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ORIGINAL ARTICLE

Coconut Oil Mitigates the Effects of Aging on the Mongolian Gerbil Prostate

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ABSTRACT

Background: Benign prostatic hyperplasia (BPH) is a disease linked to the hormonal imbalance that occurs during aging and over the last decades, complementary and alternative medicines have come on the scene as a treatment option for BPH, such as herbal medicines. Coconut oil has been shown to be capable of interfering in testosterone-induced BPH. However, until now there is no study of the effect of coconut oil during aging. The present study evaluated the effect of the intake of coconut oil on the prostate of aging gerbils (*Meriones unguiculatus*).

Methods: Two experimental groups were assigned: Gavage control (GC—animals subjected to gavage with water for 1 year, $n = 11$) and coconut oil (CO—animals subjected to gavage with coconut oil for 1 year, $n = 11$). Testosterone, and estradiol serum levels were determined by ELISA assay and histopathological analysis employed Hematoxylin–Eosin. Cell proliferation index was determined by PHH3 immunohistochemistry and TUNEL assay and receptors of androgen (AR) and estrogen (ERα and ERβ) were evaluated on the prostate.

Results: The CO group exhibited a lower prostate weight (↓16.62%), decreased thickness of the prostate muscle stroma (↓18.27%), reduced expression of both AR (↓51.32) and ERα (↓14.26%) and reduced the percentage of BPH (↓1.53%) and intraepithelial neoplasms in the prostate (↓14.24%). Coconut oil intake mitigated age-related changes and increased the rate of apoptosis in prostatic cells (↑54.32).

Conclusions: Coconut oil treatment throughout aging helped counteract the negative effects of aging on prostate health.

1 | Introduction

The elderly population is increasing and it is expected that, by 2050, 16% of the global population will be over the age of 65 [1]. Aging is a complex process, highly variable among different species and strongly controlled by many genes, being susceptible to several factors [2]. Some lifestyle-related risk factors, such as smoking, alcohol consumption, a lack of physical activity, and chronic psychological stress are pointed out as "aging accelerators" [3–5]. For this reason, the search for improvements in the aging process has grown in recent decades.

Benign prostatic hyperplasia (BPH) is an aging-related disease and affects more than 50% of men over 50 years old [6], and

recent trends in the United States, Europe and other regions suggest that its incidence and prevalence will increase due to the increase in the elderly population [7]. BPH is influenced by several hormonal mechanisms, in particular the androgens and their receptor activities, growth factors, estrogen metabolism, chronic inflammation, and metabolic syndrome [8]. Therefore, the pursuit of updated information on BPH and the formulation of innovative treatment strategies has increased.

The various options provided by the pharmaceutical industry pose the challenge of achieving the appropriate medical therapy for patients with BPH due to the final balance between efficacy, adverse effects, and costs, which is often difficult to achieve [9]. Over the last decades, complementary and alternative medicines like phytotherapeutical agents have emerged [10]. Approximately 30 phytotherapeutic compounds are used both to "maintain a healthy prostate" and for the treatment of hypertension, such as *Serenoa repens*, *Hypoxis rooperi*, *Secale cereale* [11]. The therapeutic properties of these phytotherapeutic agents in the management of BPH have been primarily ascribed to their fatty acid composition, particularly lauric acid and myristic acid [12]. Coconut oil has been shown to reduce prostate gland enlargement in animals with testosterone-induced prostatic hyperplasia, as observed in adult Sprague–Dawley rats [13] and can influence intra-prostatic testosterone levels [14]. Coconut oil, obtained from the *Cocos nucifera*, is an emerging functional food commonly used as traditional medicine and a dietary supplement in southern India and many other tropical countries and is known to reverse the condition of liver lipotoxicity and prevent insulin resistance [15]. Certain fatty acids, such as lauric acid found in coconut oil, may interfere with androgenic action mechanisms [16, 17]. Oleic and linoleic acids present in coconut oil have been shown to inhibit the proliferation of prostate cancer cells (LNCaP), with this effect primarily attributed to the inhibition of the 5α-reductase enzyme and downregulation of expression of PSA mRNA. In this sense, it is possible that inhibitory action of these fatty acids blocks the conversion of testosterone to 5α-dihydrotestosterone (DHT) and then affects the proliferation of prostate cancer cells [18].

The prostate of the Mongolian gerbil (*Meriones unguiculatus*) is responsive to the intake of oils from various sources [19]. The Mongolian gerbil spontaneously develops BPH, prostatic lesions, and adenocarcinomas during aging [20]. These prostatic disorders are linked to hormonal changes, especially of androgens and estrogens, as well as the expression of their corresponding receptors in the prostate [21]. Accordingly, it is suggested that the consumption of coconut oil may contribute to the reduction of BPH in Mongolian gerbils throughout the aging process. Our hypothesis predicts that the anti-androgenic activity of the fatty acids in coconut oil helps to inhibit proliferation by modulating steroid receptors. The present study, therefore, sought to evaluate the effect of coconut oil on the gerbil prostate throughout aging.

2 | Materials and Methods

2.1 | Ethics Approval Statement

The procedures were performed in accordance with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) and the National Council of Animal Experiment Control (CONCEA) and were authorized by the Ethics Committee on the Use of Animals (CEUA) from Institute of Biosciences, Humanities and Exact Sciences, São Paulo State University (IBILCE/UNESP) (protocol number 183/2018).

2.2 | Experimental Design and Sample Collection

The animals were kept in ventilated mini-isolators (two animals per box) under controlled light and temperature conditions (12 h light/12 h dark—24°C) with water and chow (LABIMIX-Ratos e Camundongos; Agromix Industria e Comercio de Alimentos Ltda., composed of folic acid, butylated toluene hydroxide, biotin, calcitic limestone, sodium chloride, dextrin, rice, soybean, wheat, corn, meat and bone flour, fish's flour, grass silage bicalcico phosphate, calcium iodate lysine, methionine, choline chloride, niacin, degummed soybean oil, magnesium oxide, calcium pantothenate, sodium selenite, cobalt sulphate, iron sulphate, manganese sulphate, zinc sulphate, vitamin (A, B1, B12, B2, B3, E, K3) ad libitum. All experimental procedures were performed between 7:00 a.m. and 10:00 a.m. For the experiment, 22 male Mongolian Gerbils were equally and randomly divided into two groups, Gavage control (GC, animals received 0.1 mL of water via gavage) and Coconut oil (CO, animals received 0.1 mL of coconut oil* via gavage). *Dose equivalent to 13 mL for an adult man weighing 80 kg [22]. The experimental procedures started at 100 days of age, all the animals were weighed, and the GC and CO groups received oral gavage every other day regimen for 1 year and all animals were euthanized at 465 days of age. At the end of the experiment, animals were anaesthetized with Xylazine hydrochloride (3 mg/kg) and Ketamine hydrochloride (10 mg/kg), weighed for biometric analysis ($n = 11$) and euthanized by decapitation for blood collection and prostate harvested entirely. The blood was centrifuged, and serum stored at 80°C. Prostates were weighed, and ventral lobes used for morphological and protein analysis. Tissues were either frozen immediately in liquid nitrogen ($n = 6$) or fixed by immersion in 4% formaldehyde prepared in phosphate buffer, pH 7.2, for 24 h ($n = 5$). Following the formaldehyde fixing process, samples were washed with distilled water, dehydrated in an alcohol battery, immediately processed, and included in Histosec (Merck, Germany).

2.3 | Fatty Acids Methyl Esters (FAMEs) of Coconut Oil Analysis

Coconut oil (Nature Corp LTDA, Sorocaba, Brazil) sample preparations were conducted into a derivation process using 10% (w/w) methanolic solution of boron trifluoride (Supelco, USA, CAS no. 7637-07-2) under N2 atmosphere, according to Joseph & Ackman [23]. It weighed around 6–12 mg of fat in a test tube. Then 1.5 mL of 0.5 mol L^{-1} methanolic solution of sodium hydroxide (Mallinckrodt, England, CAS no. 1310-73-2) was added to the sample tube and this mixture was kept at 100°C for 5 min, followed by cooling step at room temperature. To continue the procedure, 2 mL of BF3 in methanol was added to the sample tube and the solution was warmed at 100°C for more than 30 min. After cooling at room temperature, 1 mL of

isooctane (Merck, Germany, CAS no. 540-84-1) was added to the sample tube and it was vortexed for 30 s. Then 5 mL of saturated aqueous solution of sodium chloride (Sigma Aldrich, USA, CAS no. 7647-14-5) was added and it was vortexed again for 30 s. It was observed the formation of two phases, from which it was collected the superior phase (the organic one). Analyses were performed in triplicate ($n = 3$). For quantification reasons, it was necessary that derived samples have had 1 mg mL^{-1} of methyl tricosonoate (C23:0Me), used as internal standard (IS). Proper volume of 5 mg mL^{-1} of stock solution was added to the derivate sample to obtain a final concentration of 1 mg mL^{-1} of IS.

FAME analyses were performed using a gas chromatography system (model GC-2014, Shimadzu) with flame ionization detector (FID). Separations conditions used was as described: NukolTM capillary column (30 m × 0.25 mm d.i. × 0.25 μm); injector temperature at 220°C; injection volume of 1 μL; split ratio of 1:10; N2 flow; oven isothermal ramp at 200°C for 35 min; detector temperature at 220°C. A standard solution of FAME (GLC-85, Nu-check, EUA) was injected under the same running conditions. Chromatograms of the sample were manually integrated using the software GC Solution (Shimadzu). FAME amount present in fat was calculated using chromatogram's area for each peak (each component).

2.4 | Hormonal Analysis

Serum hormone levels of experimental groups ($n = 8$) were measured by enzyme-linked immunosorbent assay using high sensitivity specific commercial kits: Testosterone (RE 52151, Lot ETE 131, Standard range 0.2–16 ng/mL, IBL International GMBH, Hamburg, Germany) and 17-β-estradiol (RE 52041, Lot 42K079, Standard range 25–2000 pg/mL, IBL International GMBH, Hamburg, Germany). The procedures were carried out according to the manufacturer instructions and the readings determined in a microplate reader (TECAN—Infinite F50).

2.5 | Morphological Analysis

Tissues were sectioned at 4 μm, and slides were stained with hematoxylin–eosin (HE) and Gomori's reticulin for general histology. Slides were analyzed with an Olympus BX60 light microscope (Olympus, Japan), and the images were digitized with DP-BSW V3.1 software (Olympus, Japan). Stereological analysis of the prostate is well-established [24–26]. The relative frequency of prostatic compartments (epithelium, lumen, smooth muscle layer—smooth muscular layer [SML], non-muscular stroma, and blood vessels) was obtained by Image-ProPlus 6.1 software (Media Cybernetics, Silver Spring, MD) with Weibel's multipurpose graticulate with 130 points [27] and 25 test lines (5 per animal, $n = 5$). We determined the relative frequency by counting the coincident points in the test grid and dividing them by the total number of points. Height of the secretory epithelial cells (μm) and thickness of the smooth muscle layer (μm) were assessed in the histological sections using 200 measurements for each experimental group (40 per animal, $n = 5$).

2.6 | Incidence and Multiplicity of Lesions

Prostatic proliferative disorders incidence and multiplicity rates were obtained by histopathological analysis of three slides, from different histological depths, from each animal ($n = 15$/group) and tissue sections examined by investigators blindly. Samples were stained by HE and digitized at ×400 magnification using the slide scanner system (Olympus VS120-S5). Prostatic lesions classification was based on the well-established criteria of Bar Harbor [28], previously applied in the Mongolian gerbil [20, 29, 30]. The incidence was calculated as % = (number of animals with changes/number of animals per group), while the multiplicity as (number of changes in each animal/number of slides). The percentage of the area affected by prostatic lesions was calculated by dividing the area of each lesion by the total area of the histological section examined. The following prostatic lesions were considered: prostatic epithelial hyperplasia (PEH)—area of the epithelium with cell aggregation, stratification, and irregularly shaped cells; Grade I prostatic intraepithelial neoplasia (PIN I)—areas with clustering of cells with enlarged nuclei of varying size, dense chromatin, and an occasional large and prominent nucleolus.; Grade II prostatic intraepithelial neoplasia (PIN II)—Similar to PIN I but with nuclei exhibiting strongly dense chromatin and frequently large nucleoli, basal cell discontinuity, and invasion of other prostatic compartments without muscular stroma disruption; prostatic cribriform intraepithelial neoplasia (PINC)—epithelium exhibiting PIN characteristics but displayed as micro-acini.; atrophy (ATR)—area with thinning of the epithelium and muscular stroma; microinvasive carcinoma (MC)—similar to PIN II but with micro vessels and rupture of the adjacent muscular layer confirmed by immunohistochemistry staining for α-actin and reticular fibers disruption. The examples of lesions evaluated are depicted in Figure 1.

2.7 | Immunohistochemistry

Anti-α-actin (mouse monoclonal, 1A4, dilution 1:100, sc-32251, Lot BO422, Santa Cruz Biotechnology, Dallas, TX, USA); anti-PCNA (PCNA, mouse monoclonal, PC10, dilution 1:75, sc-56, Santa Cruz Biotechnology, CA, USA); anti-AR (rabbit polyclonal IgG, N-20, dilution 1:75, sc-816, Lot G1714, Santa Cruz Biotechnology, Dallas, TX, USA); anti-ERα (mouse monoclonal, IgG, D12, dilution 1:50, sc-8005, Lot IO123, Santa Cruz Biotechnology, CA, USA), and anti-ERβ (mouse monoclonal IgG, 1531, dilution 1:50, sc-53494, Lot H2013, Santa Cruz Biotechnology, CA, USA) were used as primary antibodies. For the analysis, tissue sections (4 mm) of formalin-fixed, paraffin-embedded gerbil prostatic ventral lobe collected in silanized glass slides, were deparaffinized, rehydrated through graded alcohol, and then subjected to antigen retrieval by tissue sections embedding in 10 mM citrate buffer (pH 6.0) for α-actin, PCNA, AR and ERβ; and TRIS-EDTA buffer (pH 9) for ERα. Temperature and time for retrieval were 96°C for 40 min for AR, and ERβ, 93°C for 20 min for α-actina and PCNA, and a microwave sequence (2 × 7 min at low power—400 W and 1 × 7 min at medium-low power—600 W) for ERα. Endogen peroxidases were blocked by immersing the slides in 3% H_2O_2 at room temperature for 30 min. Then, nonspecific proteins were blocked with 5% skimmed milk in TBS. Sections were incubated

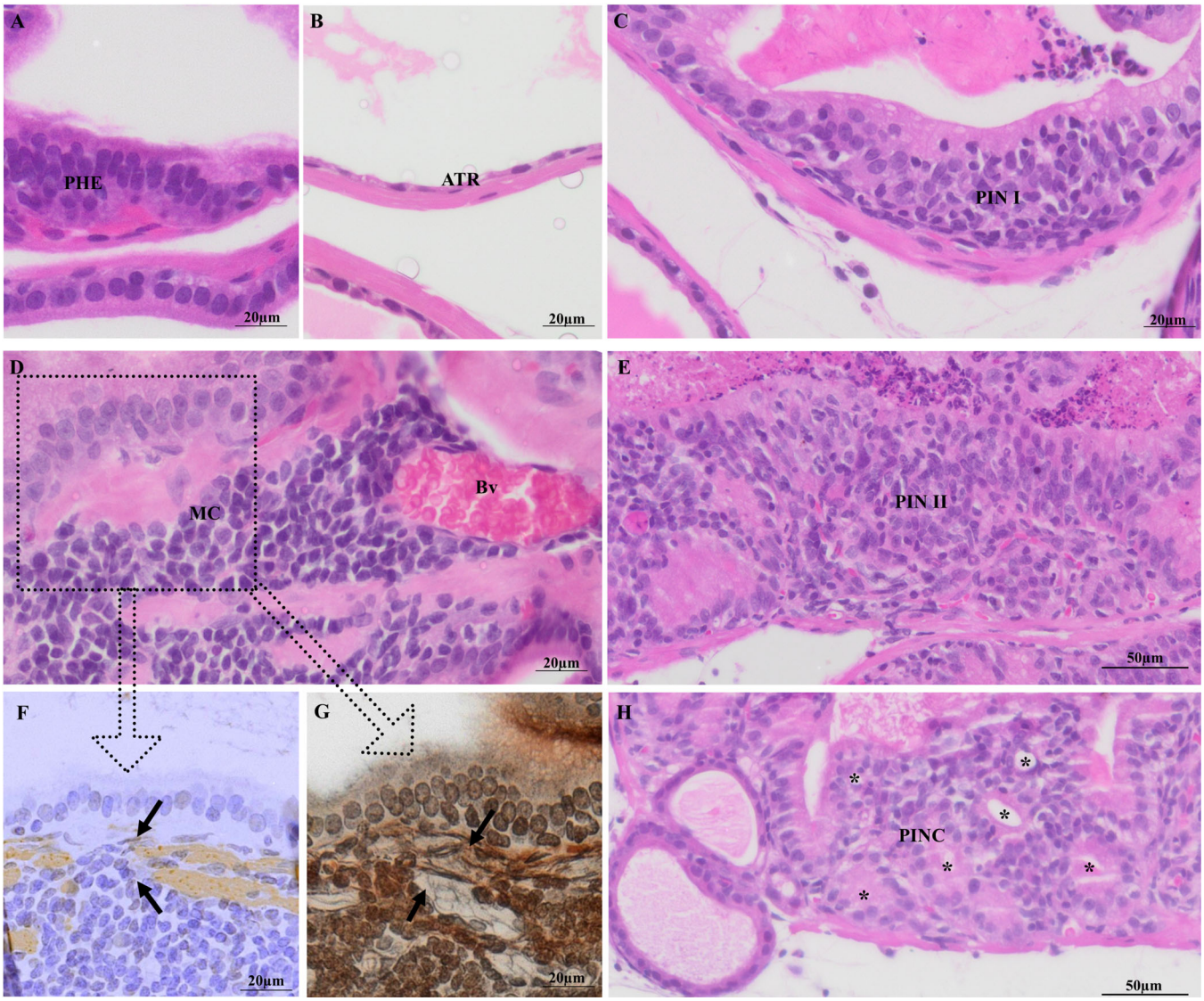


FIGURE 1 | Lesions observed in the ventral prostate of old gerbils stained with HE (A, B, C, D, E, and H), with Gomori's reticulin (G) and marking for α-actin (F). Prostatic epithelial hyperplasia—PEH (A); Epithelial atrophy—ATR (B); Grade I intraepithelial neoplasia— PIN I (C); Microinvasive carcinoma—CM (D); Grade II intraepithelial neoplasia—PIN II (E); Cribriform intraepithelial neoplasia—PINC (H) with microlumens (*). The region delimited by the dotted line in D is depicted in F and G. Arrows point to the rupture of the smooth muscle layer in F and to the rupture of reticular fibers in G. Bv indicates blood vessels. [Color figure can be viewed at wileyonlinelibrary.com]

overnight at 4°C (AR, ERα and ERβ) or for 1 h at room temperature (α-actin and PCNA) before secondary antibodies (Polymer kit Novocastra Novolink RE7230-CE, Leica Biosystems, Buffalo Grove, USA) were applied for 1 h at room temperature. Positive staining was detected using 3-30′-diaminobenzidine tetrahydrochloride (DAB) chromogen (NovolinkMaxDAB, RE7270-CE, Leica Biosystems, Buffalo Grove, USA) and counterstained with hematoxylin. The AR, ERβ and ERα staining patterns are shown in Figures 4A,B, 5AB and 7A,B, respectively.

2.8 | Western blot

Frozen ventral prostates (six per group) were smashed in liquid nitrogen in a crucible with a pistil, and after evaporation of the nitrogen, the samples were incubated for extraction (CelLytic MT cell lysis reagent, C3228, Sigma Aldrich) at 4°C, for 60 min, under agitation. Protease activity was blocked by inhibitors (Protease Inhibitor Cocktail, P8340, Sigma Aldrich), followed by centrifugation for 20 min at 14,000 rpm, 4°C. protein quantification was determined by BCA Protein Assay Kit (Pierce BCA Protein Assay Kit, 23,227, Thermo Fischer Scientific) and the absorbance measured in a microplate reader (SPECTROstar Omega, BMG Labtech). The extracts were stored at −80°C until analysis. For SDS-PAGE electrophoresis, 15 µg of sample was loaded in each lane. The bands were transferred to a nitrocellulose membrane. Blots were blocked using skimmed milk 5% in TBS + 0.1% Tween (TBSt), for 1 h, at room temperature. Overnight incubation under agitation with anti-AR (rabbit polyclonal IgG, N-20, dilution 1:300, sc-816, Lot G1714, Santa Cruz Biotechnology, Dallas, TX, USA);, ERα (mouse monoclonal, IgG, D12, dilution 1:1000, sc-8005, Lot IO123, Santa Cruz Biotechnology, CA, USA), ERβ (mouse monoclonal IgG, 1531, dilution 1:1000, sc-53494, Lot H2013, Santa Cruz Biotechnology,

CA, USA) and GAPDH (monoclonal rabbit, IgG, dilution 1:1000, Lot 2118, Cell Signalling, Danvers, MA, USA,) primary antibodies, diluted in skimmed milk 1% in TBSt. Both anti-AR and anti-ERα antibodies were incubated in the same membrane; to do this, the membranes were stripped in stripping buffer (1.5% Glycine, 1% SDS, 1% Tween in ultrapure water, pH 2.2) for 15 min at room temperature, washed thrice with TBSt and blocked again. After incubation with the primary antibody the membranes were incubated at room temperature for 1 h under agitation with horseradish peroxidase-conjugated secondary antibodies (rabbit anti-mouse dilution 1:10.000, ab6728, Abcam, Cambridge, UK, or goat anti-rabbit, dilution 1:10.000 #7074, Cell Signalling, Danvers, MA, USA). Band visualization was performed with ECL system (32,109, Thermo Fischer) using ChemiDoc (ChemiDoc MP, BioRad) followed by quantification with ImageJ. Values were shown as mean of relative density to GAPDH (loading control). The membrane images (Figures 4C, 5C and 6C) were cut and adjusted in brightness for optimal visualization of the bands.

2.9 | Cell Proliferation Index

Immunohistochemistry was performed for PCNA as a marker of cell proliferation, according to the procedures described in the section "Immunohistochemistry" mentioned thereafter. To access the apoptosis rate, TUNEL assay was performed with ApopTag commercial kit (ApopTag Plus in situ, Apoptosis Detection Kit, Millipore, S7101, CA, USA) to detect DNA fragmentation following the manufacturer instructions. Apoptosis and proliferation frequencies were determined as the ratio of marked cells to the number of total cells. At least 6000 cells were counted in the epithelium and SML per group ($n = 5$). The prostatic compartments epithelium and SML were analyzed singly (Epi and SML, respectively) and together (Epi + SML). The staining patterns were shown in Figure 3A,C for PCNA and 3B,D for TUNEL.

2.10 | Hormonal Receptor Expression Analysis

Androgen receptor (AR) and estrogen receptors alpha and beta (ERα and ERβ) expression were assessed by immunohistochemistry ($n = 5$) and Western blotting ($n = 6$). In the immunohistochemistry analysis, the positive cells frequency was determined by the ratio immunolabeled cells to the total number of cells of the epithelium (Epi), SML and epithelium and SML together (Epi + SML). Protein quantification was determined by relative density to GAPDH using ImageJ software (Wayne Rasband, Research Services Branch, National Institute of Mental Health, MD, USA).

2.11 | Statistical Analysis

Statistical analysis was performed using GraphPad Prism 6.00 software spreadsheets and graphs (GraphPad Software, San Diego, CA, USA). First, the data were checked for normality using the Kolmogorov–Smirnov test. Parametric data were analyzed by unpaired *t*-test with Welch's correction. For nonparametric data, the Mann–Whitney test was adopted. Variation in the incidence of prostate lesions was tested using the χ^2 test. Differences were considered statistically significant when $p < 0.05$.

3 | Results

3.1 | FAMEs Levels of Coconut Oil

The standard composition of coconut oil previously described [31, 32] was similar to the oil used in this experiment. The most abundant FAME in coconut oil was Methyl Laurate (50.33%), followed by Methyl Myristate (19.50%), Methyl Palmitate (9.83%), Methyl Oleate (6.56%) and Methyl Decanoate (5.86%) respectively. Other components were found in smaller amounts, such as Methyl Octanoate (6.61%), Methyl Stereate (3.03%), Methyl Linoleate (1.10%), Methyl Arachidate (0.07) and Methyl Palmitoleate (0.02). Details for the relative percentage and mass of each FAME and the correction factors employed were provided as Supporting Information.

3.2 | Weight and Hormonal Changes

At the commencement of the experiment, at the age of 100 days, no disparity in body mass among the experimental groups was observed in the animals (Table 1). At 465 days of age, the animals showed higher body mass compared to 100 days of age. No disparity in body mass among the experimental groups was observed in the animals (Table 1). Prostate weight and prostate relative weight decreased in the CO group (Mann–Whitney test; $p = 0.0035$ and *t*-test; $p = 0.0006$, respectively) (Table 1). Testosterone and estradiol levels remained unchanged (Table 1).

3.3 | General Morphological Features

All experimental groups showed PEH and PIN, resulting in the irregular appearance of the alveoli (Figure 1A,C). The SML was

TABLE 1 | Biometric and endocrine parameters of Mongolian gerbils in gavage control (GC) and coconut oil (CO) groups.

	Experimental groups	
	GC	CO
Biometric data (g)		
Body 100 days	72.33 ± 1.033	72.5 ± 1.049
Body 465 days	95.86 ± 11.90	96.59 ± 12.78
Prostate	0.0343 ± 0.0101	0.0286 ± 0.0057*
Relative prostate ($\times 10^{-3}$)	0.3552 ± 0.0702	0.2988 ± 0.0596*
Hormonal data		
Testosterone (ng/mL)	1.90 ± 0.37	1.68 ± 0.33
17β-estradiol (pg/mL)	53.41 ± 24.00	43.73 ± 18.24

Note: Values expressed as mean ± standard deviation.
*In the same row indicate statistical difference among the experimental groups.

also found to be irregular in many regions, notably proximal to the lesions (Figure 1E). In the CO group, the SML thickness decreased (*t*-test; $p = 0.0026$), as well as the SML frequency and epithelium frequency also decreased (Mann–Whitney test; $p = 0.0087$ and Mann–Whitney test; $p = 0.0108$) (Table 2).

TABLE 2 | Morphological parameters of Mongolian gerbil in the different experimental groups.

	Experimental groups	
	GC	**CO**
Morphometric data (μm)		
Epithelium height	20.58 ± 4.11	17.71 ± 1.57
SML thickness	14.01 ± 1.04	11.45 ± 1.16*
Stereological data (%)		
Epithelium	27.17 ± 10.60	18.49 ± 1.17*
Lumen	44.26 ± 11.76	53.54 ± 6.65
SML	17.38 ± 7.46	8.71 ± 2.07*
Stroma	14.92 ± 3.67	18.42 ± 6.49
Blood vessels	2.15 ± 1.93	0.83 ± 0.58

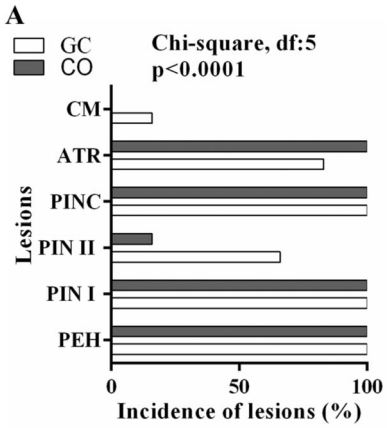
Note: Values expressed as mean ± standard deviation.
*In the same row indicate statistical difference among the experimental groups.

3.4 | Prostatic Lesions

The changes in the incidence of prostate lesions among the experimental groups was validated through the utilization of the χ^2 test (*df*: 5; $p < 0.0001$) (Figure 2A). All the experimental groups presented a high incidence of lesions, showing 100% of PEH, Grade I PIN I, and PINC (Figure 2A). However, the GC group showed the highest incidence of grade II prostatic intraepithelial neoplasia (PIN II), with a rate of 66.66%, and the lowest incidence of atrophy (ATR), with 83.33% (Figure 2A). On the other hand, the CO group had 16.66% of PIN II and 100% of ATR (Figure 2A). The incidence of microinvasive carcinoma (MC) for GC was 16.66% (Figure 2A). The multiplicity of the different lesions did not differ among the experimental groups (Figure 2B). The GC group exhibited a higher percentage of areas with PEH, PIN I, PIN II, and PINC compared to the CO group, which showed a higher percentage of ATR (Figure 3). Regarding MC, this type of lesion was only observed in the GC group (Figure 3).

3.5 | Cell Proliferation Index

The PCNA-positive cell frequency in SML of the CO group was higher than GC (Mann–Whitney test; $p = 0.0317$, but when



B

	Multiplicity	
	GC	**CO**
Lesions		
PHE	4-61 (39.83)	30-69 (46.33)
PIN I	4-15 (10)	4-13 (8.67)
PIN II	0-37 (7.33)	0-1 (0.16)
PINC	4-60 (27.17)	15-52 (25.5)
ATR	0-23 (13)	2-44 (22)
CM	0-3 (0.66)	0 (0)

FIGURE 2 | Analysis of incidence (A) and multiplicity (B) of lesions of the ventral prostate of the Mongolian Gerbil in Gavage Control (GC) or Coconut Oil (CO) groups. Incidence data (A) indicate the percentage of animals affected ($n = 5$) and statistical differences were found between the incidence of prostate lesions ($p < 0.0001$). Multiplicity data (B) corresponds to the variation in the number of lesions per animal ($n = 5$ per group); the group mean is shown in parenthesis. Prostatic epithelial hyperplasia (PEH); Grade I intraepithelial neoplasia (PIN I); Grade II intraepithelial neoplasia (PINII); Cribriform intraepithelial neoplasia (PINC); Epithelial atrophy (ATR) and Microinvasive carcinoma (CM).

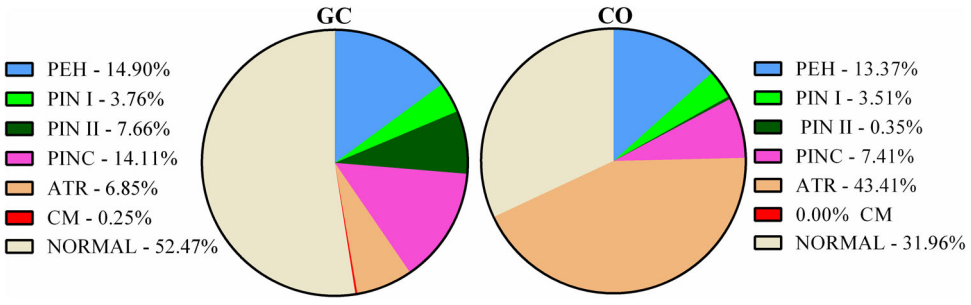


FIGURE 3 | Percentage area of PHE lesions (blue), PIN I (light green), PIN II (dark green), PINC (pink), ATR (beige), and normal prostate tissue (light gray). The percentages and corresponding graphs for the GC group (right) and CO group (left) are shown. [Color figure can be viewed at wileyonlinelibrary.com]

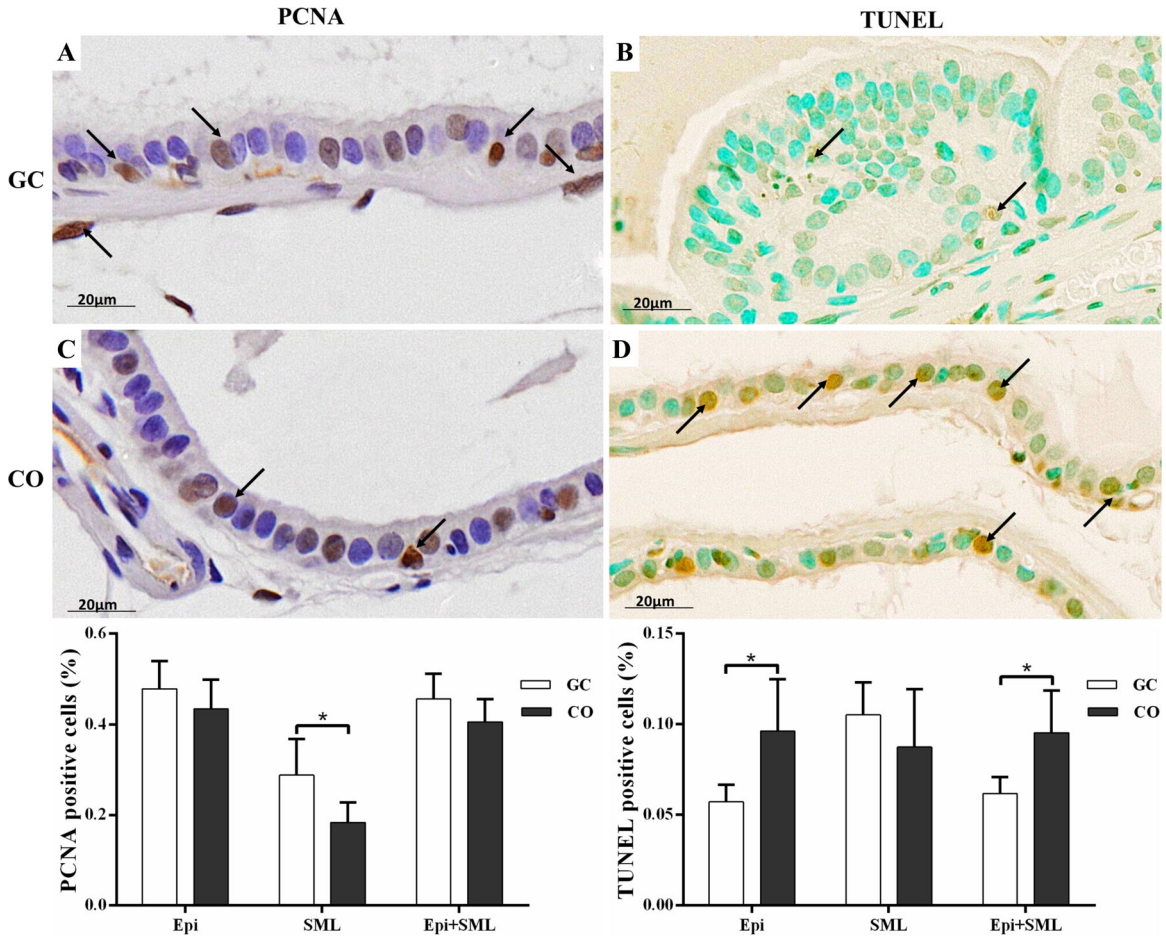


FIGURE 4 | Immunohistochemistry for cell proliferation index data using PCNA (A and C) for proliferating cells and TUNEL marking (B and D) for apoptotic cells. In E is shown the frequency of cells positive for PCNA, and in F, the frequency of cells positive for TUNEL in the epithelium (Epi), smooth muscle layer (SML) and epithelium plus smooth muscle layer (Epi + SML). *Statistical difference $p \leq 0.05$. Arrows point to positive cells. [Color figure can be viewed at wileyonlinelibrary.com]

combined with the epithelium (Epi + SML), there was no change (Figure 4E). The frequency of apoptotic cells of the CO group was higher than GC group in the epithelial compartment (*t*-test; $p = 0.0348$) and in the compartments analyzed together (Epi + SML) (*t*-test; 0.0290) (Figure 4F).

3.6 | Hormonal Receptor Expression

The CO group showed a decrease in AR expression (*t*-test; $p = 0.0351$) Figure 5E). This AR expression changed particularly in the SML (Mann–Whitney test; $p = 0.0317$) (Figure 5D). Regarding estrogen receptor beta (ERβ)-positive cells, the frequency decreased in the epithelium of the CO (Figure 6D), whereas estrogen receptor alpha (ERα)-positive cells showed a low frequency in epithelium and SML together (*t*-test; $p = 0.0481$) (Figure 7D).

4 | Discussion

The prostate is highly susceptible to aging and may respond differently to this process, leading to BPH, prostate enlargement, and prostate cancer progression [33]. In the present study we have investigated, for the first time, the effects of coconut oil consumption throughout the aging period on the prostate.

The research concerning the impact of coconut oil on weight loss is sparse. While there is some evidence indicating that the consumption of this oil may contribute to body mass reduction, this topic remains controversial [34]. A limitation in determining the potential of coconut oil for weight loss is the lack of long-term studies that provide robust evidence [35]. With advancing age, an increase in body weight is expected in gerbils [36], as observed in our experiment, and our data underscore the ineffectiveness of long-term coconut oil consumption in achieving weight loss.

In addition to increased body weight, increased prostate weight has also been observed in gerbils [36]. This increase in prostate weight is associated with the development of BPH and other prostatic lesions that occur spontaneously in old gerbils [20]. Long-term supplementation with coconut oil reduced the age-related weight increase in the prostate. The reduction in prostate weight as a result of coconut oil consumption was linked to a decrease in BPH [13]. Therefore, to determine whether these

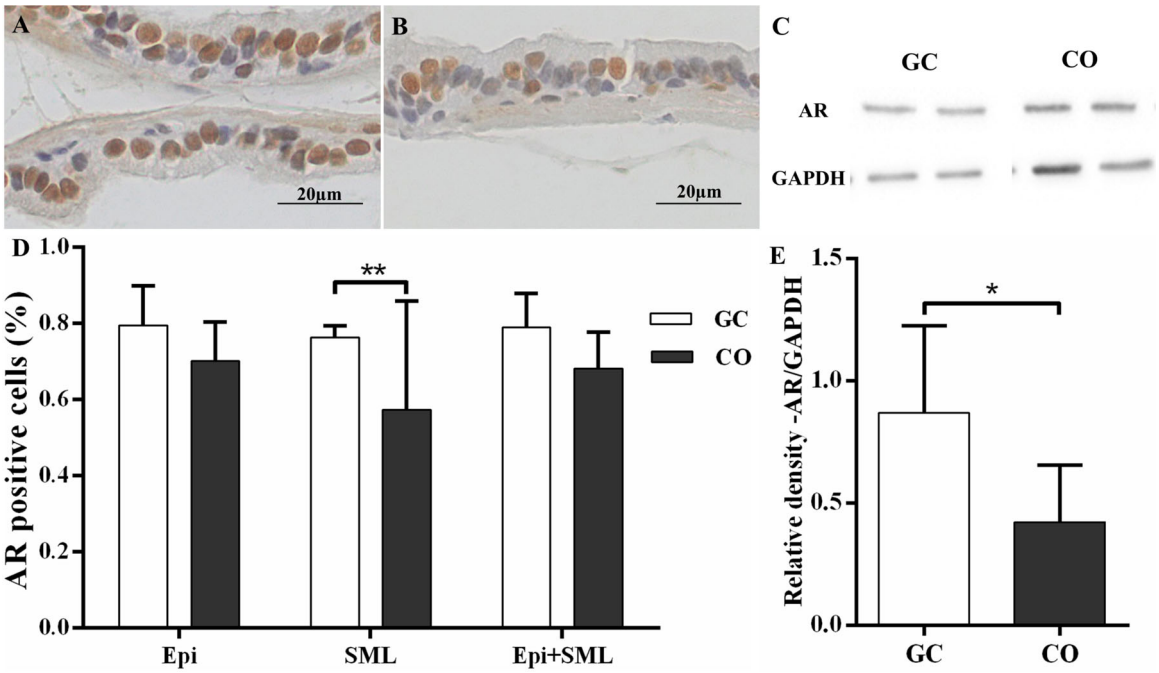


FIGURE 5 | Immunohistochemistry for androgen receptor (AR) in the ventral prostate of Mongolian gerbils, gavage control (A) and coconut oil-treated (B) groups. Frequency of cells positive for AR in the epithelium (Epi), smooth muscle layer (SML) and epithelium plus smooth muscle layer (Epi + SML) are shown in (D). Relative density of AR normalized to GAPDH, used here as a positive control (E), The blot images were cut and adjusted in brightness for optimal visualization of the bands (C). *Statistical difference $p \leq 0.05$ and **Statistical difference $p \leq 0.01$. [Color figure can be viewed at wileyonlinelibrary.com]

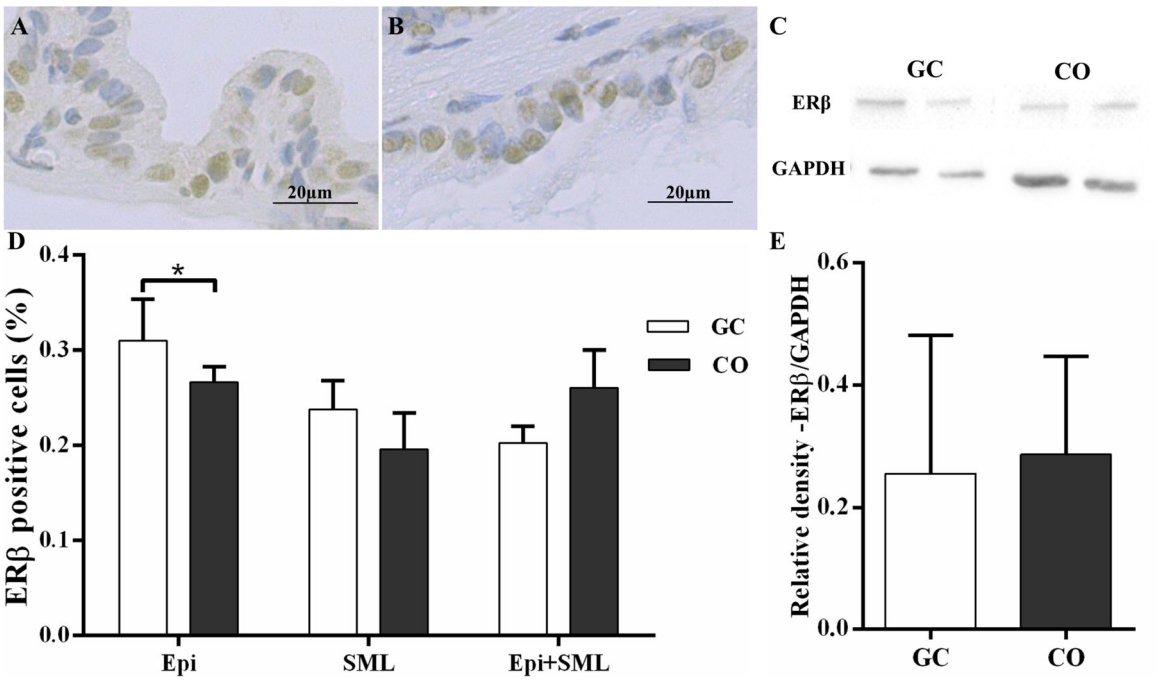


FIGURE 6 | Immunohistochemistry for estrogen receptor beta (ERβ) in the ventral prostate of Mongolian gerbils, gavage control (A) and coconut oil-treated (B) groups. Frequency of cells positive for ERβ in the epithelium (Epi), smooth muscle layer (SML) and epithelium plus smooth muscle layer (Epi + SML) are showed in (D). Relative density of ERβ normalized to GAPDH, used here as a positive control (E), The blot images were cut and adjusted in brightness for optimal visualization of the bands (C). *Statistical difference $p \leq 0.05$. [Color figure can be viewed at wileyonlinelibrary.com]

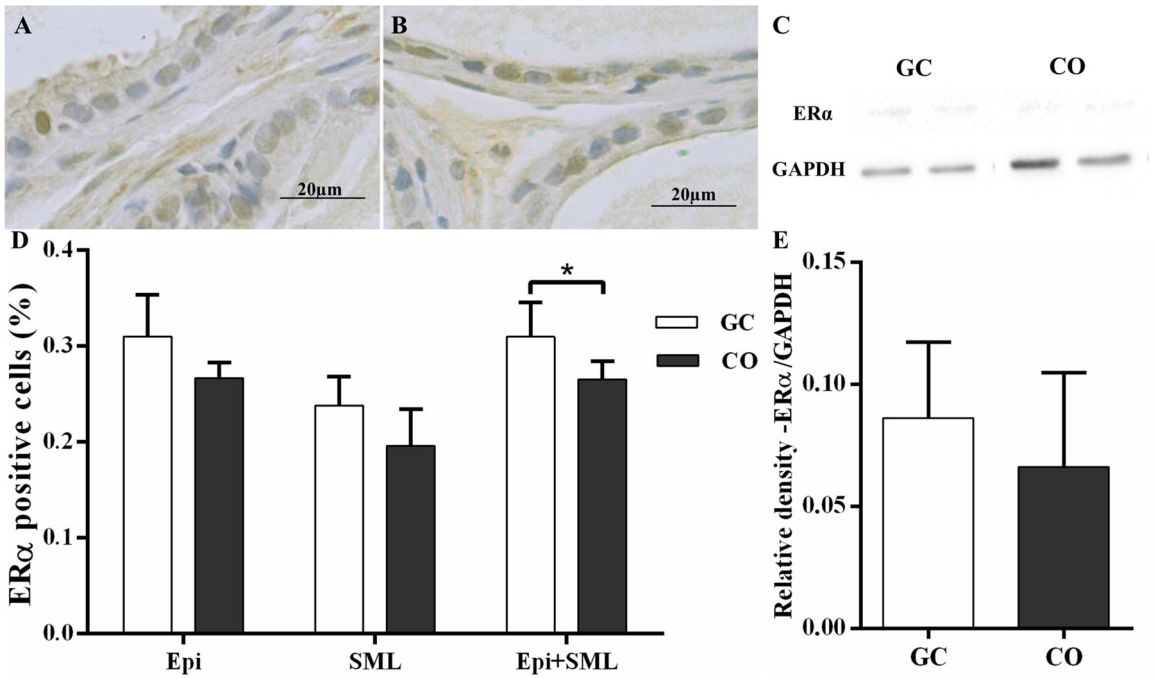


FIGURE 7 | Immunohistochemistry for estrogen receptor alpha (ERα) in the ventral prostate of Mongolian gerbils, gavage control (A) and coconut oil-treated (B) groups. Frequency of cells positive for ERα in the epithelium (Epi), smooth muscle layer (SML) and epithelium plus smooth muscle layer (Epi + SML) are showed in (D). Relative density of ERα normalized to GAPDH, used here as a positive control (E), The blot images were cut and adjusted in brightness for optimal visualization of the bands (C). *Statistical difference $p \leq 0.05$. [Color figure can be viewed at wileyonlinelibrary.com]

biometric alterations of the prostate in this study are linked to BPH, it was essential to assess prostate morphology.

The morphological characteristics of the Mongolian gerbil prostate are well established [20], these gland is highly responsive both to variations in androgen and estrogen levels, reflected in secretory gland activity [37, 38], and to interference from treatments using oils such as corn and mineral oil [19]. Some of these responses are morphological changes, such as an increase in SML thickness and an alteration in the frequency of prostatic compartments [38, 39]. Alterations in the cellular and extracellular matrix and their consequent increase are related to tumor progression [40]. Several studies have shown that variations in SML were related to smooth muscle cell activity and a change in the extracellular matrix [41, 42], and that older animals have reactive stroma associated with malignant prostatic lesions [43]. In the assessment of prostate tissue compartments, coconut oil demonstrated a beneficial effect by reducing both the height and frequency of the SML. Moreover, the elevated percentage of atrophic areas in this group may be attributed to specific underlying factors or inherent to the aging of the species.

During aging, testosterone release decreases and the prostate responds with pronounced morphological changes [44], which are also observed in Mongolian gerbil, which spontaneously exhibits prostatic lesions, including carcinoma, at an advanced age [20]. Our data corroborate the literature, since all the old animals in our study presented lesions such as PEH and PIN at distinct grades. However, coconut oil seemed to mitigate the gravity of these lesions, with a decrease in the incidence and percentage of areas of high-grade PIN, as well as the absence of adenocarcinoma.

Although spontaneous prostatic lesions are typically associated with age-related changes in hormone levels, the effects of coconut oil on the prostate appear to be mediated by mechanisms independent of systemic hormonal regulation. Possible mechanisms underlying the effects of coconut oil may be linked to its anti-inflammatory and antioxidant activities [45, 46]. Although the use of vegetable oils, such as coconut oil, may influence intra-prostatic testosterone levels [14], and lauric acid improves testosterone levels in serum and testis [47], the potential action of coconut oil on the prostate may involve its interaction with hormone receptors and their subsequent impact on cell survival. In female rats with polycystic ovarian syndrome, coconut oil restored the hypothalamic-pituitary-gonadal axis, with serum testosterone and estradiol levels similar to control rats [48]. Coconut oil supplementation activated the Nrf2 pathway to control the pro-oxidative response, resulting in higher catalase and SOD serum levels. Additionally, the anti-inflammatory properties were reported by decreasing the serum concentration of IL-1β [48].

The rate of proliferation and apoptosis is directly related to prostatic lesion development and aggressiveness in gerbils and other animals [20]. Considering the potential action of coconut oil as a lesion mitigator, the lower proliferation rate and thickness in the SML, this data suggest that this prostate compartment is more susceptible to coconut oil action. Prostate stromal components, cells and extracellular matrix are responsive to variations in hormone level and the expression of their

receptors, in addition to growth factors [49, 50]. The higher apoptosis rate when taking either the epithelium and SML together or the former alone, suggests that coconut oil also has an antiproliferative function. Its major component, lauric acid, has been reported to be an antiproliferative agent and apoptosis inducer in breast and endometrial cancer [51].

Lauric acid is thought to play a key role in the anti-BPH effect by inhibiting the activity of 5-α-reductase [52, 53]. In addition to lauric acid, other constituents of coconut oil, such as oleic acid and linoleic acid, have also been shown to inhibit the proliferation of androgen-sensitive prostate cancer cells and this effect is thought to be mediated through the inhibition of 5-α-reductase [54]. Other mechanisms through which lauric acid may contribute to the reduction of BPH include its antioxidative and anti-inflammatory properties. In rats with induced Parkinson's disease, lauric acid exhibited a neuroprotective mitigating TNF-α level, NF-κB, IL-8 mRNA expression [55]. This fatty acid also exerted a protective effect against hepatotoxicity by modulating levels of caspase-8/3, upregulating the Bcl-2 and HNF4α expressions, in addition to decreasing oxidative stress and inflammatory responses [56]. However, this is the first time the mechanism by which coconut oil and its components influence the prostate through the modulation of hormone receptor expression has been demonstrated.

The prostate is an androgen-dependent gland since its development and differentiation, as well as its maintenance in adulthood, are mediated via AR. Estrogens also influence prostate homeostasis since the prostate expresses both ERα and ERβ [57]. AR expression is directly related to prostate cell proliferation [58]. AR regulates proliferation and apoptosis through downstream signaling pathways, namely the genomic (classical) and non-genomic pathways. In the genomic pathway, upon binding to DHT, AR acts as a transcription factor, binding to androgen response elements in target genes, recruiting coactivators or corepressors, and modulating gene transcription [59]. In contrast, non-genomic pathways may involve the rapid activation of second messenger signaling cascades, such as the MAPK/ERK or PI3K/Akt pathways, or the modulation of transcription factors like AP-1 and Ets-related factors [60–62]. These processes can result in the repression of genes, including the nerve growth factor receptor (NGFR) and matrix metalloproteinases (MMP)-3, -7, and -13 [61]. In the Mongolian gerbil, under normal conditions, AR has a uniform immunolocalization between the epithelium and stroma, but this location standard can be modified by genetic and epigenetic mechanisms and as yet unknown factors [63]. Lauric acid, the main component of coconut oil, is able to inhibit the activity of 5-α-reductase and affects the AR signaling pathway [18]. Our data indicated that coconut oil influences the AR expression and, as previously mentioned, its effect was more pronounced in SML. However, about ERβ expression, the potential of coconut oil to reduce the expression of this receptor was observed only in the epithelium. Estrogenic action within the prostate is complex, and both have been reported to play different roles. ERα is related to the development and increase of proliferation, inflammation and cancer, while ERβ has antiproliferative, anti-inflammatory and anti-carcinogenic potential [64]. ERα expression thus consolidated our hypothesis that coconut oil plays an anti-lesional role since the CO group showed a lower frequency of ERα-positive cells. Interestingly, their immunolocalization was different from that of AR-positive cells. Studies regarding the localization of AR, ERα and ERβ expression in the gerbil ventral prostate suggest that AR is expressed in both the epithelium and SML, ERβ is found principally in the epithelium, and ERα in the SML [63, 65].

5 | Conclusions

As expected, the prostate in older gerbils showed PHE, PINs and carcinoma, which are aging-related alterations. However, the long-term coconut oil treatment shows potential benefits to the prostate and minimized the impact of aging. Although more studies are needed to elucidate the key mechanisms involved, our results are provocative, especially with regard to changes in morphology and receptor expression, principally in the stroma. Stromal alterations are common in older animals [43] and may drive malignant transformation in human prostatic epithelial cells [40]. Furthermore, it is important to note that the mechanisms of action of coconut oil still need to be better understood, suggesting that future research should explore potential site-specific, anti-inflammatory, and antioxidant mechanisms that may contribute to the reduction of lesions, without necessarily depending on changes in hormone levels and the expression of their receptors. Therefore, the effectiveness of a natural product, such as coconut oil, in mitigating the impact of aging on the prostate encourages further studies on this issue.

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Ethics Statement

The methodology for this study was approved by the Institutional Committee for Ethics in Animal Experimentation of the Institute of Biosciences, Letters and Exact Sciences/São Paulo State University (183/2018-CEUA-São José do Rio Preto).

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data used to support the findings of this study are available from the corresponding author upon request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.