layer composed of loose areolar tissue (2, 3). Primary adenocarcinoma of seminal vesicles are rare but neoplastic lesions spreading from adjacent organs such as the prostate, urinary bladder and rectum are commonly observed (4, 5). Seminal vesicles play a key role in sperm synthesis, consequently diseases affecting this gland result in male infertility. Therefore, it is important to have an available animal model that mimics the alterations of this gland.

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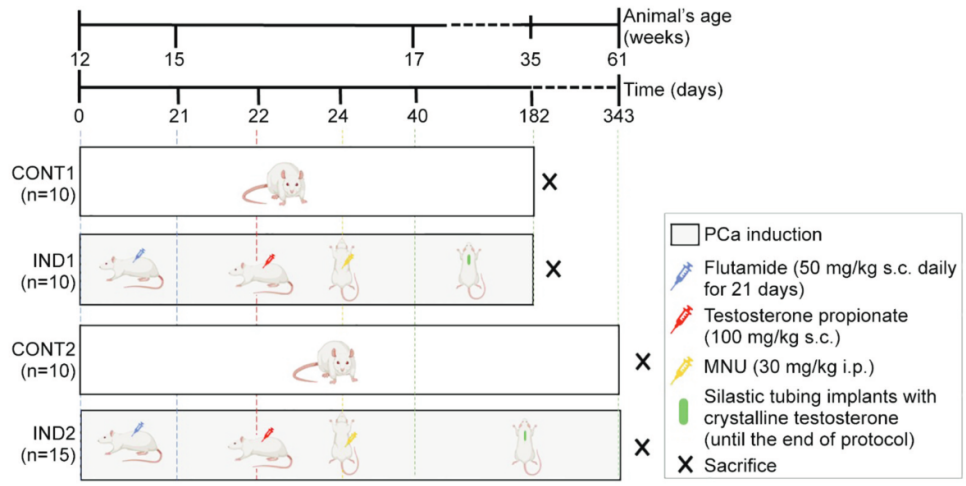

Figure 1. *Experimental protocol for prostate cancer induction and follow-up of prostate and seminal vesicle lesions.*

In rodents, seminal vesicles are bilateral glands located dorsolaterally to the urinary bladder, curled, with caudally directed anterior tips and attached to the anterior prostate (6). The secretions of the seminal vesicles and coagulating gland clot after ejaculation in the genital tract of female rat forming a copulatory plug that is critical in transcervical sperm transport (7). This saccular-like organ has a muscular wall surrounding a large central lumen and a complex infolded mucosa coated by columnar cell, forming irregular anastomosing channels that communicate in a central cavity (8). Adenocarcinomas of the seminal vesicles are rare spontaneous tumors in most strains of rats and mice (9, 10). However, seminal vesicle lesions, mainly adenocarcinomas, are frequently found in animal models of chemically and hormonally induced prostate cancer (PCa) (11, 12). Despite this, the development of PCa lesions have rarely been correlated with seminal vesicle lesions. To fill this gap, the present study aimed to characterize the lesions of seminal vesicles associated with a protocol of PCa induction in rats through the administration of hormones and chemical carcinogens to contribute to better characterization of this model.

Materials and Methods

Animals. All procedures were performed according to the European Directive 2010/63/EU and National Decree-Law No. 113/2013 on the protection of animals used for scientific purposes and approved by the Portuguese competent authority (Direcção Geral Alimentação e Veterinária, Approval no. 021326). Forty-five male Wistar Unilever rats (*Rattus norvegicus*) of 4 weeks of age were obtained from Charles River Laboratories (Écully, France). The animals were maintained under controlled conditions of temperature (23±2˚C), humidity (50±10%), and light:dark cycle (12-h:12-h under an air system filtration (10-20 ventilations/hour). They had *ad libitum* access to water and a standard laboratory diet (Mucedola 4RF21®, Milan, Italy).

Experimental design. After a quarantine and a period of acclimatization to the laboratory conditions, at 12 weeks of age, the animals were randomly divided into four groups (Figure 1): Two control groups, CONT1 (n=10) and CONT2 (n=10); and two induced PCa groups, IND1 (n=10) and IND2 (n=15).

A multistep protocol was carried out in animals of the IND1 and IND2 groups to induce PCa development. Firstly, the rats received a subcutaneous injection of flutamide (50 mg/kg; TCI Chemicals, Portland, OR, USA) for 21 consecutive days. One day after the last flutamide administration, testosterone propionate (TCI Chemicals) was dissolved in corn oil and subcutaneously administered (100 mg/kg). Two days later, these rats were intraperitoneally injected with the carcinogen *N*-methyl-*N*-nitrosourea (Isopac®; Sigma Chemical Co., Madrid, Spain), at a dose of 30 mg/kg. Two weeks later, silastic tubes were filled with crystalline testosterone (Sigma Chemical Co.) and subcutaneously implanted in the interscapular region of the animals, which were previously anesthetized with ketamine (75 mg/kg, Imalgene® 1000; Merial S.A.S., Lyon, France) and xylazine (10 mg/kg, Rompun® 2%; Bayer Healthcare S.A., Kiel, Germany). The implants remained until the end of the experimental protocol. Animals from the CONT1 and IND1 groups were euthanized at 35 weeks of age, while the animals from the CONT2 and IND2 groups were sacrificed later at 61 weeks of age. All animals were sacrificed by an intraperitoneal administration of ketamine (75 mg/kg, Imalgene® 1000; Merial S.A.S.) and xylazine (10 mg/kg, Rompun® 2%; Bayer Healthcare S.A.), followed by exsanguination by cardiac puncture. At sacrifice, animals' body weights were measured and seminal vesicles were collected and weighed separately from prostatic lobes; the dorsal and lateral prostate lobes surrounding prostatic urethra were weighed as a block (KERN® PLT 6200-2A scale; Dias de Sousa S.A., Alcochete, Portugal). After this, the glands were immersed in 10% buffered formalin for at least 24 hours.

Histological analysis. After fixation, seminal vesicles and prostates were processed for routine histological evaluation. Paraffin sections of 3-μm thickness were stained with hematoxylin and eosin and observed under a light microscopy by a pathologist. The lesions of the seminal vesicles were histologically classified according to

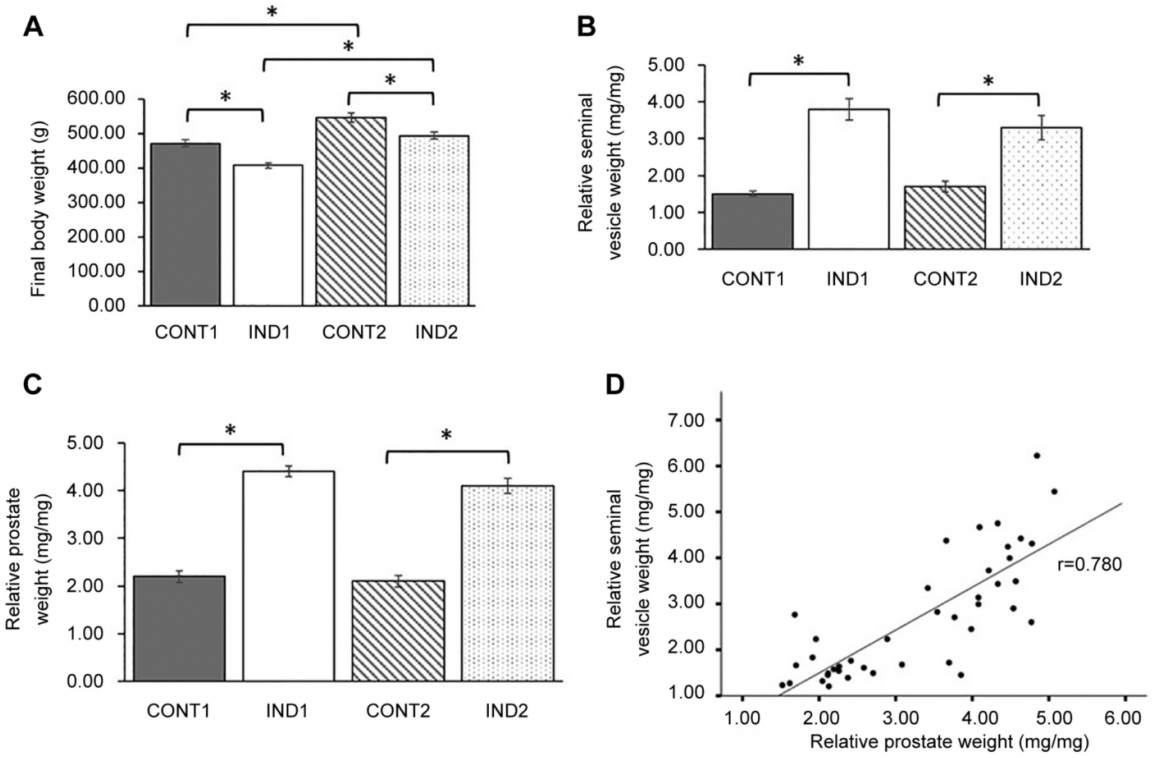

Figure 2. *Body weight and relative organ weight of experimental prostate cancer (IND1/2) and control (CONT1/2) groups. Final body weight (A), and relative seminal vesicle (B) and prostate (C) weights in all experimental groups. D: Correlation between seminal vesicle relative weight and relative prostate weight. The correlation was statistically significant ($p<0.0001$). Data are presented as the mean±standard error. *Significantly different at $p<0.05$.*

Suwa *et al*. (8) and those of the dorsolateral prostate were classified according to Bosland (13).

Immunohistochemical analysis. The immunohistochemical detection of Ki-67 was performed using the standard protocol of the NovoLink Polymer Detection System® (Leica Biosystems, Newcastle upon Tyne, UK). Sections were incubated overnight at 4°C with primary antibody for Ki-67 (clone MIB-5; Dako, Glostrup, Denmark) at a dilution of 1:50. Ki-67 immunoexpression (proliferation index) was evaluated as the percentage of stained cells out of the total number of cells counted in three randomly selected, high-power (400×) fields in normal and hyperplastic seminal vesicles and prostates.

Statistical analysis. Statistical analysis was performed using the SPSS program (version 25; IBM, Armonk, NY, USA). Statistical differences among groups were assessed by one-way analysis of variance with Bonferroni correction for multiple comparisons. Histological results were analyzed using chi-square test. All data are presented as the mean±standard error. For each animal, the relative seminal vesicle weight was calculated by dividing the tissue weight (g) by the final body weight (g). Pearson correlation test was used to evaluate the association between relative seminal vesicle and prostate weights. Spearman correlation test was used to evaluate the association between the number of seminal vesicles and prostatic lesions. Differences with *p*-values lower than 0.05 were considered statistically significant.

Results

General findings. One animal from the CONT1 group was sacrificed before the experiment due to a skin lesion in the facial region incompatible with animal welfare and survival. One animal from the IND2 group died suddenly at 56 weeks of age without exhibiting signs of discomfort or disease. Data from both animals were excluded from the study. The mean final body weight of animals with PCa (IND1 and IND2) was significantly lower when compared to the age-matched controls. Moreover, the mean body weight of the animals sacrificed at 35 weeks of age (CONT1 and IND1) was significantly lower when compared with those sacrificed at 61 weeks of age (CONT2 and IND2), for both control and induced groups (Figure 2A; $p<0.05$).

The mean relative seminal vesicle and prostate weights were significantly higher in PCa-induced groups when compared with their respective control groups (Figure 2B and C; $p<0.05$). Relative seminal vesicle and prostate weights were significantly positively corelated (Figure 2D; r=0.780 and $p<0.0001$).

Histological analysis. The incidence of lesions in seminal vesicle and dorsolateral prostate at both times of sacrifice are

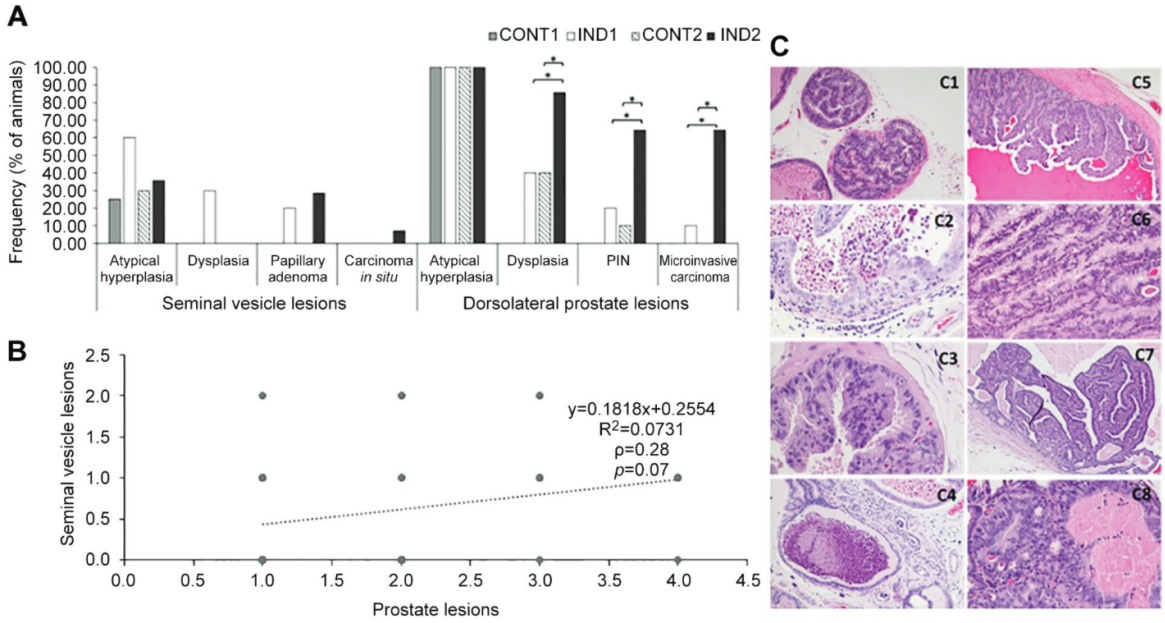

Figure 3. *Histopathological analysis of the seminal vesicle and dorsolateral prostate lobe in experimental prostate cancer (IND1/2) and control (CONT1/2) groups. A: Incidence of lesions in seminal vesicle and dorsolateral prostate lobe. The percentage represents the number of animals with lesions per total number of animals in the group. B: Correlation between the number of lesions in seminal vesicles and dorsolateral prostate lobe. C: Histological images of the lesions in dorsolateral prostate (C1-C4) and seminal vesicle (C5-C8). Hyperplasia in CONT1 (C1 and C5); dysplasia in IND1 (C2 and C6); prostatic intraepithelial neoplasia (PIN) (C3) and microinvasive carcinoma (C4) in CONT2; papillary adenoma (C7) and carcinoma in situ (C8) in IND2. Haematoxylin and eosin staining. Magnification of ×400 in all images. *Significantly different at $p<0.05$.*

shown in Figure 3A. The lesions observed in the seminal vesicle were classified as atypical hyperplasia, dysplasia, papillary adenoma, and carcinoma *in situ*. Invasive carcinoma was not observed in any group. Atypical hyperplasia was observed in all groups, dysplasia was only observed in the IND1 group, papillary adenomas were observed in both PCa groups, and carcinoma *in situ* was only observed in the IND2 group. The IND2 group presented more papillary adenomas and carcinomas *in situ* than the IND1 group (28.6% *versus* 20.0% and 7.14% *versus* 0.00%, respectively). Despite this, the number of lesions did not significantly after between groups ($p>0.05$).

Dorsolateral prostate lesions were classified as atypical hyperplasia, dysplasia, prostatic intraepithelial neoplasia (PIN) and microinvasive carcinoma (Figure 3A). Similar to that observed in seminal vesicle, atypical hyperplasia was observed in all groups. Dysplasia and PIN were observed in both PCa groups and the CONT2 group, while microinvasive carcinoma was only observed in both PCa groups. In the IND1 group, 40% of animals developed dysplasia, 20% PIN and 10% invasive PCa. The IND2 group developed more preneoplastic and neoplastic lesions of the dorsolateral prostate, with 85.7% exhibiting dysplasia, 64.3% PIN and 64.3% invasive carcinoma ($p<0.05$).

Although not statistically significant, a positive correlation was observed between the number of seminal vesicle and prostatic lesions (Figure 3B, $\varrho=0.28$, $p=0.07$). Some examples of the lesions observed in the seminal vesicles and dorsolateral prostate lobes are shown in Figure 3C.

Immunohistochemistry. Nuclear immunolabelling for Ki-67 was observed in both prostate and seminal vesicles. In hyperplastic seminal vesicles, the proliferation index was higher in the CONT2 group when compared with IND2 group ($p<0.05$). PCa-Induced animals sacrificed at 61 weeks of age (IND2) had a lower proliferation index in normal and hyperplastic seminal vesicles than those sacrificed at 35 weeks of age (IND1). Despite this, statistically significant differences were not found ($p>0.05$) (Table I and Figure 4).

In prostate glands, the proliferation index was lower in the CONT1 group than in IND1 ($p<0.05$) in normal and hyperplastic tissue (Table I and Figure 4). The same pattern was observed in groups sacrificed at 61 weeks of age (CONT2 and IND2), but without reaching statistically significance ($p>0.05$). Moreover, the IND1 group had a higher proliferation index in hyperplastic prostatic tissue than the IND2 group ($p<0.05$) (Table I and Figure 4).

Table I. *Immunohistochemical evaluation of the proliferation index in tissue from two control groups (CONT1 and CONT2) and two groups in which prostate cancer was induced (IND1 and IND2).*

			Sacrificed at 35 weeks		Sacrificed at 61 weeks	
			CONT1 (n=4)	IND1 (n=5)	CONT2 (n=5)	IND2 (n=5)
Proliferation index (%)	Seminal vesicle	Normal tissue	10.40±0.09	13.16±0.06	10.29±0.07	4.41±0.01
		Hyperplasia	14.50±0.01	16.56±0.02	23.94±0.03	9.48±0.05*
	Prostate	Normal tissue	2.16±0.00	6.69±0.01*	3.14±0.01	4.64±0.01
		Hyperplasia	4.41±0.01	22.66±0.01*#‡	6.96±0.01	12.71±0.03#

Statistically significantly different at $p<0.05$ from: *corresponding control, ‡IND2, and #normal tissue.

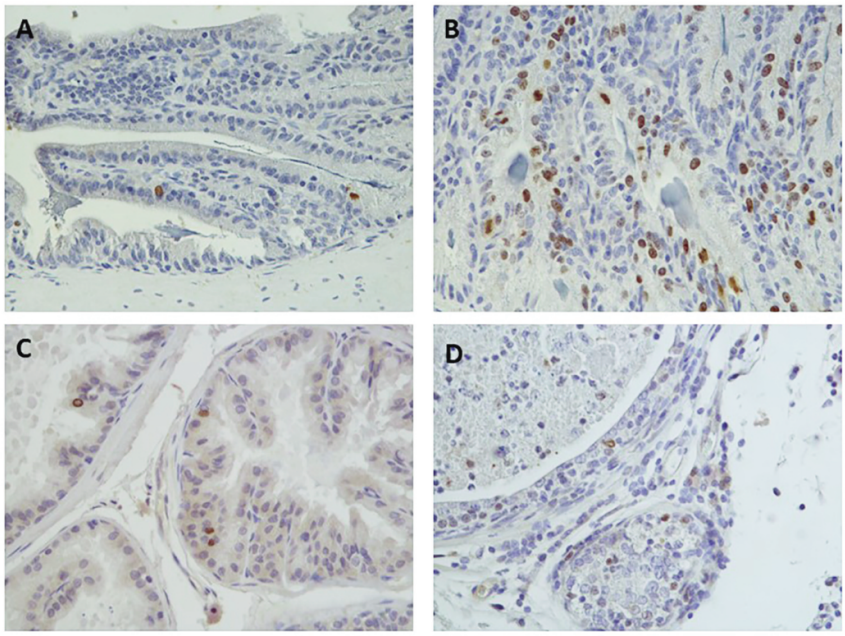

Figure 4. *Immunohistochemical staining for Ki-67. A: Normal seminal vesicle. B: Hyperplastic seminal vesicle. C: Normal prostate tissue. D: Hyperplastic prostate. Magnification of ×400 in all images.*

Discussion

This research aimed to characterize and correlate lesions of the seminal vesicles and prostate identified in a rat model of chemically and hormonally induced PCa. The protocol carried out in this study to induce PCa development was based on that previously published by Bosland (11, 14). This multistep protocol induces lesions in the dorsolateral prostate lobe of the rat, which is considered homologous to the peripheral zone of the prostate in humans, where most carcinomas develop (15). Associated with this protocol, the seminal vesicles also develop preneoplastic and neoplastic lesions, such as atypical hyperplasia and adenocarcinoma (11, 12). The present article is innovative by presenting specific results of seminal vesicle lessons, correlating them with prostate lesions, and characterizing their proliferation index.

PC-induced groups (IND1 and IND2) presented higher mean relative seminal vesicle and prostate weights than their respective control groups, which can be related to the PCa induction protocol that induced cell proliferation and hyperplasia. Kumar *et al*. used a multistep protocol to induce PCa by administering testosterone for three days, and a single dose of *N*-methyl-*N*-nitrosourea, followed by intraperitoneal injection of testosterone for 60 days (16). Their results showed that seminal vesicle weight was significantly increased in PCa-induced rats rather than controls, which our results are in accordance with. However, Kumar and co-workers did not address the

correlation between mean relative seminal vesicle and prostate weights, as was performed by our team. In our study, we found a significant correlation between relative seminal vesicle and prostate weights, indicating that as the relative prostate weight increases, so does the seminal vesicle weight. The protocol implemented for PCa induction affects not only the prostate, but also clearly interferes with seminal vesicle characteristics.

In this work, the histological lesions observed in the seminal vesicles were classified as atypical hyperplasia, dysplasia, papillary adenoma, and carcinoma *in situ*. Atypical hyperplasia of the seminal vesicles is characterized by an increase in the number of glandular epithelial cells, without distention of the acini or compression of the surrounding tissues (9, 17, 18). In dysplasia there is a disturbance of normal glandular architecture but without acinar lumen obliteration (9). Papillary adenoma is histologically characterized by papillary architecture (8, 9). In carcinoma *in situ* there is a disruption of the tissue architecture, with atypical cells but without spread to surrounding tissue (9). As this spectrum of lesions is similar to that identified in Man, we can state that this model has an added value for the study of the seminal vesicle and translation of results to humans.

The animals from the IND2 group developed more seminal vesicle lesions than did the IND1 group and the respective control group (CONT2). The animals from the IND2 group developed papillary adenomas and carcinomas *in situ* in the seminal vesicles, which were not observed in any other group. Based on our results, there is a correlation between the development of lesions in the prostate and in the seminal vesicles. Similarly, there was a higher incidence of dorsolateral lesions in animals with PCa sacrificed at 61 weeks (IND2) than those with PCa sacrificed at 35 weeks (IND1). This can be explained by a longer exposure to testosterone *via* slow-release implants in the IND2 group. The higher incidence of lesions in the prostate was accompanied by an increase of the incidence of lesions in the seminal vesicles The control group sacrificed at 61 weeks (CONT2) also had a higher incidence of prostate dysplasia and PIN than the control group sacrificed at 35 weeks (CONT1). These lesions in the control group may be explained by animals' age at sacrifice. The relationship between age in humans and rats is not linear (19), but we consider that animals at 61 weeks of age are old and, like mild-aged men, are more susceptible to alterations in the prostate and the development of lesions. Although there are few reported cases of lesions and cancer of seminal vesicles in men, the reported cases are mostly described in older adults (20-22). Therefore, we can say that in the rat, age matters for the development of lesions not only in the prostate, but also in seminal vesicles, as described for Man.

Ki-67 is a nuclear protein associated with cell proliferation and is a good marker for cancer cell proliferation (23). Ki-67 is highly expressed in malignant PCa lesions when compared with benign lesions, and is considered a good prognostic factor (24, 25). As far as we are aware, in literature there are few Ki-67 immunoreactivity studies in seminal vesicles primary tumors in rat (26) and men (27, 28). However, in these articles, there does seem to be a relationship between the Ki-67 index and tumor aggressiveness. Looking at our results, we observed that an increase in histological grade of seminal vesicle and prostate lesions was accompanied by an increase in their proliferation rate, hyperplasic lesions had a higher proliferation index than normal tissue whether located in a seminal vesicle or in the prostate.

Conclusion

We observed that the longer the exposure to testosterone, the greater was the incidence of preneoplastic and neoplastic lesions in both seminal vesicles and the prostate, suggesting that testosterone exposure affects the spectrum of lesions developed. This is in accordance with the slow, stepwise, and cumulative effect of a carcinogenic agent on the initiated cell leading to neoplastic transformation. This model for PCa induction can be simultaneously used to study seminal vesicle lesions and, if well planned, can help to reduce the number of animals used in cancer experiments. We can also conclude that age indeed matters, not only in Man but also in the rat, because older rats had more severe lesions than those sacrificed at a younger age.

Conflicts of Interest

All Authors declare no actual, potential, or perceived conflicts of interest that would prejudice the impartiality of the study.

Authors' Contributions

ENG conducted experiments with live animals and wrote the article; FS performed the histopathological evaluation, the immunohistochemical analysis and wrote the article; BMMO and JET performed the immunohistochemical analysis and wrote the article; AIFR conducted the experiments with live animals and wrote the article; BC participated in animals sacrifice and samples processing and wrote the article; RF performed the experimental design, participate in animals sacrifice and wrote the article; PAO conceived the experimental design, supervised the animal experiments, participated in animal sacrifice and wrote the article.

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