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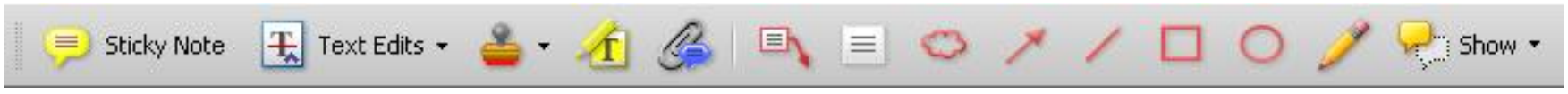

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1. Replacement Text Tool — For replacing text.

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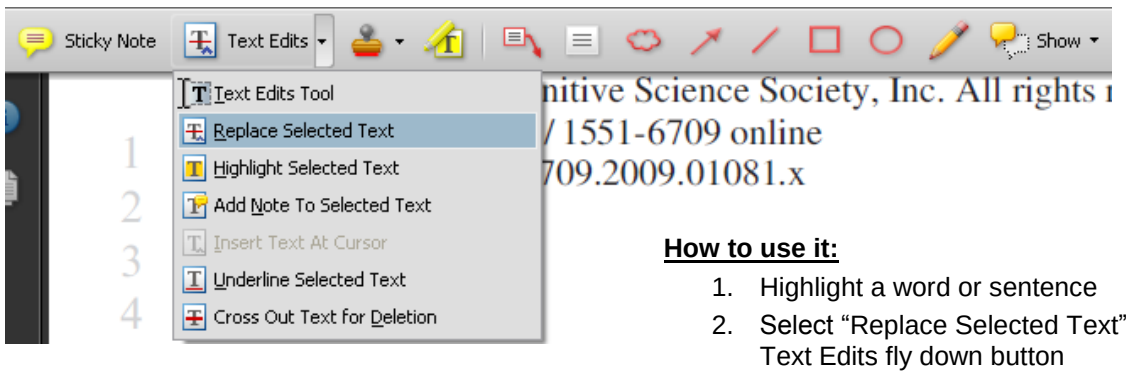

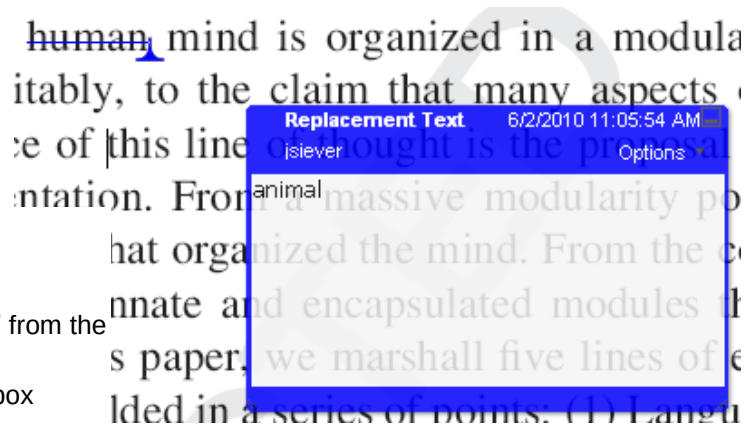

How to use it:

1. Highlight a word or sentence
2. Select “Replace Selected Text” from the Text Edits fly down button
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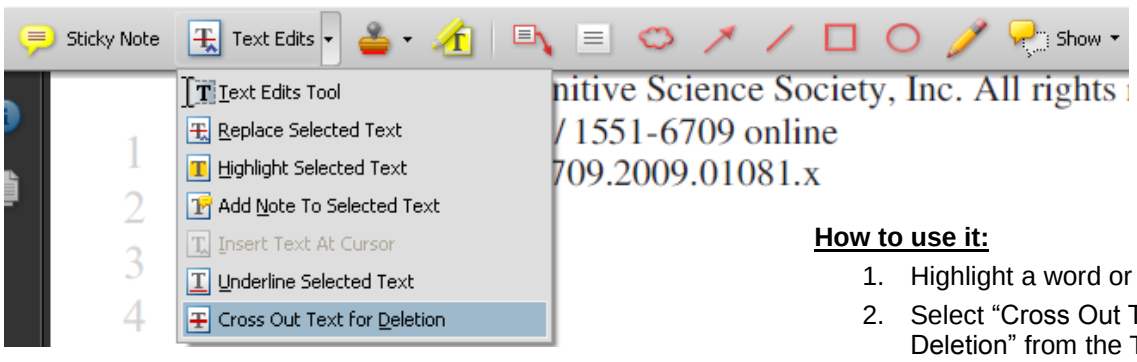

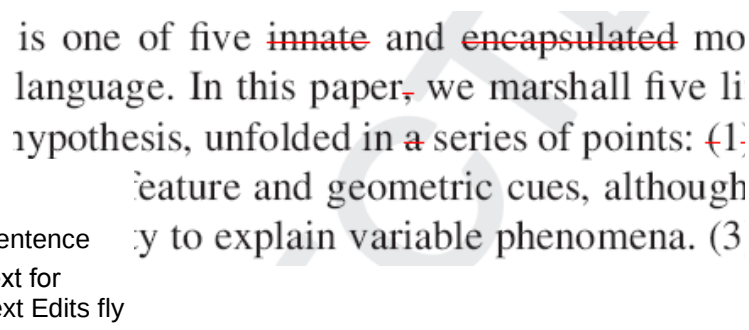

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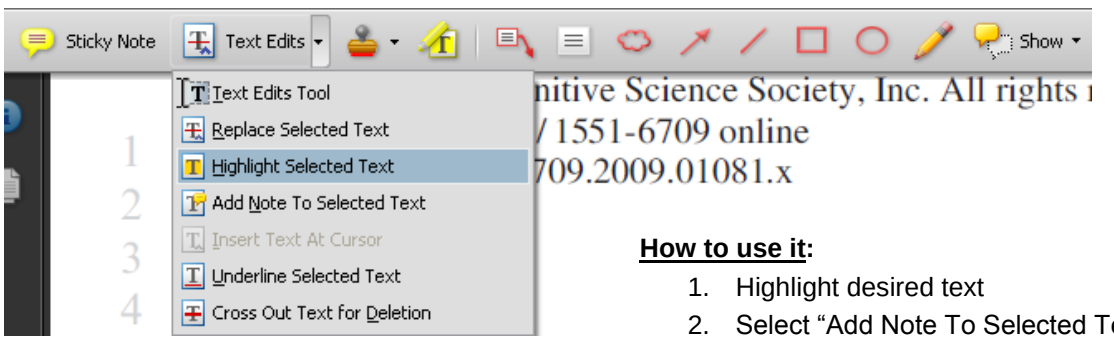

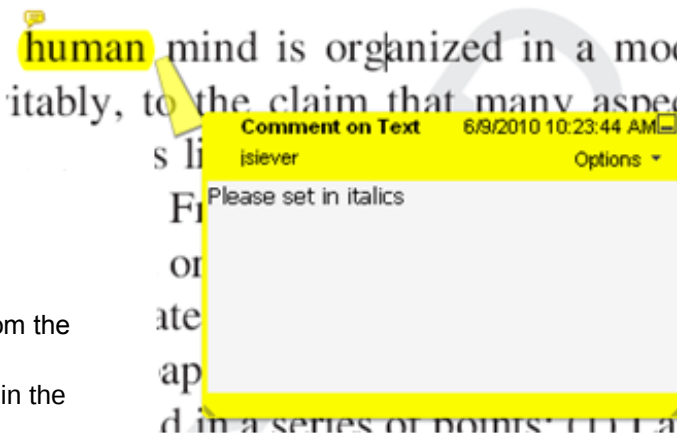

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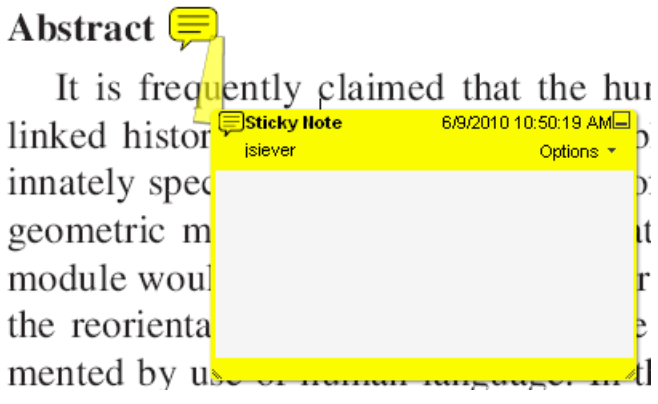

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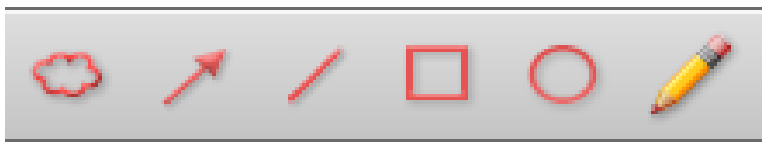

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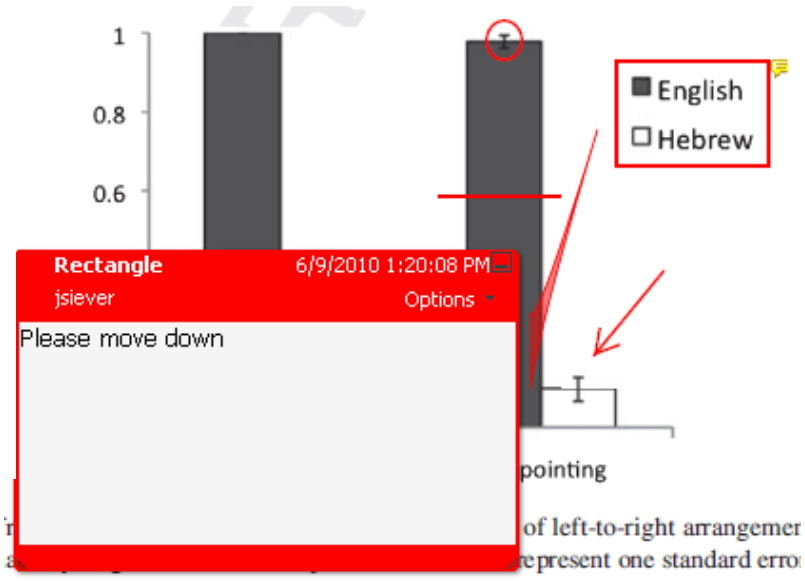

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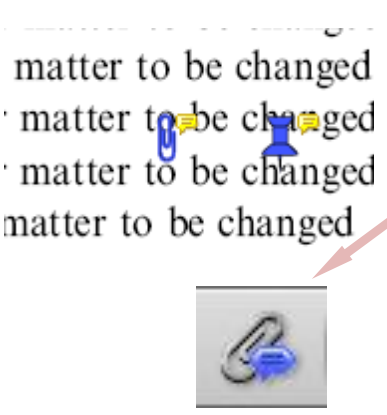

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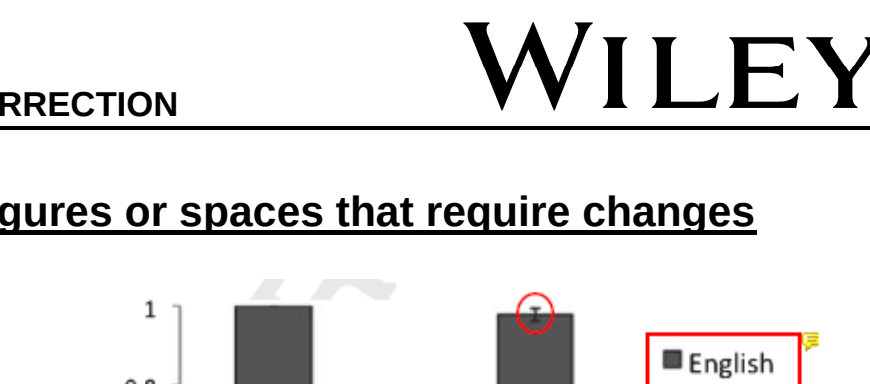

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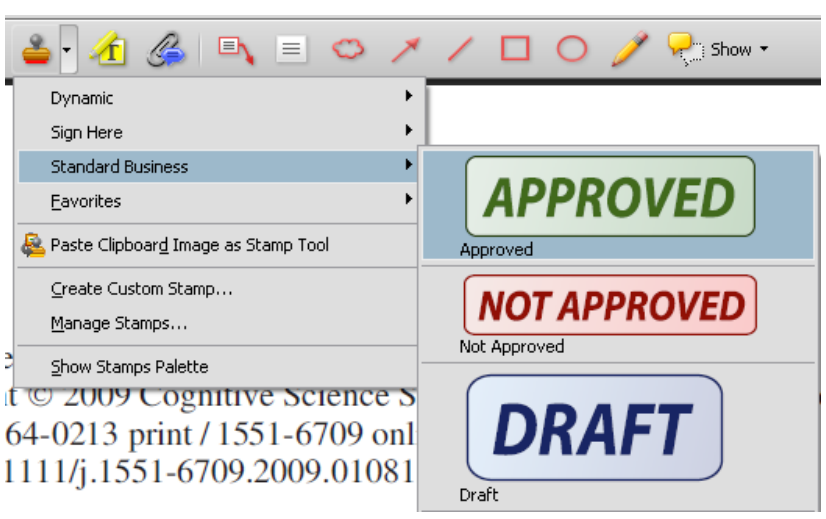

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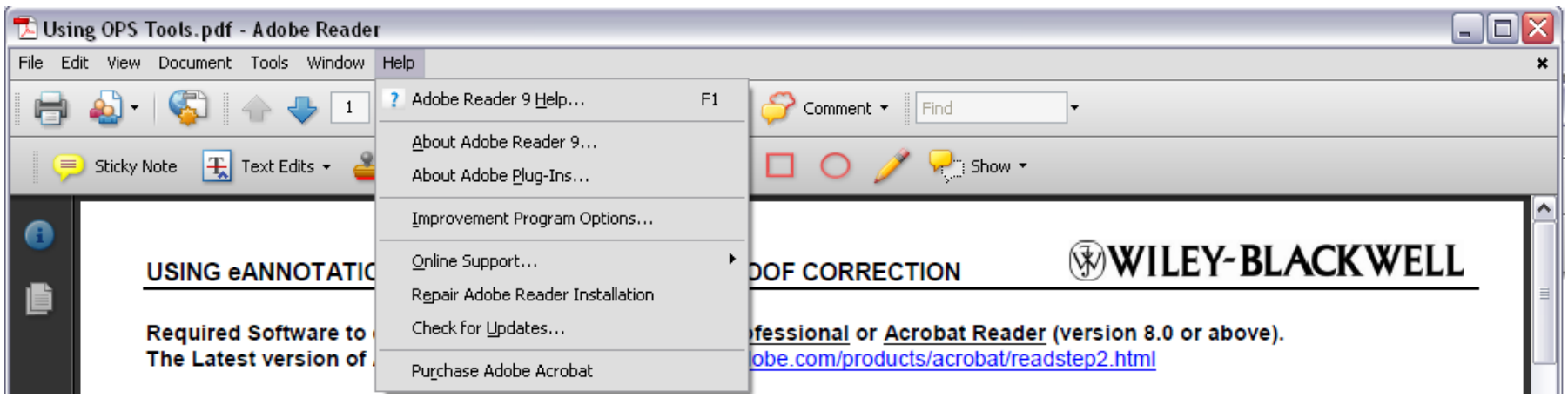

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RESEARCH ARTICLE

Steroidal hormone and morphological responses in the prostate anterior lobe in different cancer grades after Celecoxib and Goniothalamin treatments in TRAMP mice

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Abstract

Prostate cancer is the second most diagnosed cancer in the world, and alternative methods to prevent and treat different lesion grades need to be evaluated. The objective was to evaluate the morphological, hormonal, and inflammatory responses in the prostate anterior lobe in transgenic adenocarcinoma of the mouse prostate (TRAMP), following Celecoxib and Goniothalamin (GTN) treatments. All animals were treated for 4 weeks, from 8 weeks of age and euthanized either immediately after treatment (12-week-old mice: immediate response) or later (22-week-old mice: late response). The results showed a significant increase of high-grade prostatic intraepithelial neoplasia (HGPIN) and well-differentiated adenocarcinoma (WDA), according to the age in the control groups. Celecoxib treatment decreased the WDA incidence in the late response group. GTN led to a significant healthy tissue increase, and an LGPIN and HGPIN decrease in the immediate response group. In the late response group, GTN led to healthy area increase and there was no occurrence of WDA. AR and ERα immunoexpressions were reduced by both treatments in the immediate response groups. However, only GTN was able to decrease the ERα level in the late response group. Regarding COX-2 immunoreactivity, both treatments reduced the frequency of this enzyme. We can conclude that the prostate anterior lobe is a good model to study prostate cancer, considering its slow progression. Both treatments led to cancer delay in the prostate anterior lobe. However, GTN pointed towards a better treatment spectrum in the signaling pathways in the prostate microenvironment, particularly in ERα.

Keywords: anterior prostate; anti-inflammatory; cancer; Celecoxib; Goniothalamin; hormones

Introduction

It is known that different types of cancer are responsible for the great majority of deaths worldwide. In addition, it is expected that cancer incidence will increase rapidly according to population growth and life style habits such as smoking, overweight, among others (Torre et al., 2016).

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Abbreviations: AR, Androgen Receptor; CCL-3, Chemokine (C-C motif) ligand 3; CEL 2, Late response Celecoxib group; CEL1, Immediate response Celecoxib group; CMC, Carboxymethylcellulose; COX-1, Cyclooxygenase-1; COX-2, Cyclooxygenase-2; DAB, 3,3′-diaminobenzidine; ERα, Estrogen Receptor alpha; ERβ, Estrogen Receptor beta; GTN, Goniothalamin; GTN 2, Late response Goniothalamin group; GTN1, Immediate response Goniothalamin group; HGPIN, High-Grade Prostatic Intraepithelial Neoplasia; HRP, Horseradish peroxidase; IL1-β, Interleukin 1beta; IL-6, Interleukin 6; INOS, Inducible nitric oxide synthase; LAPC-4, Los Angeles Prostate Cancer-4 cell line; LGPIN, Low-Grade Intraepithelial Neoplasia; LNCaP, Androgen-sensitive human prostate adenocarcinoma cell line; MCF-7, Estrogen-sensitive breast cancer cell line; MIP-2, Macrophage inflammatory protein 2-alpha; NCI/ ADR-RES, Ovarian tumor cell line; NfkB, Nuclear factor kappa B; NSAIDs, Nonsteroidal anti-inflammatory drugs; OVCAR-3, Cisplatin-resistant ovarian cancer cell line; PC-3, Human prostate cancer cell; PCNA, Proliferating cell nuclear antigen; T12, Transgenic Adenocarcinoma of the Mouse Prostate with 12 weeks-old; T22, Transgenic Adenocarcinoma of the Mouse Prostate with 22 weeks-old; T8, Transgenic Adenocarcinoma of the Mouse Prostate with 8 weeks-old; TBS-T, Tris-buffered saline, 0.1% Tween 20; TNF-α, Tumor necrosis factor alpha; TRAMP, Transgenic Adenocarcinoma of the Mouse Prostate; WDA, Well-differentiated adenocarcinoma

Prostate cancer is the most diagnosed cancer in 87 countries from North, South America, Northern, Western and Southern Europe and Oceania and also, the highest prostate cancer incidence is in the USA and the estimated number of new prostate cancer cases was 161,360 in 2017 (Torre et al., 2016; Siegel et al., 2017). In Brazil, prostate cancer was the second most frequent type of cancer in 2016 and also around 61.200 new prostate cancer cases were predicted in 2016 (INCA, 2015).

The rat and mouse prostate is subdivided into paired lobes and named according to their position around the urethra, being classified as ventral, lateral, dorsal, and anterior lobes or coagulating glands (Hayashi et al., 1991). Due to differences in lobe-specific branching morphogenesis, each lobe has distinct features (Marker et al., 2003).

Considering prostate cancer development and progression, different transgenic models have been developed to study this disease (Greenberg, 2000). One of these models is the transgenic adenocarcinoma of the mouse prostate or TRAMP mice, which develops progressive stages of prostate tumors, presenting cases from prostatic intraepithelial neoplasia (PIN) to invasive adenocarcinoma, and metastatic lesions (Gingrich and Greenberg, 1996). Moreover, proliferative lesions progress at a different rate in each lobe, being least often in the anterior prostate (Kaplan-Lefko et al., 2003). On the other hand, the anterior prostate morphological features are described by columnar epithelium projections into the lumen, showing central nuclei and granular cytoplasm (Kaplan-Lefko et al., 2003).

Literature has shown the involvement of inflammation in various types of cancer progression and development, such as in prostate cancer (De Marzo et al., 1999; Vendramini-Costa and Carvalho, 2012; Thapa and Ghosh, 2015;). It is known that prostatic inflammation generates free radicals, such as nitric oxide and reactive oxygen species (Palapattu et al., 2005; Sciarra et al., 2008). Also, inflammatory cell infiltration provides another source of free radicals that can generate pre-cancerous transformations through oxidative DNA damage, as well as genetic alterations, apoptosis, structural, and functional protein changes (Palapattu et al., 2005). In addition, genotypic and phenotypic changes in the stromal cells, during inflammatory responses, may trigger stromal reactivity in the prostate (Rowley 1999; Tuxhorn et al., 2001; Barron and Rowley 2012).

Another important factor for the structural and functional maintenance and overall prostate homeostasis are the androgenic and estrogenic hormones (Prins et al., 1991; Weihua et al., 2001; Cooke et al., 2017). It is known that estrogens act synergistically to testosterone, influencing prostate function under normal and pathological conditions, and so could lead to the development of glandular lesions (Cunha et al., 2001; Weihua et al., 2001). The estrogenic action on the prostate is mediated by estrogen receptors, α (ERα) and β (ERβ) receptors, which are members of the nuclear receptor superfamily (Christoforou and Christopoulos, 2014). ERα is known to be related to epithelial cell differentiation and proliferation, which may be a precursor for prostatic lesions (Cunha et al., 2001; Härkönen and Mäkelä, 2004; Ho, 2004). According to Robinette (1988), estrogens may enhance proinflammatory factors and are accompanied by proliferative processes of fibromuscular tissue. Harris et al. (2000) demonstrated that estrogen administration stimulates the transcription of pro-inflammatory factors such as IL-1β, IL-6, MIP-2, and iNOS in the prostate lateral lobe of Wistar rats. Another study showed that estrogen administration for 30 days increased the prostatic levels of inflammatory markers such as TNF-α, COX-2 and CCL-3 in castrated Sprague–Dawley rats (Jia et al., 2015).

Different authors have evaluated alternative therapies for cancer treatment, including drugs with anti-inflammatory properties and cytotoxicity activity such as Goniothalamin (GTN). GTN is a styryl lactone originated from plants of the *Goniothalamus* genus, which has presented toxicity against different lineages of cancer cells (Sam et al., 1987; Al-Qubaisi et al., 2011). In the prostate, GTN effects were able to decrease the inflammatory process and improve glandular morphology during senescence (Kido et al., 2017).

Other anti-inflammatory drugs, as of non-steroidal (NSAID's), also have been the target of studies involving prevention and treatment of several types of cancer (Wakabayashi 2000). These drugs have a common mechanism of action that inhibits cyclooxygenases (COX) pathways, a property that confers an anti-inflammatory role due to blockade of prostaglandin synthesis (Calatayud and Esplugues, 2016). COX-1 is expressed in most tissues and mediates prostaglandin synthesis, controlling normal physiological functions, whereas COX-2 is not expressed in normal tissue, being induced by proinflammatory and mitogenic stimuli [Q2](Herschman, 1996; Calatayud and Esplugues, 2016). Therefore, selective COX-2 inhibitors, such as Celecoxib, are more recommended than COX-1 inhibitors, since this selectivity confers less side effects when compared to classical NSAID's (Jendrossek, 2013).

Thus, the main objective of this study was to evaluate the morphological, hormonal and inflammatory responses in the microenvironment of the prostate anterior lobe in the TRAMP mouse model, following Celecoxib and Goniothalamin treatments in different periods of prostatic cancer progression.

Materials and methods

Drugs

Goniothalamin (GTN) was obtained from Laboratory of Organic Synthesis (Institute of Chemistry/University of

Campinas, São Paulo, Brazil) under the supervision of Dr. Ronaldo Aloise Pilli, and prepared according to de Fátima et al. (2005) and Vendramini-Costa et al. (2014). Celecoxib was obtained from CELEBRA (Pfzer Pharmaceuticals LLC, Caguas, Puerto Rico), and diluted in carboxymethylcellulose (CMC) 0.05% (Sozer et al., 2011). The acute toxicity of GTN has been evaluated in a previous study (Vendramini-Costa et al., 2015).

Animals and experimental procedure

Seventy transgenic male TRAMP mice (C57BL/6-Tg (TRAMP) 8247Ng/JX FVB/Unib F1/J) were used and divided into seven experimental groups. All the mice were provided by the Multidisciplinary Center for Biological Investigation on Laboratory Animal Science (CEMIB) at the University of Campinas and received water and solid diet ad libitum. The control mice were divided into three groups of different ages: 8 (T8), 12 (T12), and 22 (T22) weeks of age, and orally received the vehicles CMC 0.05% or phosphate-buffered saline (PBS) + 1% Tween 80 (10 mL/kg), following a similar protocol for the treated groups. In order to assess the effects of short and long-term treatment, the animals were treated from 8 to 12 weeks of age, and euthanized at different times: the immediate-response groups at 12-week-old (CEL1 or GTN1) and the late-response groups at 22 weeks-old (CEL2 or GTN2). Celecoxib Treatment: CEL1 (n = 10) and CEL2 (n = 10) groups received a 10 mg/kg Celecoxib dose orally five times a week for 30 days only (from 8 to 12-week old mice [Kido et al., 2016]). Goniothalamin Treatment: GTN1 (n = 10) and GTN2 (n = 10) groups received a 150 mg/kg GTN dose orally, three times a week for 30 days only (from 8 to 12-weeks old mice) (Kido et al., 2016).

After the experimental treatments, the mice were anesthetized with 2% xylazine hydrochloride (5 mg/kg; Konig, São Paulo, Brazil) and 10% ketamine hydrochloride (60 mg/kg; Fort Dodge, IA), then euthanized and the prostate anterior lobes were collected. This study was approved by the institutional Committee for Ethics in Animal Research (University of Campinas—UNICAMP, protocol n°. 3458-1) and the experiments were carried out in agreement with the Ethical Principles for Animal Research established by the Brazilian College for Animal Experimentation (COBEA).

Morphological analyses

Anterior prostate samples were collected from five animals per group, fixed in Bouin's solution, washed in 70% ethanol, dehydrated in increasing concentration of ethanol, and embedded in plastic polymers (Paraplast®, Sigma–Aldrich, St Louis, MO, USA). After that, the samples were cut into 5 μm thick sections (Microtome Hyrax M60 Zeiss, Munich, Germany) and stained with hematoxylin–eosin and Masson's Trichrome (Junqueira et al., 1979). The photomicrographs were obtained using a Nikon Eclipse E-400 photomicroscope (Nikon, Tokyo, Japan).

To quantify the incidence of different prostatic lesion 10 random fields per animal were evaluated at 400X magnification. Each field was divided in four quadrants, and in each quadrant the predominant morphological feature was classified according to Kido et al. (2016) methodology: (1) Healthy tissue; (2) Low-grade prostatic intraepithelial neoplasia (LGPIN); (3) High-grade prostatic intraepithelial neoplasia (HGPIN); (4) Well-differentiated adenocarcinoma (WDA) (Figure 1a–d). The morphological classification of different prostatic lesion grades in TRAMP mice was partially based on Berman-Booty et al. (2011).

Morphometrical analysis

Prostatic samples of five animals from each experimental group, the same used for the light microscopy evaluation, were assessed and the cytoplasmic and nuclear areas were evaluated. The analysis was performed in healthy and hyperplastic regions from the epithelium, where the areas of the nucleus and cytoplasm were measured in at least 1000 cells per group. The software used was Image-Pro Plus 5 (Media Cybernetics, USA).

Immunohistochemical analysis

The antigens were detected using the following primary antibodies: rabbit polyclonal anti-AR (sc-816) (Santa Cruz Biotechnology, EUA), rabbit polyclonal anti-α-actin (ab5694) (Abcam, EUA), mouse polyclonal anti-COX-2 (sc-376861) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit polyclonal anti-ERα (sc-542) (Santa Cruz Biotechnology), mouse polyclonal anti-PCNA (ab29) (Abcam, EUA). For HRP-conjugated secondary antibodies we used goat anti-mouse IgG (W4021; Promega), and goat anti-rabbit IgG (W4018; Promega). To perform the immunohistochemical evaluation, four anterior prostate samples were used and the procedures followed the same protocol showed in previous studies (Kido et al., 2016). All primary antibodies were diluted in a 1:50 ratio, except for PCNA (1:300), and the secondaries were diluted in 1:100. The peroxidase activity was detected using a 3,3′-diaminobenzidine (DAB-Sigma–Aldrich) in the tissue, which was counter-stained with Harris' hematoxylin. Prostatic sections from each experimental group were evaluated through the DAB precipitate (brown), which indicated the immunoreactivity. The scoring of immunolabeled tissue was performed for AR, α-actin, COX-2 and ER-α using a multipoint system with 165 points of intersection, whereas for PCNA 792 points of intersection

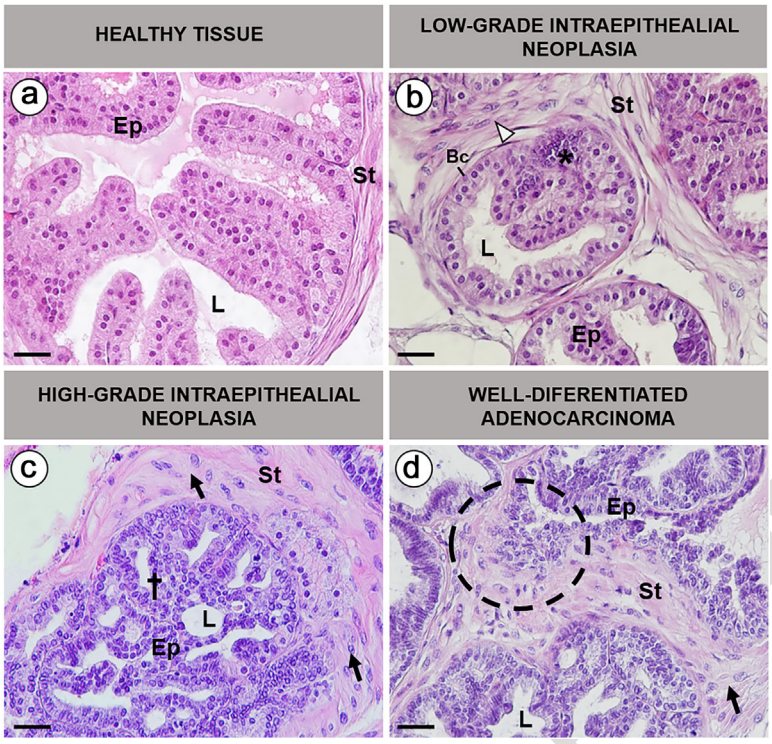

Figure 1 Photomicrographs of the different lesions in the prostate anterior lobe from TRAMP mice. (a) Prostate without lesion. Acini covered by simple epithelium with columnar cells and central nuclei. (b) Low-grade prostatic intraepithelial neoplasia (LGPIN) (asterisk). Epithelial stratification showing larger cellular nucleus and cytoplasm. Hypertrophied and hyperplastic stroma (white arrowhead). (c) High-grade prostatic intraepithelial neoplasia (HGPIN). Epithelial stratification and cribriform architectural pattern in the glandular lumen (cross), larger cellular nucleus and cytoplasm (arrow). Hypertrophied stroma (black arrow). (d) Well-differentiated adenocarcinoma (WDA). Membrane basal discontinuity and invasion of epithelial cells through the stroma (discontinued circle). Hypertrophied stroma (black arrow). Ep, Epithelium; ST, Stroma; L, Lumen; Bc, Basal Cell. Hematoxylin-Eosin (a–d). (scale bar = 25 μm).

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were considered (Weibel, 1963). Ten random fields were captured under 400× magnification and relative frequency was determined by the brown marking coincident with the point of intersection in the grid. Immunoreactivity was graded as 0 (zero) for negative staining (0%), 1 weak (low frequency of positivity) (<10%), 2 moderate (mean frequency of positivity) (10–20%), and three intense (high frequency of positivity) (>20%) (modified from Tuxhorn et al., 2002)

Western blotting evaluation

Prostate anterior lobe samples from five animals were frozen and then homogenized by the Polytron homogenizer (Kinematica Inc., Lucerne, Switzerland) in a protein extraction buffer (50 μL/mg). Then, the extracts were centrifuged at 14000 rpm for 20 min at 4°C, and protein quantification was performed using the Bradford method. A total of 75 μg protein was applied and separated by electrophoresis to the SDS–polyacrylamide gel under reducing conditions. Subsequently, the proteins were electrically transferred (120V) to nitrocellulose membranes for 1 h and 30 min (Amersham Life Science, Arlington Heights, IL, USA). The membranes were blocked with 3% bovine serum albumin (BSA) diluted in tris-buffered saline and tween 20 (TBS-T) for 1 h and incubated overnight with the primary antibodies in a dilution range of 1:350–1:1000: anti-AR (sc-816) (Santa Cruz Biotechnology), rabbit polyclonal anti-α-actin (ab5694) (Abcam, EUA), rabbit polyclonal anti-ERα (sc-542) (Santa Cruz Biotechnology). After that, the membranes were incubated for 2 h with secondary HRP conjugate anti-rabbit and anti-mouse antibodies in a dilution range of 1:4000–1:10000 diluted in 1% BSA. The peroxidase activity was detected through the incubation of the membranes with a chemiluminescent solution (Pierce Biotechnology, Rockford, IL, USA) for 5 min and captured by Gene Gnome equipment and the GeneSys image acquisition software (Syngene Bio Imaging, Cambridge, UK). The antibody for mouse monoclonal anti-β-actin (sc-81178) (Santa Cruz Biotechnology) was used as endogenous control for comparison among groups. The intensity of antigen bands was quantified by densitometry using the Image J (Image Analysis and Processing in Java)

software for image analyses and was expressed as the mean percentage in relation to β-actin band intensity.

Statistical analysis

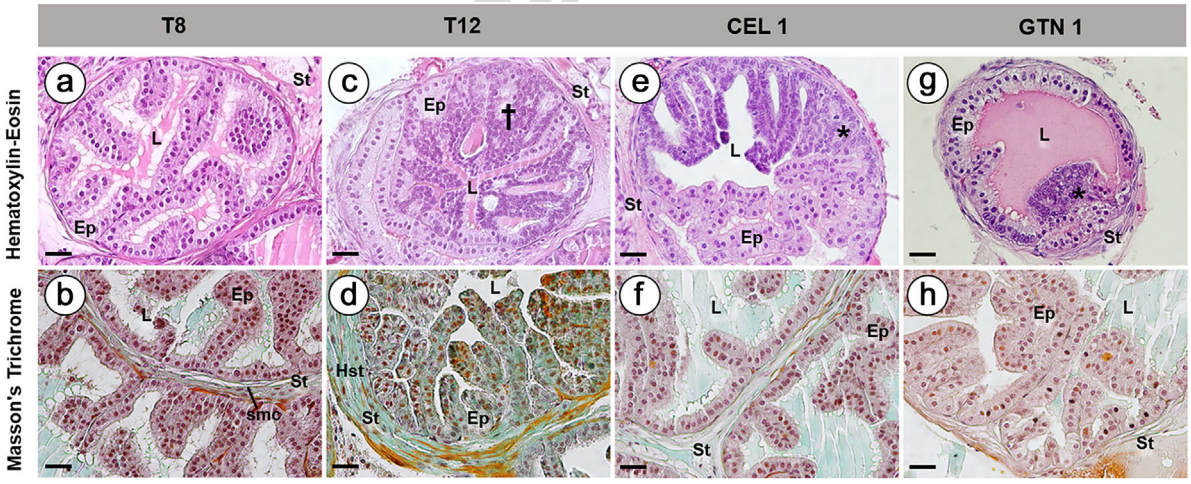
The comparative statistical analysis of proliferative lesion incidence, morphometry, AR, α-actin, and ER-α protein levels, among the experimental groups was carried out by analysis of variance (ANOVA) followed by Tukey multiple range test, with the level of significance set at 5% . The results were expressed as the mean standard deviation (Zar 1999). All statistical analyses were performed by the software GraphPad Prism (version 5.0).

Results

Morphological analyses

The control TRAMP mice showed both alterations and healthy glandular regions in the prostate anterior lobe, however, there was a different proportion between these characteristics according age of the mice (Figures 2a–d, 3a and 3b, 4a, and 5a). Q3The glandular tissue without alterations was characterized by secretory epithelium that presented columnar cells with central nuclei covering the acini and intermingled with basal cells with pyramidal form, which were organized discontinuously (Figures 1a and 2a and 2b). The prostatic stroma presented collagen fibers and smooth muscle cells, concentrically placed around of the acini (Figures 2a and 2b). On the other hand, there were different grades of prostatic lesions in the TRAMP mice at 8, 12, and 22 weeks of age such as LGPIN, HGPIN, and WDA (Figures 1b–d). The LGPIN showed epithelial cell stratification with increased nucleus and cytoplasm areas, characterizing hypertrophied and hyperplastic cells (Figures 1b). Occasional hypertrophied stroma regions were especially verified underlying to epithelium proliferation regions (Figures 1b). The HGPIN was characterized by epithelial cell stratification, projected towards the glandular lumen (Figures 1c). A significant increase of the nuclear and cytoplasmic areas was verified in proliferative regions (Figures 6a–d). Also, stromal thickening was observed adjacent to altered morphological glandular regions (Figure 3b). Finally, the WDA was characterized by epithelial cell infiltration towards glandular stroma and basal membrane discontinuity (Figure 1d). The cells in the proliferative regions showed cellular atypia with larger nuclear and cytoplasmic areas. Hypertrophied and hyperplastic stroma was observed surrounding proliferative glandular areas, as demonstrated by the presence of stromal smooth muscle cells (Figure 1d).

The results showed an increase in different glandular alterations, particularly in the T8-T12 groups. (Figure 4a) Also, an increase in HGPIN and WDA incidence was seen in the T12-T22 groups, characterizing the disease progression in TRAMP model (Figure 5a). There is no occurrence of WDA in the prostate anterior lobe in T8 group. These results were confirmed by means of a decrease in PCNA immunolabeling (Figures 4a and 4b, 5a and 5b; Table 1).



COLOUR

Figure 2 Photomicrographs of the prostate anterior lobe of TRAMP mice. T8 group (a and b): acini with folded mucosa and simple secretory epithelium with columnar cells. Stroma with smooth muscle cells (smc) and collagen fibers. T12 group (c and d): Secretory epithelium with columnar cells, HGPIN presence (cross) and stromal thickening (Hst). CEL 1 group (e and f): LGPIN regions (asterisk) in the glandular acini and stromal features similar to the T8 group. GTN 1 group (g and h): Healthy tissue predominance and occasional LGPIN regions (asterisk) in the prostate anterior lobe. Stromal morphological characteristics similar to the T8 group (a and b). Ep, Epithelium; St, Stroma; L, Lumen. Hematoxylin–eosin staining (a, c, e, and g); Masson's trichrome staining (b, d, e, and f) (scale bar = 25 μm).

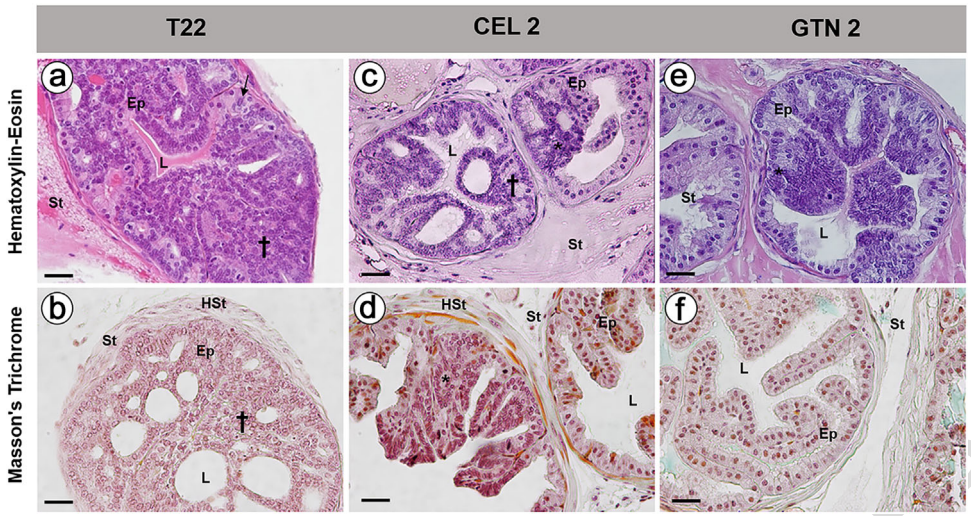


Figure 3 Photomicrographs of the prostate anterior lobe of TRAMP mice from T22 (a and b); CEL 2 (c and d); GTN 2 (e and f) groups. T22 group (a and b): Glandular cellular proliferation indicated by higher HGPIN (†) frequency. Hyperplastic and hypertrophied prostatic stroma (HSt), particularly close to epithelial proliferation regions. CEL 2 group (c and d): Proliferative areas, showing LGPIN (asterisk) and HGPIN (cross). Stroma showing fibromuscular hypertrophy (HSt). GNT 2 group (e and f): Occasional areas of proliferative lesions such as LGPIN (asterisk) and prevalence of healthy tissue. Stroma features were similar to T1GTN group. Ep, Epithelium; St, Stroma; L, Lumen. Hematoxylin–eosin staining (a, c, and e); Masson's trichrome staining (b, d, and f) (scale bar = 25 μm).

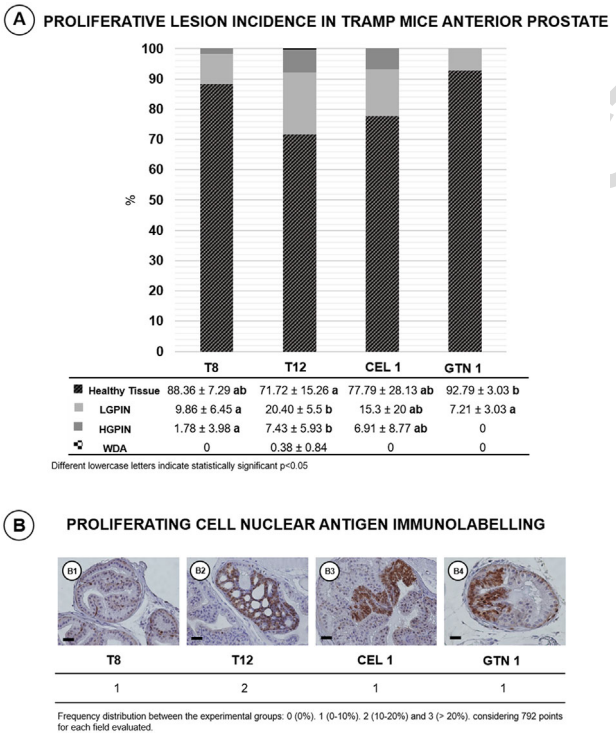


Figure 4 PCNA immunolableling and quantification of proliferative lesion incidence in the prostate anterior lobe of TRAMP mice. a: Graphic of proliferative lesion incidence. b: PCNA immunolableling from T22, CEL2, GTN2 groups. PCNA, Proliferating Nuclear Cell Antigen (scale bar = 25 μm).

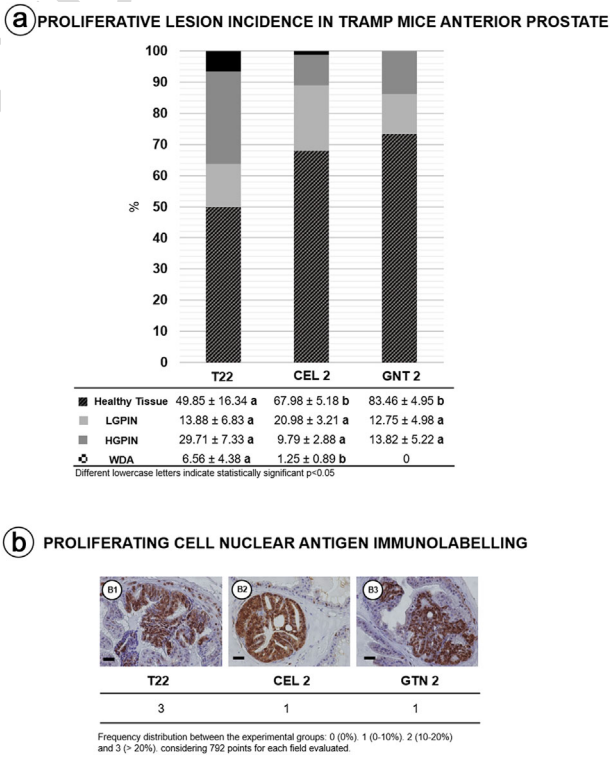


Figure 5 Morphometric analysis of nuclear and cytoplasmic areas in healthy and hyperplastic glandular epithelium from prostate anterior lobe from T8, T12, CEL 1, and GTN 1 (a and b) and T22, CEL 2 and GTN 2 (c and d).

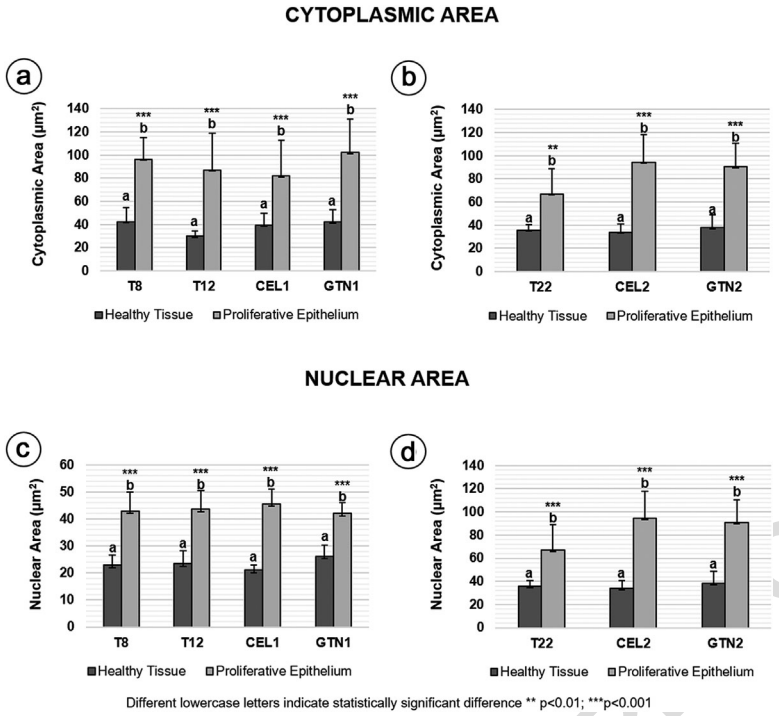


Figure 6 PCNA immunolableling and quantification of proliferative lesion incidence in the prostate anterior lobe of TRAMP mice. a: Proliferative lesion incidence graphic. b: PCNA immunolabeling from different experimental groups. PCNA, Proliferating Nuclear Cell Antigen (scale bar = 25 μm).

Immediate response Celecoxib group (CEL 1)

The prostate anterior lobe in this group showed a trend towards decreased incidence of LGPIN and HGPIN lesions when compared with the T12 control group, although not statistically significant. The healthy glandular tissue was predominant in this group (Figures 2e and 2f and 4a). The proliferative glandular areas showed atypical cells with increased cellular cytoplasm and nucleus (Figures 6a and 6c). There was no occurrence of WDA in this group in relation to T12 group. Regarding PCNA immunolabeling, CEL 1 group was classified as weak, confirming lesion numerical incidence reduction observed by morphological analysis (Figures 4a and 4b).

Table 1 AR, ERα, α-actin, and COX-2 immunoreactivities in the prostate anterior lobe of TRAMP mice in the immediate response groups.

	T8	T12	CEL1	GTN1
AR	1	2	1	1
ERα	1	2	1	1
α-actin	1	2	2	2
COX-2	1	2	1	1

Frequency distribution between the experimental groups: 0 (0%). 1 (0-10%). 2 (10-20%) and 3 (> 20%). considering 165 points for each field evaluated.

Late response Celecoxib group (CEL 2)

There was a significant healthy glandular increase and a WDA decrease in the late response group treated with Celecoxib when compared to the T22 group (Figures 3c and 3d and 5a). The prostatic stroma showed thickening of collagen fibers, characterizing stromal hypertrophy (Figure 3d). Celecoxib treatment also resulted in anti-proliferative action in the late response group, leading to a reduction in the PCNA frequency, classified as weak (Figure 5b).

Immediate response Goniothalamin group (GTN 1)

Compared to the T12 group, there was a higher incidence of healthy tissue and decreased incidence of LGPIN in the immediate response GTN group. There were no occurrences of HGPIN and WDA in the immediate response GTN group in comparison with T12 group (Figures 2c, d, g, h and 4a). The morphological features of prostate anterior lobe in the immediate response GTN group were similar to that found in the T8 group (Figures 2a, b, g, h). The PCNA immunolabeling was classified as weak in the GTN 1 group, indicating reduction of the proliferative process (Figure 4b).

Late response Goniothalamin group (GTN 2)

The healthy glandular tissue was better maintained in the late response GTN group in relation to T22 group (Figures 3a, b, e, f and 5a), with no presence of WDA (Figure 5a). The

morphology of the secretory epithelium from prostate anterior lobe was similar to that found in the T12 group (Figures 2c and 2d and 3e and 3f). Similarly to the GTN 1 group, the PCNA frequency decreased in the late response GTN group (Figure 5b).

Immunohistochemistry and Western blotting analyses

Androgen receptor (AR)

The AR immunolabeling was weak in the T8 group, which was observed in both prostatic compartments (Figure 7a; Table 1). On the other hand, the prostate from T12 group showed moderate epithelial and stromal AR staining (Figure 7b; Table 1). Goniothalamin and Celecoxib reduced the AR frequency in the immediate response groups, showing the same tendency in the AR protein level evaluation (Figures 7c and 7d and 8a; Table 1).

The late response group showed moderate AR immunolabeling considering both prostatic compartments, and there were no changes after both experimental treatments (Figure 9a–c; Table 2). The same pattern was seen by Western blotting (Figure 8b).

Estrogen receptor (ERα)

Weak ERα reactivity was detected in both prostatic compartments of the T8 group, particularly in cellular cytoplasmic region (Figure 7e, Table 1). However, moderate ERα reactivity was verified in the T12 group, showing cytoplasmic and nuclear immunolabeling in epithelial and stromal cells (Figure 7f; Table 1). In both immediate response treatments there was reduced ERα reactivity in the cytoplasm of epithelial cells, as well as significantly decreased protein levels (Figures 7g and 7h and 8a; Table 1).

Intense ERα immunolabeling was identified in the late response group (T22), located in cellular nuclei and cytoplasm of cells from stroma and epithelium, especially in prostatic lesion areas (Figure 9d; Table 2). On the other hand, GTN treatment reduced ERα reactivity frequency at

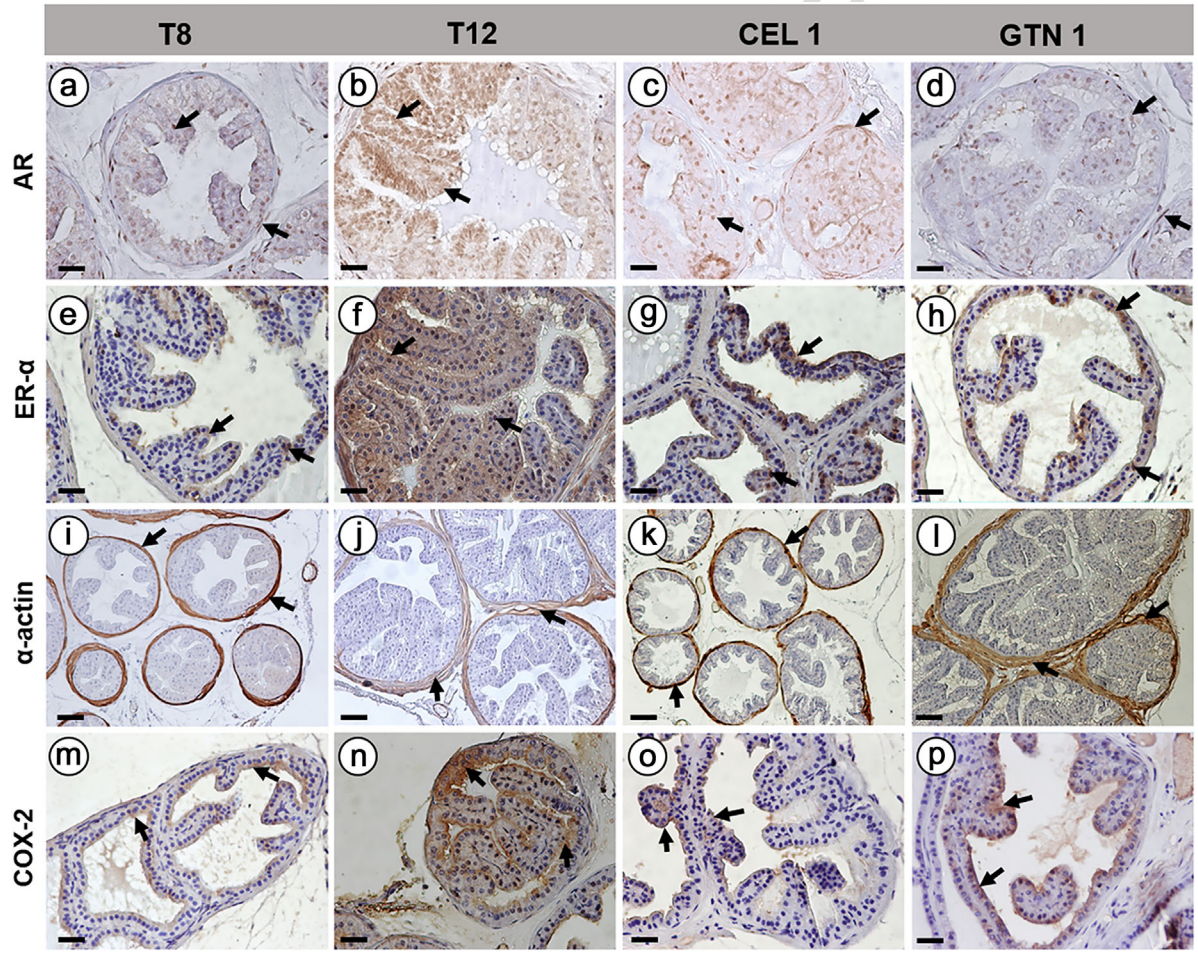


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Figure 7 AR, ERα, α-actin and COX-2 immunolabeling in the prostate anterior lobe (black arrow) from T8 (a, e, i, m), T12 (b, f, j, n), CEL 1 (c, g, k, o), and GTN 1 (d, h, i, p) groups. Semi-quantitative evaluation was shown in the Table X. Q4Ep, Epithelium; St, Stroma; L, Lumen (scale bar = 25 μm).

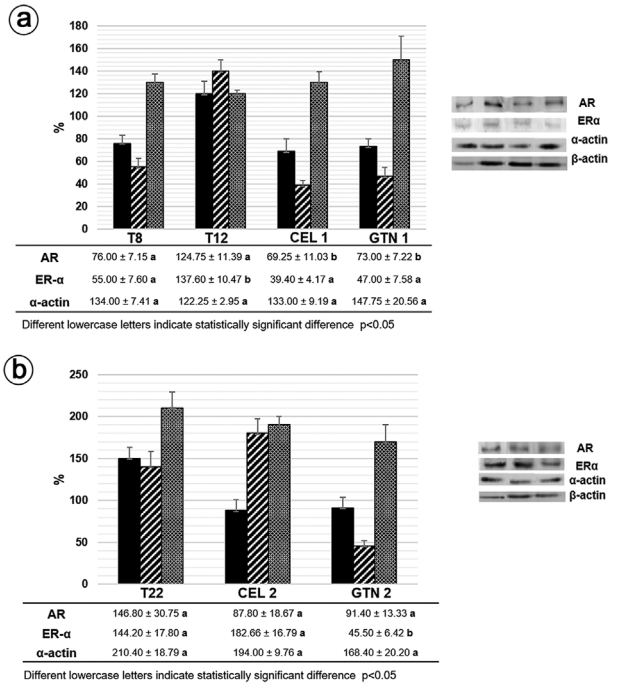

Figure 8 AR, ERα, and α-actin protein level quantification of the anterior prostate lobe. a: T8, T12, CEL 1, and GTN 1. b: T22, CEL 2, and GTN 2. β-actin was the endogenous control.

prostate cancer advanced stages, whereas in late response group to Celecoxib treatment there were no changes in relation to T22 group. ERα protein levels determined by Western blotting were in agreement with the immunohistochemical findings (Figures 8b and 9e and 9f; Table 2).

α-actin

Weak α-actin immunoreactivity was verified in the stroma from T8 group, whereas moderate α-actin reactivity frequency was found in the T12 group, characterizing a α-actin immunoreactivity increase (Figures 7i and 7j; Table 1). In addition, both immediate response treatments neither change the α-actin immunoreactivity nor the protein levels in the prostate anterior lobe (Figures 7k and 7l; Table 1).

Intense α-actin immunoreactivity in T22 group was detected, which is in accordance with the thickening layers of smooth muscle cells reported through morphological analysis (Figure 9g; Table 2). Goniothalamin was the only treatment able to reduce α-actin immunoreactivity in the late response groups (Figure 9h; Table 2). However, the Western blotting results did not show any statistical difference between the treated and control groups, despite having numerical decrease (Figure 8b).

COX-2

Weak COX-2 cellular cytoplasmic immunoreactivity was identified in both prostatic compartments from T8 group animals (Figure 7m; Table 1). Moderate COX-2 frequency was identified in the T12 group in both epithelial and stromal cells when compared to T8 group (Figure 7n; Table 1). On the other hand, both immediate response treatments led to a COX-2 reduction, showing weak immunoreactivity in the prostate anterior lobe epithelium and stroma (Figures 7o and 7p; Table 1).

Intense COX-2 immunoreactivity was observed in the advanced stages of prostate cancer (T22), which was detected throughout the prostatic compartments, particularly, in the cell apical area (Figure 9j; Table 2). The late response treatments led to a slight COX-2 immunoreactivity reduction classified as moderate (Figures 9k and 9l; Table 2).

Discussion

The results herein showed aging-dependent lesion progression in the anterior prostate from TRAMP mice, highlighting the increase of WDA frequency at 12- and 22-week-old mice. The animals from the immediate response treatment with Celecoxib presented no occurrence of WDA, whereas in the late response group, a healthy glandular tissue increase and a WDA glandular decrease were observed. Moreover, the proliferating cells were less frequent in both response periods after Celecoxib treatment. The GTN immediate response group, in its turn, showed no occurrence of HGPIN and WDA, besides a higher incidence of healthy glandular tissue. Similarly, the late GTN response presented no WDA occurrence and a significant healthy tissue increase. The proliferation marker also confirmed the decrease of glandular cells proliferation.

Different studies pointed out that almost 100% of TRAMP mice, from 24 to 30 weeks of age, developed prostatic poorly-differentiated adenocarcinoma, showing different types of metastasis (Gingrich and Greenberg, 1996; Greenberg et al., 1995; Kaplan-Lefko et al., 2003). It is also known that there is a difference in cancer progression among the prostate lobes (Suttie et al., 2003). According to Greenberg et al. (1995) the highest PB-Tg transgene levels were detected in the prostate ventral and dorsal lobes in adult mice. However, transgene expression in the prostate anterior lobe and seminal vesicle was less frequent (Gingrich et al., 1999). Also, Suttie et al. (2003) evaluated cancer progression in different prostate lobes in the TRAMP mouse model, verifying that there were more multifocal lesions, papillary or cribriform patterns in the prostate ventral and lateral lobes than in the prostate anterior lobe. According to Berman-Booty et al. (2011) the prostate anterior lobe showed the lowest incidence of poorly-differentiated adenocarcinoma in relation to the other prostate lobes, and the HGPIN was the most important prostate lesion in this lobe in 18–24-week-old mice. The prostate anterior lobe presented a lower lesion frequency in relation to the prostate ventral, lateral, and dorsal lobes,

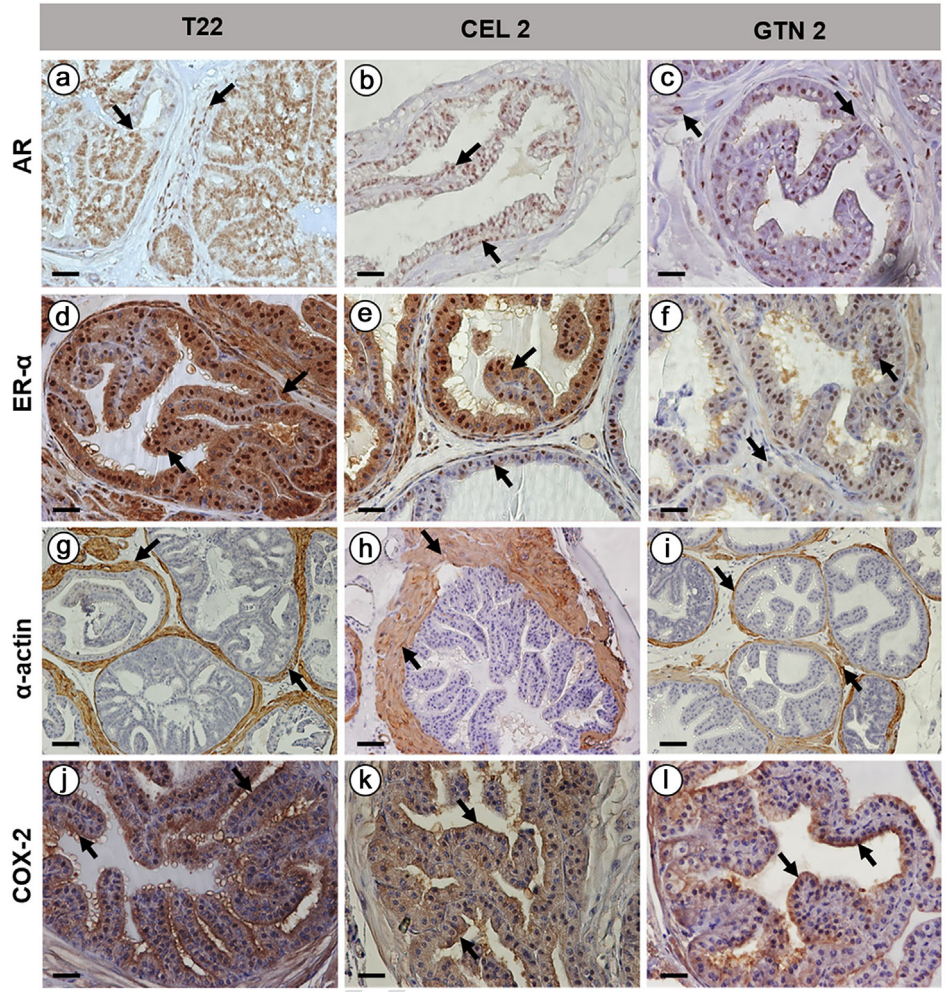

Figure 9 AR, ERα, α-actin, and COX-2 immunolabeling in the prostate anterior lobe (black arrow) from T22 (a, d, g, j), CEL 2 (b, e, h, k), GTN 2 (c, f, i, l) groups. Semi-quantitative evaluation was shown in the Table X. Ep, Epithelium; St, Stroma; L, Lumen (scale bar = 25 μm).

showing a predominantly normal epithelium as the TRAMP mice aged (Kaplan-Lefko et al., 2003). Recent studies from our research group also confirmed that lesion progression in the other prostatic lobes is more pronounced than in the prostate anterior lobe (Kido et al., 2016; Silva et al., 2017). Kido et al. (2016) showed that the presence of LGPIN and HGPIN lesions in the prostate ventral lobe of 8- and 12-week-old TRAMP mice was two to five times greater than those found in the present study. Furthermore, the same authors verified a particular predominance of HGPIN in the prostate ventral lobe in 22-week-old TRAMP mice, representing approximately 50%, whereas in the present results, the HGPIN incidence was 13.88% at the same age (Kido et al., 2016). In addition, the seminal vesicles are also susceptible to lesion development in TRAMP mice (Dal Pozzo et al., 2016). However, higher proliferative lesion frequency, as well as WDA were observed only in the seminal vesicle from 22-week-old TRAMP mice (Dal Pozzo et al., 2016).

Table 2 AR, ERα, α-actin and COX-2 immunoreactivities in the prostate anterior lobe of TRAMP mice in the late response groups.

	T22	CEL2	GNT2
AR	2	2	2
ERα	3	3	2
α-actin	3	3	2
COX-2	3	2	2

Frequency distribution between the experimental groups: 0 (0%). 1 (0–10%). 2 (10–20%) and 3 (> 20%). considering 165 points for each field evaluated.

Thus, we concluded that the prostate anterior lobe presented lower cancer severity with late lesion progression even in 22-week-old TRAMP mice. Nevertheless, the prostate anterior lobe is a good model to study slow progression of prostate cancer. This is true taking into

consideration the diversity and severity of tumoral grades, which can be seen in the prostate of human beings, due to prostatic microenvironment interaction dynamics.

Regarding natural product-derived compounds, different studies have shown an antitumoral activity in different cancer cell lines treated with compounds such as GTN (de Fátima et al., 2005). Barcelos et al. (2014) verified that compounds derived from GTN presented antiproliferative activity, leading to reactive oxygen species occurrence and apoptosis in the human prostate cancer cells (PC-3). Also, antiproliferative effects on human renal cancer cells were seen after administering (R)-GTN and also (S)-GTN (non-natural isomer) in vitro (de Fátima et al., 2008). Studies in vivo confirmed the antiproliferative and anti-inflammatory role of GTN and its derivatives, such as Ehrlich ascitic and solid tumor inhibition, cell proliferation reduction in TRAMP mice, and colitis and colon cancer prevention (Barcelos et al., 2014; Kido et al., 2016; Vendramini-Costa et al., 2017). According to Kido et al. (2016), GTN led to prostate ventral lobe cancer delay when administered to early grade lesions. In addition, these same authors verified that GTN decreased HGPIN and WDA in the prostate ventral lobe in the immediate response group, by means of the same procedures of the study herein (Kido et al., 2016). However, GTN was not efficient to decrease HGPIN and WDA in the prostate ventral lobe, considering the late response in 22-week-old TRAMP mice, despite having a healthy tissue increase (Kido et al., 2016).

Celecoxib, a COX-2 selective inhibitor, is involved in the conversion of arachidonic acid to prostaglandin and is related to inflammatory processes (Dannenberg et al., 2001). Also, COX-2 expression is associated to tumorigenesis and tumor progression (Sano et al., 1995; Tsujii et al., 1997). Previous studies have shown that different doses of Celecoxib supplementation administered to TRAMP mice promoted a decrease in prostate dorsolateral tumors, simultaneously with an increase in apoptosis rate, which was proportional to the Celecoxib dose (Narayanan et al., 2006). Also, proliferation was decreased and apoptosis was increased in the prostate ventral lobe of TRAMP mice at 12 weeks of age after Celecoxib treatment, using the same protocol of the present study (Kido et al., 2016). The same authors verified decrease in undifferentiated tissue in the prostate ventral lobe of 22-week-old TRAMP mice. On the other hand, Flamiatos et al. (2017) did not observe positive effects, such as increase in apoptosis in the prostate adenocarcinoma in men who received Celecoxib at a 400 mg twice daily dose for 4 weeks, before radical prostatectomy surgery.

Therefore, we concluded that Celecoxib treatment was efficient in delaying prostate cancer progression in the prostate anterior lobe, leading to a severity decrease, and to healthy glandular tissue improvement, especially in the late response group. Also, GTN treatment led to a remarkable prostate anterior lobe progression delay in both immediate and late response groups, showing cellular proliferation decrease and tumorigenic process decrease in the TRAMP model. GTN treatment could be pointed out as a promising drug for cancer progression delay and also tissue repair, considering both experimental conditions and slow cancer progression.

It is known that androgens have an important role in morphogenesis and prostate maintenance (Gelmann, 2002). However, androgens and their receptor (AR) have been strongly suggested to be risk factors involved in prostate cancer development (Pienta and Esper, 1993; Gelmann, 2002). The results herein reported showed a significant protein level decrease and AR immunolabeling frequency in the immediate response groups, treated with both Celecoxib and GTN. Niu et al. (2008) demonstrated that AR knockout TRAMP mice had smaller prostate tumors with low rates of cell proliferation. According to Abedinpour et al. (2011), androgen ablation in association with Celecoxib treatment led to tumor regression through angiogenesis reduction, apoptosis increase and mitosis interruption. Celecoxib administration in the human prostate cell lineage, LNCaP and LAPC-4, not only displayed decreased AR, but also the inhibition of these genes at the transcriptional level, due to the increased expression of c-jun protein levels (Pan et al., 2003). In addition, the same authors suggested that AR inhibition induced by Celecoxib might have occurred despite COX-2 inhibition, suggesting an alternative mechanism of action of this non-steroidal anti-inflammatory drug.

The results presented herein may suggest this correlation of decreased AR immunostaining with a concomitant decrease in PCNA immunostaining by both treatments, especially those in the immediate response groups. Thus, we concluded that both Celecoxib and GTN were effective in the AR level decrease, contributing to the delay of lesion progression in the anterior prostate. Moreover, we highlight the GTN action in the androgenic hormonal pathway which is described for the first time in literature.

Regarding estrogens, high ER-α frequency was observed in the early prostate anterior lobe lesion development in the present study. Similar to the AR response in the anterior prostate, decreased ER-α levels were found after both immediate-response treatments. However, only GTN treatment was able to decrease the levels of this receptor in the late response groups. Different authors reported that ER-α is related to the cancerous process in the prostate (Härkönen and Mäkelä, 2004; Hetzl et al., 2014). In addition, ERα activation also promotes inflammation, which may stimulate the aromatase enzyme and ERα additional activation, leading to prostatic adenocarcinoma development (Ellem and Risbridger, 2007). Ellem et al. (2009) using AROM+ mice, which overexpress aromatase, showed an increase in

the estrogen rate concomitant to its activation and increase in mast cells frequency. Sĺusarz et al. (2012) verified the protumorigenic effects of ERα, showing lower incidence of poorly differentiated prostatic adenocarcinoma in ERα knockout model in TRAMP mice compared to wild type mice.

There are no studies in literature showing Celecoxib effects on the estrogenic pathway in the prostate, however, some studies have already found these effects on estrogen-dependent breast cancer cells (Bocca et al., 2011; Jeon et al., 2015). Bocca et al. (2011) observed that Celecoxib treatment in the estrogen-dependent breast cancer cell line (MCF-7) led to cell growth inhibition, which was associated to reduced ER-α expression and activation. It is important to note that Celecoxib presented a slight effect on COX-2 levels in the MCF-7 line, However this drug was able to reduce aromatase expression (Bocca et al., 2011). Regarding GTN, literature also does not provide data about the action of this compound in the estrogenic pathway. However, other studies have demonstrated the cytotoxic activity of GTN in estrogen-dependent cancer cell lines such as breast (MCF-7) and ovary (OVCAR-3, NCI/ADR-RES) (Ali et al., 1997; Pihie et al., 1998; Inayat-Hussain et al., 2003; de Fátima et al., 2005; de Fátima et al., 2006). Therefore, we concluded that Celecoxib and GTN treatments were beneficial and interfered in the estrogenic pathway signaling, especially in the immediate response groups. Furthermore, GTN treatment stood out due to more effectively interfering in the estrogenic pathway in the late response group, contributing to a delay in tumor progression.

In relation to inflammation, the results herein showed COX-2 increased levels in the anterior prostate, according to the aging in the TRAMP model. Also, a COX-2 decrease, after treatments with Celecoxib and Goniotalamin in the immediate and late response groups, was verified. It is known that the inflammatory process leads to a favorable environment for prostatic lesion development (De Marzo et al., 2007). Kido et al. (2016) showed that the control of the inflammatory process at early stages of prostate cancer was fundamental for the negative regulation of the signaling pathways involved in proliferation processes in late stages of cancer development. These same authors verified a COX-2 immunoexpression decrease after treatment with GTN and Celecoxib in the short and long term treatments. Although the study by Kido et al. (2016) was performed in the ventral prostate, which presents more severe lesions than the prostate anterior lobe, the same response tendency was observed in the present study, confirming the anti-inflammatory action of both drugs. Furthermore, GTN was more effective than Celecoxib in decreasing other pro-inflammatory mediators in the TRAMP model, such as Nf-kB levels in the prostate and IL1-β and TNF-α plasmatic levels. Recently, a study concerning Celecoxib and GTN effect on the senile mice prostatic microenvironment also revealed the modulatory action of both drugs on inflammatory reduction and prostatic morphology maintenance, highlighting their chemopreventive role in the prostate (Kido et al., 2017)

In relation to α-actin, the results showed that none of the treatments was able to alter the immunoexpression of this molecule. However, the present results showed that α-actin increases according to the proliferative lesion progression in the prostate anterior lobe, confirming data already presented in literature. Montico et al. (2015) observed that α-actin increased in the dorsolateral prostate in elderly and TRAMP mice, indicating stromal reaction linked to increase of age and neoplasia onset (Montico et al., 2015). In addition, Yu et al. (2011) showed that differences in α-actin expression are linked to AR activity in smooth muscle cells. Therefore, stromal remodeling during neoplasia development in the prostate could be understood as an adaptive response of the prostatic microenvironment in relation to the onset of lesions (Tuxhorn et al., 2001).

Conclusions

Therefore, we concluded that the prostate anterior lobe is a good model to study prostate cancer which presents slow progression, being indicated for studies that involve chemopreventive treatments. Furthermore, both treatments influenced different signaling pathways in the prostate microenvironment, highlighting the inflammatory and hormonal processes, which are crucial for glandular balance. This study suggests that anti-inflammatory drugs are indeed beneficial for prostate cancer prevention. Finally, this is the first report demonstrating the potential of GTN in the modulation of androgenic and estrogenic pathways in the prostate.

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References

Abedinpour P, Baron VT, Welsh J, Borgstrom P (2011) Regression of prostate tumors upon combination of hormone ablation therapy and celecoxib in vivo. Prostate 71(8): 813–23.

Ali AM, Mackeen MM, Hamid M, Aun QB, Zauyah Y, Azimahtol HL, Kawazu K (1997) Cytotoxicity and electron microscopy of cell death induced by goniothalamin. Planta Med 63(1): 81–3.

Al-Qubaisi M, Rozita R, Yeap SK, Omar AR, Ali AM, Alitheen NB (2011) Selective cytotoxicity of goniothalamin against hepatoblastoma HepG2 cells. Molecules 16(4): 2944–59.

Barcelos RC, Pastre JC, Vendramini-Costa DB, Caixeta V, Longato GB, Monteiro PA, De Carvalho JE, Pilli RA (2014) Design and synthesis of N-acylated aza-goniothalamin derivatives and evaluation of their in vitro and in vivo antitumor activity. ChemMedChem 9(12): 2725–43.

Barron DA, Rowley DR (2012) The reactive stroma microenvironment and prostate cancer progression. Endocr Relat Cancer 19(6): 187–204.

Berman-Booty LD, Sargeant AM, Rosol TJ, Rengel RC, Clinton SK, Chen C-S, Kulp SK (2011) A review of the existing grading schemes and a proposal for a modified grading scheme for prostatic lesions in TRAMP Mice. Toxicol Pathol 40(1): 5–17.

Bocca C, Bozzo F, Bassignana A, Miglietta A (2011) Antiproliferative effects of COX-2 inhibitor celecoxib on human breast cancer cell lines. Mol Cell Biochem 350(1-2): 59–70.

Calatayud S, Esplugues JV (2016) Chemistry, pharmacodinamics, pharmacokinetics of NSAID's. In: Lanas A ed. NSAIDs and aspirin: Recent advances and implications for clinical management. Spain: Springer, pp. 3–16.

Christoforou P, Christopoulos P (2014) The role of estrogen receptor β in prostate cancer. Mol Med 20(1): 1.

Cooke PS, Nanjappa MK, Ko C, Prins GS, Hess RA (2017) Estrogens in male physiology. Physiol Rev 97(3): 995–1043.

Cunha GR, Wang YZ, Hayward SW, Risbridger GP (2001) Estrogenic effects on prostatic differentiation and carcinogenesis. Reprod Fertil Dev 13(4): 285–96.

Dal Pozzo CFS, Kido LA, Montico F, Gonçalves MP, Cagnon VHA (2016) Morphology and MMP-9, AR and IGFR-1 responses of the seminal vesicle in TRAMP mice model. Tissue Cell 48(3): 217–23.

Dannenberg AJ, Altorki NK, Boyle JO, Dang C, Howe LR, Weksler BB, Subbaramaiah K (2001) Cyclo-oxygenase 2: A pharmacological target for the prevention of cancer. Lancet Oncol 2(9): 544–51.

de Fátima Â, Kohn LK, Antônio MA, de Carvalho JE, Pilli RA (2005) (R)-Goniothalamin: Total syntheses and cytotoxic activity against cancer cell lines. Bioorg Med Chem 13(8): 2927–33.

de Fátima Â, Kohn LK, De Carvalho JE, Pilli RA (2006) Cytotoxic activity of (S)-goniothalamin and analogues against human cancer cells. Bioorg Med Chem 14(3): 622–31.

de Fátima Â, Zambuzzi WF, Modolo LV, Tarsitano CAB, Gadelha FR, Hyslop S, Carvalho JEd, Salgado I, Ferreira CV, Pilli RA (2008) Cytotoxicity of goniothalamin enantiomers in renal cancer cells: Involvement of nitric oxide, apoptosis and autophagy. Chem Biol Interact 176(2-3): 143–50.

De Marzo AM, Marchi VL, Epstein JI, Nelson WG (1999) Proliferative inflammatory atrophy of the prostate. Am J Pathol 155(6): 1985–92.

De Marzo AM, Platz EA, Sutcliffe S, Xu J, Grönberg H, Drake CG, Nakai Y, Isaacs WB, Nelson WG (2007) Inflammation in prostate carcinogenesis. Nat Rev Cancer 7(4): 256–69.

Ellem SJ, Risbridger GP (2007) Treating prostate cancer: A rationale for targeting local oestrogens. Nat Rev Cancer 7(8): 621–7.

Ellem SJ, Wang H, Poutanen M, Risbridger GP (2009) Increased endogenous estrogen synthesis leads to the sequential induction of prostatic inflammation (prostatitis) and prostatic premalignancy. Am J Pathol 175(3): 1187–99.

Flamiatos JF, Beer TM, Graff JN, Eilers KM, Tian W, Sekhon HS, Garzotto M (2017) Cyclooxygenase-2 (COX-2) inhibition for prostate cancer chemoprevention: Double-blind randomised study of pre-prostatectomy celecoxib or placebo. BJU Int 119(5): 709–16.

Gelmann EP (2002) Molecular biology of the androgen receptor. J Clin Oncol 20(13): 3001–15.

Gingrich JR, Barrios RJ, Foster BA, Greenberg NM (1999) Pathologic progression of autochthonous prostate cancer in the TRAMP model. Prostate Cancer Prostatic Dis 2(2): 70–5.

Gingrich JR, Greenberg NM (1996) A transgenic mouse prostate cancer model. Toxicol Pathol 24(4): 502–4.

Greenberg NM (2000) Androgens and growth factors in prostate cancer: A transgenic perspective. Prostate Cancer Prostatic Dis 3(4): 224–8.

Greenberg NM, DeMayo F, Finegold MJ, Medina D, Tilley WD, Aspinall JO, Cunha GR, Donjacour AA, Matusik RJ, Rosen JM (1995) Prostate cancer in a transgenic mouse. Proc Natl Acad Sci USA 92(8): 3439–43.

Härkönen PL, Mäkelä SI (2004) Role of estrogens in development of prostate cancer. J Steroid Biochem Mol Biol 92(4): 297–305.

Harris MT, Feldberg RS, Lau KM, Lazarus NH, Cochrane DE (2000) Expression of proinflammatory genes during estrogen-induced inflammation of the rat prostate. Prostate 44(1): 19–25.

Hayashi N, Sugimura Y, Kawamura J, Donjacour AA, Cunha GR (1991) Morphological and functional heterogeneity in the rat prostatic gland. Biol Reprod 45(2): 308–21.

Hetzl AC, Montico F, Lorencini RM, Kido LA, Cândido EM, Cagnon VHA (2014) Prostatic microenvironment in senescence: Fibroblastic growth factors x hormonal imbalance. Histochem Cell Biol 141(5): 531–42.

Ho SM (2004) Estrogens and anti-estrogens: Key mediators of prostate carcinogenesis and new therapeutic candidates. J Cell Biochem 91(3): 491–503.

Inayat-Hussain SH, Annuar BO, Din LB, Ali AM, Ross D (2003) Loss of mitochondrial transmembrane potential and caspase-9 activation during apoptosis induced by the novel styryl-lactone goniothalamin in HL-60 leukemia cells. Toxicol In Vitro 17(4): 433–9.

INCA (2015) Estimate 2016: Cancer Incidence in Brazil. National Cancer Institute José Alencar Gomes da Silva, Rio de Janeiro.

Jendrossek V (2013) Targeting apoptosis pathways by Celecoxib in cancer. Cancer Lett 332(2): 313–24.

Jeon YW, Ahn YE, Chung WS, Choi HJ, Suh YJ (2015) Synergistic effect between celecoxib and luteolin is dependent on estrogen receptor in human breast cancer cells. Tumour Biol 36(8): 6349–59.

Jia Y, Liu X, Yan JY, Chong LM, Li L, Ma AC, Zhou L, Sun ZY (2015) The alteration of inflammatory markers and apoptosis

on chronic prostatitis induced by estrogen and androgen. Int Urol Nephrol 47(1): 39–46.

Junqueira LCU, Bignolas G, Brentani RR (1979) Picrosirius staining plus polarization microscopy, a specific method for collagen detection in tissue sections. Histochem J 11((4)): 447–55.

Kaplan-Lefko PJ, Chen TM, Ittmann MM, Barrios RJ, Ayala GE, Huss WJ, Maddison LA, Foster BA, Greenberg NM (2003) Pathobiology of autochthonous prostate cancer in a pre-clinical transgenic mouse model. Prostate 55(3): 219–37.

Kido LA, Montico F, Sauce R, Macedo AB, Minatel E, Costa DB, Carvalho JE, Pilli RA, Cagnon VH (2016) Anti-inflammatory therapies in TRAMP mice: Delay in PCa progression. Endocr Relat Cancer 23(4): 235–50.

Kido LA, Montico F, Vendramini-Costa DB, Pilli RA, Cagnon VHA (2017) Goniothalamin and celecoxib effects during aging: Targeting pro-inflammatory mediators in chemoprevention of prostatic disorders. Prostate 77(8): 838–48.

Marker PC, Donjacour AA, Dahiya R, Cunha GR (2003) Hormonal, cellular, and molecular control of prostatic development. Dev Biol 253(2): 165–74.

Montico F, Kido LA, San Martin R, Rowley DR, Cagnon VHA (2015) Reactive stroma in the prostate during late life: The role of microvasculature and antiangiogenic therapy influences. Prostate 75(14): 1643–61.

Narayanan BA, Narayanan NK, Pttman B, Reddy BS (2006) Adenocarcina of the mouse prostate growth inhibition by celecoxib: Downregulation of transcription factors involved in COX-2 inhibition. Prostate 66(3): 257–65.

Niu Y, Altuwaijri S, Yeh S, Lai K-P, Yu S, Chuang K-H, Huang S-P, Lardy H, Chang C (2008) Targeting the stromal androgen receptor in primary prostate tumors at earlier stages. Proc Natl Acad Sci USA 105(34): 12188–93.

Palapattu GS, Sutcliffe S, Bastian PJ, Platz EA, De Marzo AM, Isaacs WB, Nelson WG (2005) Prostate carcinogenesis and inflammation: Emerging insights. Carcinogenesis 26(7): 1170–81.

Pan Y, Zhang JS, Gazi MH, Young CYF (2003) The cyclooxygenase 2-specific nonsteroidal anti-inflammatory drugs celecoxib and nimesulide inhibit androgen receptor activity via induction of c-Jun in prostate cancer cells. Cancer Epidemiol Biomarkers Prev 12(8): 769–74.

Pienta KJ, Esper PS (1993) Risk factors for prostate cancer. Ann Intern Med 118(10): 793–803.

Pihie AH, Stanslas J, Din LB (1998) Non-steroid receptor-mediated antiproliferative activity of styrylpyrone derivative in human breast cancer cell lines. Anticancer Res 18(3a): 1739–43.

Prins GS, Birch L, Greene GL (1991) Androgen receptor localization in different cell types of the adult rat prostate. Endocrinology 129(6): 3187–99.

Robinette CL (1988) Sex-hormone-induced inflammation and fibromuscular proliferation in the rat lateral prostate. Prostate 12(3): 271–86.

Rowley DR (1999) What might a stromal response mean to prostate cancer progression? Cancer Metastasis Rev 17(4): 411–9.

Sam TW, Sew-Yeu C, Matsjeh S, Gan EK, Razak D, Mohamed LA (1987) Goniothalamin oxide: An embryotoxic compound from Goniothalamus macrophyllus (Annonaceae). Tetrahedron Lett 28(22): 2541–4.

Sano H, Kawahito Y, Wilder RL, Hashiramoto A, Mukai S, Asai K, Kimura S, Kato H, Kondo M, Hla T (1995) Expression of cyclooxygenase-1 and -2 in human colorectal cancer. Cancer Res 55(17): 3785–9.

Sciarra A, Mariotti G, Salciccia S, Autran A, Monti S, Toscano V, Di F (2008) Prostate growth and inflammation. J Steroid Biochem Mol Biol 108: 254–60.

Siegel RL, Miller KD, Jemal A (2017) Cancer statistics, 2017. CA Cancer J Clin 67(1): 7–30.

Silva RF, Nogueira-Pangrazi E, Kido LA, Montico F, Arana S, Kumar D, Raina K, Agarwal R, Cagnon VHA (2017) Nintedanib antiangiogenic inhibitor effectiveness in delaying adenocarcinoma progression in Transgenic Adenocarcinoma of the Mouse Prostate (TRAMP). J Biomed Sci 24(1): 31.

SÍusarz A, Jackson GA, Day JK, Shenouda NS, Bogener JL, Browning JD, Fritsche KL, MacDonald RS, Besch-Williford CL, Lubahn DB (2012) Aggressive prostate cancer is prevented in ERÍKO mice and stimulated in ERαKO TRAMP mice. Endocrinology 153(9): 4160–70.

Sozer S, Diniz G, Lermioglu F (2011) Effects of celecoxib in young rats: histopathological changes in tissues and alterations of oxidative stress/antioxidant defense system. Arch Pharm Res 34(2): 253–9.

Suttie A, Nyska A, Haseman JK, Moser GJ, Hackett TR, Goldsworthy TL (2003) A grading scheme for the assessment of proliferative lesions of the mouse prostate in the TRAMP model. Toxicol Pathol 31(1): 31–8.

Thapa D, Ghosh R (2015) Chronic inflammatory mediators enhance prostate cancer development and progression. Biochem Pharmacol 94(2): 53–62.

Torre LA, Siegel RL, Ward EM, Jemal A (2016) Global cancer incidence and mortality rates and trends—An update. Cancer Epidemiol Biomarkers Prev 25(1): 16–27.

Tsujii M, Kawano S, DuBois RN (1997) Cyclooxygenase-2 expression in human colon cancer cells increases metastatic potential. Proc Natl Acad Sci USA 94(7): 3336–40.

Tuxhorn JA, Ayala GE, Rowley DR (2001) Reactive stroma in prostate cancer progression. J Urol 166(6): 2472–83.

Tuxhorn JA, Ayala GE, Smith MJ, Smith VC, Dang TD, Rowley DR (2002) Reactive stroma in human prostate cancer: Induction of myofibroblast phenotype and extracellular matrix remodeling. Clin Cancer Res 8(9): 2912–23.

Vendramini-Costa DB, Carvalho JE (2012) Molecular link mechanisms between inflammation and cancer. Curr Pharm Des 18(26): 3831–52.

Vendramini-Costa DB, de Castro IB, Ruiz AL, Marquissolo C, Pilli RA, de Carvalho JE (2010) Effect of goniothalamin on the development of Ehrlich solid tumor in mice. Bioorg Med Chem 18(18): 6742–7.

Vendramini-Costa DB, Francescone R, Posocco D, Hou V, Dmitrieva O, Hensley H, de Carvalho JE, Pilli RA, Grivennikov SI (2017) Anti-inflammatory [Q5]natural product goniothalamin reduces colitis-associated and sporadic colorectal tumorigenesis. Carcinogenesis 38(1): 51–63.

Vendramini-Costa DB, Monteiro KM, Iwamoto LH, Jorge MP, Tinti SV, Pilli RA, de Carvalho JE (2014) Gastroprotective effects of goniothalamin against ethanol and indomethacin-nduced gastric lesions in rats: Role of prostaglandins, nitric oxide and sulfhydryl compounds. Chem Biol Interact 224: 206–12.

Vendramini-Costa DB, Spindola HM, de Mello GC, Antunes E, Pilli RA, de Carvalho JE (2015) Anti-inflammatory and antinociceptive effects of racemic goniothalamin, a styryl lactone. Life Sci 139: 83–90.

Wakabayashi K (2000) NSAIDs as cancer preventive agents. Asian Pac J Cancer Prev 1(2): 97–113.

Weibel ER (1963) Principles and methods for the morphometric study of the lung and other organs. Lab Invest 12: 131–55.

Weihua Z, Makela S, Andersson LC, Salmi S, Saji S, Webster JI, Jensen EV, Nilsson S, Warner M, Gustafsson JA (2001) A role for estrogen receptor beta in the regulation of growth of the ventral prostate. Proc Natl Acad Sci USA 98(11): 6330–5.

Yu S, Zhang C, Lin CC, Niu Y, Lai KP, Chang HC, Yeh SD, Chang C, Yeh S (2011) Altered prostate epithelial development and IGF-1 signal in mice lacking the androgen receptor in stromal smooth muscle cells. Prostate 71(5): 517–24.

Zar JH (1999) Biostatistical Analysis. New Jersey: Prentice Hall Upper.

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