Fitoterapia 124 (2018) 137–144

Contents lists available at ScienceDirect

Fitoterapia

journal homepage: www.elsevier.com/locate/fitote

Prepubertal chrysin exposure upregulates either AR in male ventral prostate or AR and ERα in Skene's paraurethral gland of pubertal and adult gerbils

Naiara C.S. Ribeiro[a], Mônica S. Campos[b], Mariana B. Santos[c], Gabriela M. Ayusso[c], Patrícia S.L. Vilamaior[b], Luis O. Regasini[c], Sebastião R. Taboga[b], Manoel F. Biancardi[a], Ana P.S. Perez[d], Fernanda C.A. Santos[a,*]

[a] Department of Histology, Embryology and Cell Biology, Laboratory of Histophysiology, Institute of Biological Sciences, Federal University of Goiás, Goiânia, Goiás 74001970, Brazil
[b] Department of Biology, Laboratory of Microscopy and Microanalysis, State University of São Paulo – UNESP, Rua Cristóvão Colombo, 2265, São José do Rio Preto, São Paulo 15054000, Brazil
[c] Department of Chemistry and Environmental Sciences, Laboratory of Green and Medicinal Chemistry, Institute of Biosciences, Humanities and Exact Sciences (Ibilce), São Paulo State University (Unesp), Rua Cristóvão Colombo, 2265, São José do Rio Preto, São Paulo, Brazil
[d] Special Academic Unit of Health Sciences, Medicine Course, Federal University of Goiás, Jataí, Goiás 75804-020, Brazil

ARTICLE INFO

Keywords:
Flavonoids
Female prostate
Ventral prostate
Gerbil
Reproduction

ABSTRACT

Chrysin is a plant-derived polyphenol that has the potential to increase endogenous testosterone levels both by inhibiting the aromatase enzyme and by stimulating testicular steroidogenesis. The effects of chrysin on the prostate are unknown, especially during its development and functional maturation. Thus, the aim of this study was to evaluate the effects of chrysin prepubertal exposure on the male and female prostates of both pubertal and adult gerbils. To evaluate the possible androgenic responses of chrysin, gerbils were also exposed to testosterone. Male and female gerbils were exposed to chrysin or to testosterone cypionate from postnatal day 15 to 42. Male and female gerbils were euthanized at either 43 days or 90 days age. The prostates were collected for biometrical, morphological and immunohistochemical analysis. The results showed that prepubertal exposure to chrysin had differential effects on the prostate of both pubertal and adult animals. The prostates of male and female pubertal gerbils showed no histological alterations, although there was increased frequency of androgen receptor (AR) in males and females, and estrogen receptor alpha (ERα) in females. Adult males and females presented developed prostate glands, with higher cell proliferative rate. In addition, AR and ERα frequency remained high in the prostate of adult animals. These results demonstrated that prepubertal exposure to chrysin disrupts steroid receptors regulation in the prostate, potentiating the response of this gland to the biological effects of endogenous steroids. In this context, excessive consumption of phytoestrogens during the critical stages of development should be considered with caution.

1. Introduction

Chrysin (5, 7-dihydroxyflavone) is an active compound of the flavone class, naturally found in several plant foods, such as medicinal herbs, propolis, honey, chamomile, mushrooms and fruit bark [1,2]. This flavone has been considered beneficial for health due to its anti-inflammatory, antioxidant, anti-allergic, antiviral, anxiolytic and anticancerogenic properties [3,4].

Food supplements rich in chrysin are related to improving serum testosterone levels. Chrysin stimulates testicular steroidogenesis by upregulating the expression of StAR gene with concomitant increase of testosterone production [5,6]. Moreover, chrysin also boosts testosterone *via* inhibition of aromatase, an enzyme that converts testosterone to estradiol [7,8].

Based on its hormonal properties, chrysin has been employed for the treatment of reproductive disorders. Studies with rodents have demonstrated that chrysin causes a delay in testosterone decline during aging, increases either the libido or the quantity and motility of seminal sperms [3,5,9]. Indeed, due to its potential to inhibit the bioavailability of endogenous estrogens, chrysin has been suggested for gynecomastia,

Abbreviations: C, control group; Chr, chrysin group; T, testosterone group; AR, androgen receptor; ERα, estrogen receptor alpha; PCNA, proliferating cell nuclear antigen

* Corresponding author at: Department of Histology, Embryology and Cell Biology, Laboratory of Histophysiology, Federal University of Goiás, Campus II Samambaia, Goiânia, Goiás 74001970, Brazil.
E-mail address: fernanda_alcantara@ufg.br (F.C.A. Santos).

https://doi.org/10.1016/j.fitote.2017.11.003
Received 5 September 2017; Received in revised form 29 October 2017; Accepted 1 November 2017
Available online 10 November 2017
0367-326X/

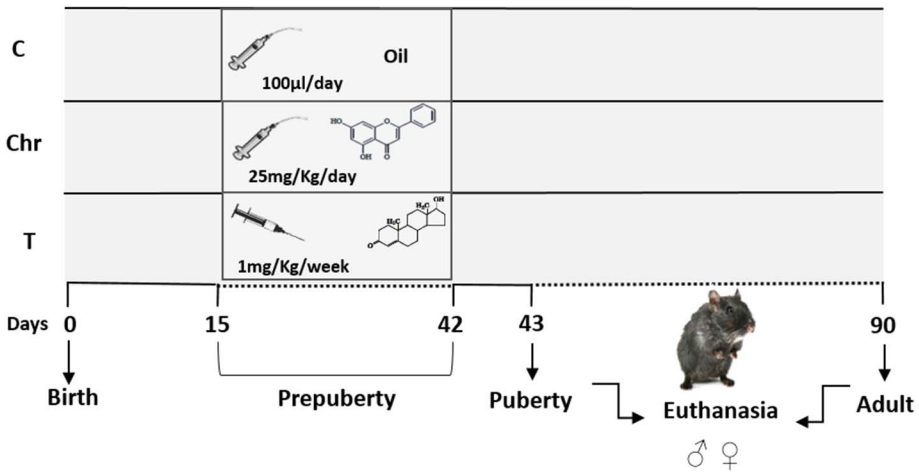

Fig. 1. Schematic representation of the experimental protocol employed in this study.

breast and prostate cancer treatment [10,11]. Despite its promising benefits, the studies regarding chrysin effects upon male and female reproductive systems are scarce. Thus, the effects of chrysin on the reproductive accessory glands, especially the prostate, are unknown.

The prostate is an accessory gland of the mammal reproductive system, and its function is to synthesize and to secrete an alkaline liquid that promotes nutrition and sperm survival [12]. Contrary to classical descriptions, the prostate is not a male-exclusive organ, since it can also be found in women and in several rodent species (also termed as Skene's paraurethral gland) [13,14]. Hormonal regulation and prostate functional identity acquirement are established either during intrauterine or postnatal development [15]. Thus, the early exposure to biologically natural compounds may change the intrinsic developmental prostate program, leading to permanent morphophysiological disorders.

Since chrysin is able to increase testosterone levels and also to decrease estrogen bioavailability, we hypothesize that intake of flavonoid-enriched foods may change the prostate morphophysiology, especially in critical phases of glandular development. Therefore, the purpose of this study was to evaluate the effects of chrysin prepubertal exposure on the male and female prostates of both pubertal and adult gerbils. Indeed, in order to investigate the androgenic potential of chrysin, we analyzed, comparatively and under the same experimental conditions, the effects of testosterone upon gerbil prostate.

2. Material and methods

2.1. Chemical procedures

The chrysin synthesis was performed in two steps, according to protocols described by Ramesh and co-authors, with modifications [16]. First, we have prepared chalcone intermediate by using Claisen-Schmidt condensation between trihydroxyacetophenone and benzaldehyde [17]. This reaction was performed in 60% sodium hydroxide in ethanol at room temperature for 24 h. The reaction mixture was poured into crushed ice and acidified with HCl at pH 3. The crude product was subject to liquid-liquid partition with ethyl acetate. The combined organic phases were concentrated under reduced pressure and chromatographed over silica gel, to furnish 2′,4′,6′-trihydroxychalcone in 44% yield. Second, the conversion from chalcone to flavone was achieved by intramolecular nucleophilic substitution [18]. Solution of 2′,4′,6′-trihydroxychalcone in glycerol and iodine was refluxed for 8 h. After complete conversion confirmed by TLC analysis, the reaction mixture was extracted with ethyl acetate by liquid-liquid partition. The crude product was purified over silica gel, yielding chrysin (23%). Chrysin was obtained as pale yellow solid. ^{1}H NMR (600 MHz, DMSO-d_6): 12.82 (br s, 1H), 8.06 (d, 2H), 7.59 (m, 3H), 6.96 (s), 6.54 (d) and 6.23 (d). ^{13}C NMR (125 MHz, DMSO-d_6): 182.3, 164.9, 163.7, 161.9, 157.9, 132.5, 131.3, 129.6, 126.8, 106.6, 105.0, 99.5 and 94.6.

2.2. Animals

The male and female gerbils were maintained in the Histophysiology Laboratory of the Federal University of Goiás (UFG; Goiânia – GO) under controlled room temperature (25 °C) on a 12 h light/dark cycle. All animals were housed in new polyethylene cages and filtered water was provided from glass bottles. Gerbils were fed with standard rodent food *ad libitum* (Labina-Purina®; composition: 23% protein, 4% fat, 5% fiber and 12% minerals). Animal handling and experiments were performed according to the ethical guidelines of the Federal University of Goiás (CEUA/UFG, protocol n° 110/15), following the Guide for Care and Use of Laboratory Animals.

2.3. Experimental design

We used 30 adult female and 30 adult male gerbils (*Meriones unguiculatus*), all between 90 and 120 days old, for mating. We randomly matched one male to one female to form independent families. The mating day was determined by the presence of spermatozoa in the vaginal smears; this day was considered as day zero, being the initial day of the gestational period [19]. After birth, pups (48 male and 48 female) were destined to form the following groups: Control group (C) – animals received oral doses of the dilution vehicle (corn oil; 100 µL/animal) from the 15th until 42nd day of postnatal life (impubertal or prepubertal phase according to Pinto-Fochi et al. [20]; Chrysin group (Chr) – animals received oral doses of chrysin (25 mg/kg/day; according to the dose used by Darwish et al. [6]) from the 15th until the 42nd day of postnatal life; Testosterone group (T-positive control group) – animals received subcutaneous injections of testosterone cypionate (1 mg/kg/week; Deposteron/EMS) from the 15th until the 42nd day of postnatal life. Male and female gerbils were euthanized at either 43 days (puberty onset) or 90 days (adult onset) of postnatal life. In this way, 12 experimental subgroups were formed (Fig. 1). All animals (n = 8/subgroup) were euthanized by a lethal dosage of anesthesia (100 µL/100 g), which was prepared with a mixture (proportion of 1/1) of anesthetic (Cetamin, Syntec) and muscle relaxant (Xylazine, Vetbrands). The body, gonadal and whole prostatic complex (correspondent urethral segment, ventral, dorsolateral and dorsal prostate lobes in males; and vaginal segment, corresponding urethral segment and prostatic tissue in females) were weighed.

2.4. Light microscopy

The whole prostatic complex (n = 8 glands/group) were fixed by immersion in methacarn (proportions: methanol 60%, chloroform 30%,

Table 1
[1]Body, prostate complex and testicular weight in all experimental groups (n = 8/group). [2]Stereological data obtained for the ventral prostate in all experimental groups (n = 30 fields in 3 animals/group). Values are means ± standard error of mean. Superscript letters (a,b,c) represent statistically significant differences between the experimental groups ($p \leq 0.05$).

	PUB			ADULT		
	C	Chr	T	C	Chr	T
[1]Biometry						
Body weight (g)	27.2 ± 2.4	29.6 ± 1.8	25.2 ± 1.4	64.4 ± 3.2	57.6 ± 1.5	68.4 ± 3.5
Prostate complex weight (g)	0.06 ± 0.01	0.07 ± 0.01	0.06 ± 0.01	0.8 ± 0.04	0.6 ± 0.03	0.8 ± 0.02
Testes weight (g)	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	1.0 ± 0.06[a]	0.7 ± 0.05[b]	1.5 ± 0.10[c]
[2]Stereology (%)						
Epithelium	28.3 ± 1.0	28.7 ± 0.9	26.9 ± 1.4	17.9 ± 0.8[a]	25.3 ± 1.5[b]	28.7 ± 1.1[b]
Lumem	7.4 ± 0.6[a]	7.9 ± 0.7[a]	29.7 ± 2.6[b]	56.4 ± 1.5[a]	48.6 ± 2.2[b]	41.0 ± 2.1[c]
Muscle stroma	27.4 ± 1.1[a]	25.5 ± 1.4[ab]	21.1 ± 1.7[b]	8.1 ± 0.5[a]	10.9 ± 0.6[b]	11.4 ± 0.5[b]
Non-muscle stroma	36.8 ± 1.3[a]	37.8 ± 1.4[a]	22.1 ± 1.9[b]	17.4 ± 1.3	15.1 ± 1.0	18.6 ± 1.8

Table 2
[1]Body, prostate complex and ovarian weight in all experimental groups (n = 8/group). [2]Stereological data obtained for the female prostate in all experimental groups (n = 30 fields in 3 animals/group). Values are means ± standard error of mean. Superscript letters (a,b,c) represent statistically significant differences between the experimental groups ($p \leq 0.05$).

	PUB			ADULT		
	C	Chr	T	C	Chr	T
[1]Biometry						
Body weight (g)	26.0 ± 1.5	28.4 ± 1.0	25.6 ± 0.7	46.8 ± 4.0	51.6 ± 2.3	54.4 ± 1.2
Prostate complex weight (g)	0.07 ± 0.006	0.04 ± 0.008	0.06 ± 0.006	0.14 ± 0.03	0.14 ± 0.03	0.31 ± 0.01
Ovary weight (g)	0.01 ± 0.002	0.02 ± 0.001	0.02 ± 0.004	0.02 ± 0.003	0.03 ± 0.001	0.01 ± 0.003
[2]Stereology (%)						
Epithelium	26.7 ± 1.1[a]	23.7 ± 1.5[a,b]	28.2 ± 1.0[a,c]	22.6 ± 0.9[a]	33.3 ± 1.3[b]	27.8 ± 1.3[c]
Lumem	26.2 ± 2.8[a]	28.07 ± 3.4[a]	38.5 ± 1.8[b]	34.9 ± 2.2[a]	24.4 ± 2.2[b]	26.0 ± 1.8[b]
Muscle stroma	20.4 ± 1.2[a]	22.2 ± 1.5[a]	13.8 ± 0.5[b]	12.5 ± 0.6	12.0 ± 0.5	11.4 ± 0.6
Non-muscle stroma	27.2 ± 2.2[a]	25.9 ± 2.2[a]	19.7 ± 1.4[b]	29.7 ± 1.8	30.1 ± 1.7	34.6 ± 1.1

and acetic acid 10%) for four hours at 4 °C, or in 4% paraformaldehyde (buffered in 0.1 M phosphate, pH 7.2) for 24 h. Then, the tissues were dehydrated through an ethanol series, embedded in paraffin (Histosec, Merck, Darmstadt, Germany), and sectioned at 5 μm on a Leica microtome (Leica RM2155, Nussloch, Germany). The sections were stained by hematoxylin-eosin (HE). The specimens were analyzed and digitized using a Zeiss Axioscope A1 light microscope (Zeiss, Germany).

2.5. Stereology

The stereological analyses were carried out using Weibel's multipurpose graticulate with 130 points and 10 test lines [21]. The relative frequency of each component of the prostatic tissue (epithelium, lumen, non-muscle stroma and muscle stroma) was determined. We chose 30 microscopic fields at random from each experimental group (six fields 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. Stereological analysis was performed using Image-Pro Plus software v6.1 for Windows (Media Cybernetics Inc., Silver Spring, MD, USA).

2.6. Immunohistochemistry

Female and ventral male prostate sections were subjected to immunohistochemistry to detect androgen receptor (AR), estrogen receptor-alpha (ERα) and proliferating cell nuclear antigen (PCNA) (n = 3 animals/group). The sections were deparaffinized in xylene and rehydrated through a decreasing alcohol series. Antigen retrieval was performed in a citrate buffer (pH 6.0). For detection of primary antibodies, the Leica BIOSYSTEMS NovoLink Polymer Detection System (RE7150-K, United Kingdom) kit was used. Endogenous peroxidase activity was neutralized with Novocastra Peroxidase Block, and afterwards Novocastra Protein Block was used to reduce non-specific binding of the primary antibody and polymer. Primary antibodies reactive to AR (rabbit polyclonal IgG, N-20, sc-816, Santa Cruz Biotechnology, Santa Cruz, CA, USA), ERα (rabbit polyclonal IgG, MC-20, sc-542, Santa Cruz Biotechnology), and PCNA (mouse monoclonal IgG2a, SC 56, Santa Cruz Biotechnology, CA, USA) were employed at a dilution of 1:100 overnight at 4 °C. On the next day, Novocastra Post Primary and Polymer were used as secondary antibodies. The sections were stained with DAB Chromogen and DAB Substrate Buffer (in a proportion of 1:20), and finally counterstained with hematoxylin. The histological sections were analyzed using a Zeiss Axioscope A1 light microscope (Zeiss, Germany).

2.7. AR, ERα and PCNA quantification

For AR, ERα, and PCNA quantification, 30 microscopic fields (n = 3 animals/group; magnification of 400 ×) were used for each experimental group. In each field, the total number of positive epithelial and stromal cells was obtained as a relative frequency (%) in relation to the total number of cells. All these analyses were performed using the image analysis system previously described.

2.8. Statistical analyses

The hypothesis tests employed to determine statistical significance were the Kruskal-Wallis test for non-parametric distributions and the ANOVA for parametric distributions. Further determination of the significant statistical differences between experimental groups was done using Dunn's test for non-parametric distributions and the Tukey's test for parametric distributions. The data were analyzed using Statistica 7.0 (StatSoft, Inc., Tulsa, OK, USA). The level of significance was set at 5% ($p \leq 0.05$). Values are presented as mean ± standard error of the mean (SEM).

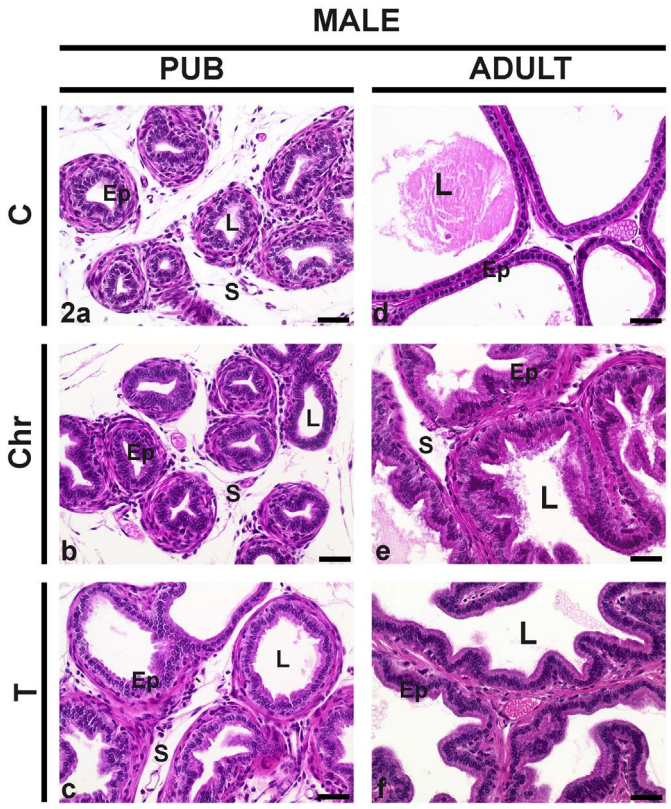

Fig. 2. Histological sections of ventral male prostate stained by HE method. Epithelium (Ep), lumen (L), stroma (S). Scale bar: 20 μm.

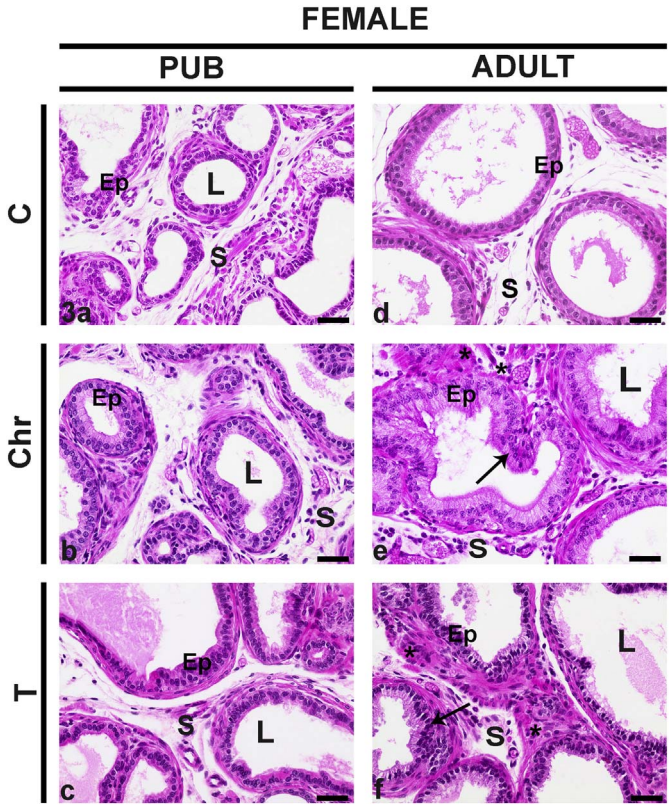

Fig. 3. Histological sections of female prostate stained by HE method. Epithelium (Ep), lumen (L), stroma (S), epithelial hyperplasia (arrows), stromal reshuffling (*). Scale bar: 20 μm.

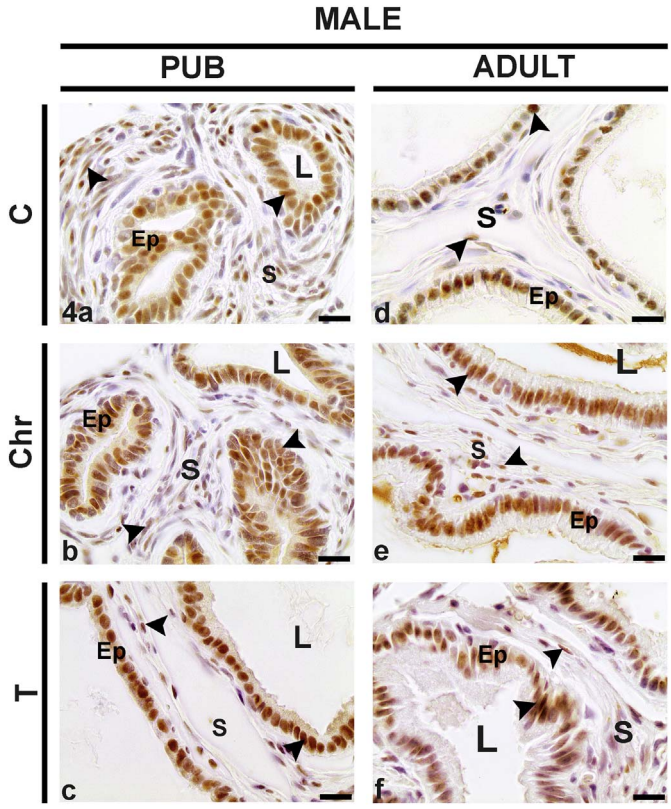

Fig. 4. AR immunolocation in male ventral prostate. Arrowheads indicate AR-positive cells in epithelium (Ep) and stroma (S)·Lumen (L). Scale bar: 10 μm.

2.9. Plasma total testosterone dosage

Blood samples were obtained immediately after the euthanasia, by cardicac puncture of gerbils. Considering the small size of gerbils, this procedure allows to obtain an adequate volume to perform the serum dosages. The serum was separated by centrifugation (3000 rpm) and stored at − 20 °C for subsequent hormone analysis. Circulating serum testosterone levels were determined by Competitive Enzyme immunoassay (Monobind Inc., AccuBind, Lake Forest, USA). The sensitivity was 0.0576 ng/mL.

3. Results

3.1. Biometry

Pubertal exposure to chrysin and to testosterone did not change body, testicular and prostatic weight in pubertal males (Table 1). However, testosterone-treated adult males showed increased testis weight, whereas chrysin-treated adult males had a reduction of this organ ($p \leq 0.05$). We found no biometrical changes for the analyzed females (Table 2).

3.2. Morphology and stereology

Prepubertal exposure to chrysin did not change ventral prostate morphology in pubertal male gerbils (Fig. 2a–b, Table 1). However, testosterone exposure caused an increase in the prostate alveolus in pubertal males (Fig. 2c, Table 1). In adult males, either chrysin or testosterone promoted an increase in epithelium and muscle stroma frequency, besides a reduction in the luminal compartment (Fig. 2d–f; Table 1).

In pubertal females, only testosterone changed morphological and stereological parameters of the prostate, increasing the luminal

Table 3
Frequency (%) of AR and PCNA-positive cells in the male ventral prostate of all experimental groups. Values are means ± standard error of mean. Superscript letters[(a,b)] represent statistically significant differences between the experimental groups ($p \leq 0.05$).

	PUB			ADULT		
	C	Chr	T	C	Chr	T
AR						
Epithelium	72.7 ± 0.8[a]	78.8 ± 0.6[b]	89.1 ± 0.6[c]	72.9 ± 0.7[a]	80.9 ± 0.6[b]	84.8 ± 0.5[c]
Stroma	32.9 ± 1.6[a]	28.2 ± 1.0[a]	38.2 ± 1.5[b]	21.4 ± 1.1[a]	26.7 ± 1.3[b]	25.9 ± 0.9[b]
PCNA						
Epithelium	8.3 ± 0.4[a]	9.2 ± 0.3[a]	11.4 ± 0.3[b]	6.2 ± 0.2[a]	9.0 ± 0.3[b]	7.6 ± 0.2[c]
Stroma	6.5 ± 0.5[a]	7.6 ± 0.3[a]	8.6 ± 0.2[b]	7.5 ± 0.3[a]	8.3 ± 0.2[a]	9.6 ± 0.3[b]

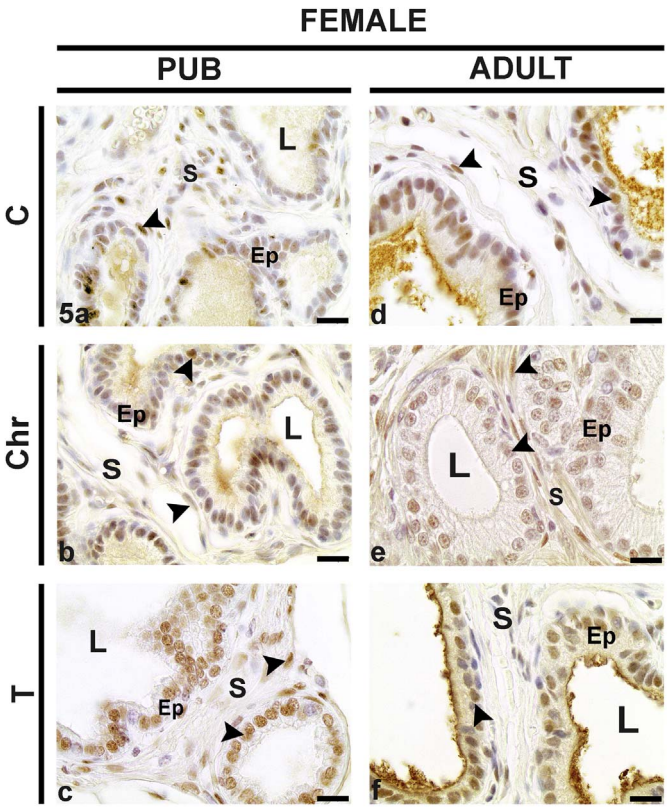

Fig. 5. AR immunolocation in female prostate. Arrowheads indicate AR-positive cells in epithelium (Ep) and stroma (S)·Lumen (L). Scale bar: 10 μm.

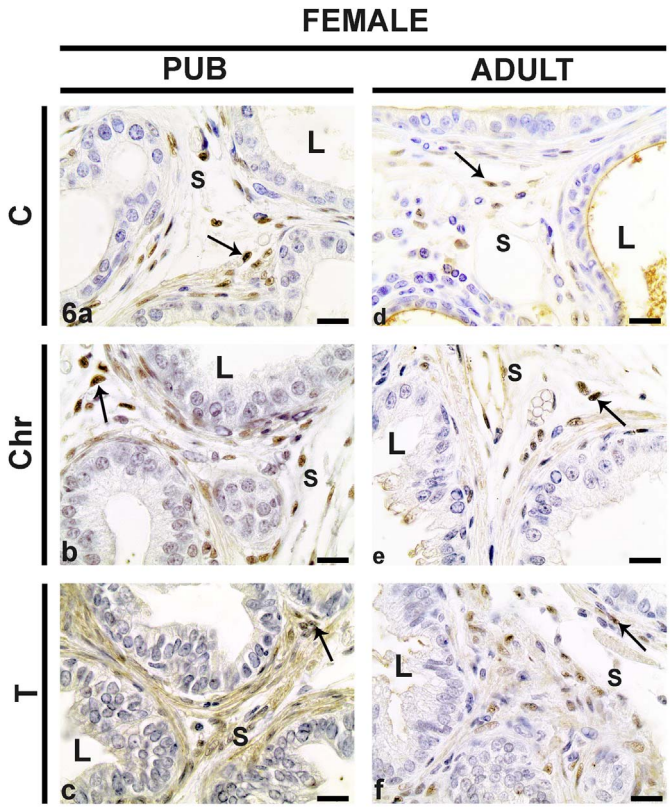

Fig. 6. ERα immunolocation in female prostate. Arrowheads indicate AR-positive cells in stroma (S).Epithelium (Ep), Lumen (L). Scale bar: 10 μm.

compartment, and decreasing muscle and non-muscle stroma (Fig. 3a–c; Table 2). However, in adult females, both drugs promoted similar alterations, including epithelial hyperplasia and stromal reshuffling (Fig. 3d–f; Table 2).

3.3. Androgen receptor

Prepubertal exposure to chrysin increased AR immunostaining in epithelium of the pubertal and adult males, and in the stroma of adult males. However, pubertal and adult testosterone-treated males showed

Table 4
Frequency (%) of AR, ERα and PCNA-positive cells in the female prostate of all experimental groups. Values are means ± standard error of mean. Superscript letters[(a,b)] represent statistically significant differences between the experimental groups ($p \leq 0.05$).

	PUB			ADULT		
	C	Chr	T	C	Chr	T
AR						
Epithelium	49.4 ± 1.3[a]	63.2 ± 1.0[b]	79.2 ± 0.9[c]	50.3 ± 0.7[a]	74.6 ± 0.9[b]	84.6 ± 0.9[c]
Stroma	24.0 ± 1.5[a]	31.0 ± 1.5[b]	32.9 ± 2.1[b]	19.7 ± 1.0[a]	31.7 ± 1.1[b]	27.2 ± 1.0[c]
ERα						
Stroma	18.3 ± 0.8[a]	30.1 ± 1.0[b]	22.7 ± 0.6[c]	20.6 ± 0.8[a]	28.9 ± 0.9[b]	25.3 ± 0.5[c]
PCNA						
Epithelium	8.8 ± 0.3[a]	9.8 ± 0.3[a]	11.0 ± 0.4[b]	5.9 ± 0.3[a]	9.7 ± 0.3[b]	7.1 ± 0.2[c]
Stroma	8.9 ± 0.3[a]	12.6 ± 0.5[b]	10.2 ± 0.3[c]	6.7 ± 0.3	7.4 ± 0.2	6.9 ± 0.1

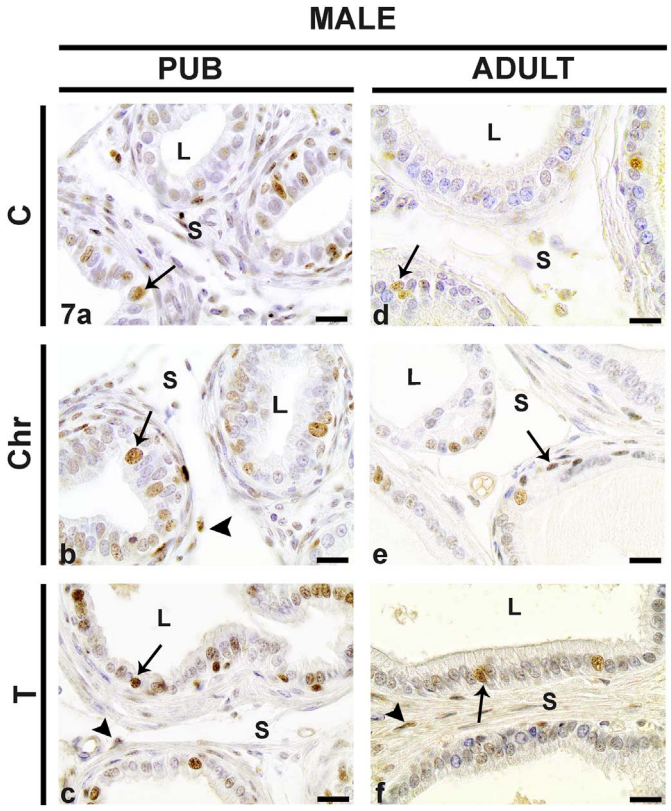

Fig. 7. Immunohistochemistry for PCNA in male ventral prostate of the all experimental groups. Arrowheads indicate PCNA-positive cells in epithelium (Ep) and stroma (S). Lumen (L). Scale bar: 10 μm.

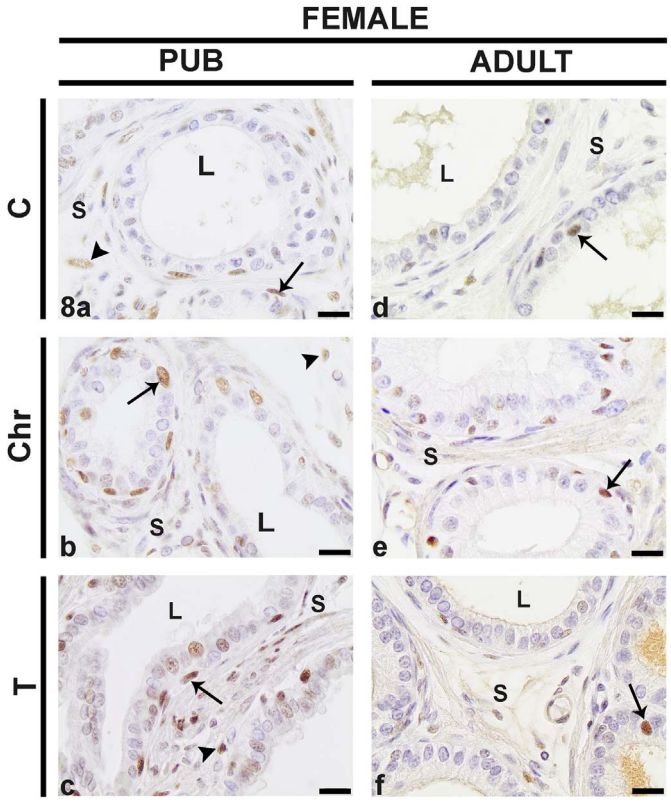

Fig. 8. Immunohistochemistry for PCNA in female prostate of the all experimental groups. Arrowheads indicate PCNA-positive cells in epithelium (Ep) and stroma (S). Lumen (L). Scale bar: 10 μm.

higher epithelial immunostaining for AR when compared with the control and chrysin groups (Fig. 4; Table 3).

In females, either chrysin or testosterone were able to increase AR immunostaining in both the epithelium and prostate stroma of pubertal and adult animals (Fig. 5; Table 4). However, testosterone exposure promoted a superior increase in AR-positive epithelial cells when compared with chrysin treatment (Table 4).

3.4. Estrogen receptor alpha

In this study, we did not succeed in achieving ERα reactions in male gerbils. In females, either chrysin or testosterone caused a significant increase in stromal immunostaining in pubertal and adult glands (Fig. 6; Table 4). However, pubertal and adult females exposed to chrysin showed an increased ERα immunostaining when compared with testosterone-exposed females (Table 4).

3.5. Proliferating cell nuclear antigen receptor

PCNA-positive cells were observed in both epithelium and stroma compartments of all experimental groups (Figs. 7 and 8). In pubertal males, we observed that chrysin did not change the proliferative rate of the ventral prostate (Fig. 7a–b; Table 3). However, animals exposed to testosterone showed an increase of epithelial and stromal PCNA-positive cells (Fig. 7c; Table 3). Adult males exposed to chrysin showed an increase in epithelial PCNA, whereas testosterone-exposed males had an increase in epithelial and stromal PCNA immunostaining (Fig. 7d–f; Table 3).

Pubertal females exposed to chrysin showed an increase in stromal PCNA, whereas females exposed to testosterone had an increase in epithelial and stromal immunostaining (Fig. 8a–c, Table 4). In adult females, only the epithelial compartment showed an increase in the frequency of PCNA-positive cells, with this increase being more expressive in females exposed to chrysin (Fig. 8d–f, Table 4).

3.6. Testosterone plasma levels

Chrysin supplementation did not cause statistically significant changes in the testosterone serum levels of the male and female gerbils (pub and adult). However, testosterone treatment caused a significant increase of the testosterone levels in males and females of the pub group (Fig. 9; $p \leq 0.05$).

4. Discussion

In this study we demonstrated the morphology, and the immunolocation of AR, ERα, and PCNA in the prostate of pubertal and adult gerbils exposed to chrysin or testosterone during prepubertal development. As expected and also reported in previous assays regarding androgenization of gerbil prostate development [22,23], pubertal males and females exposed to testosterone during the prepubertal phase showed an increase in cellular proliferation, intense prostate development and higher testosterone plasma levels. However, in the present study we did not observe similar results for the pubertal animals exposed to chrysin. Male and female pubertal gerbils exposed to chrysin did not show changes of body, prostate, or gonadal weight. Testosterone plasma levels also did not change in Chr-treated gerbils. The prostate of the Chr animals did not show any structural or proliferative change, but exhibited a higher AR (male and female) and ERα (female) immunostaining.

Several *in vivo* studies have shown that chrysin causes increased bioavailability of testosterone, both by inhibiting the conversion of testosterone to estrogen by the aromatase enzyme [3,7,8,11] and by stimulating androgen synthesis *via* increased StAR gene expression in Leydig cells [5,6]. However, in pubertal gerbils treated with chrysin we did not find testosterone boosting or histological alterations similar to

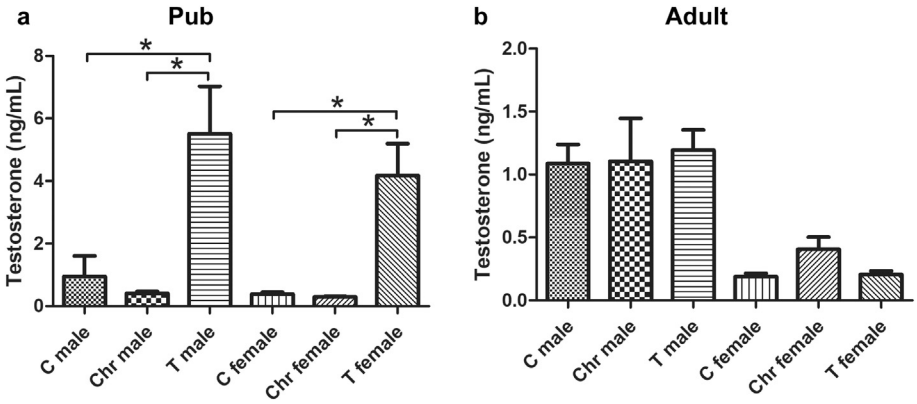

Fig. 9. Testosterone plasma levels (ng/mL) in all experimental groups (n = 5 animals/group). Values are means ± standard error of mean. Asterisks represent statistically significant differences between the experimental groups ($p \leq 0.05$).

those observed in rodents submitted to androgenic stimuli [24,25]. Studies demonstrated an increase in serum testosterone levels when rodents were exposed to chrysin doses ranging between 25 and 80 mg/kg/day [3,6,26]. Still, in a recent study, roosters supplemented with chrysin (25, 50 or 75 mg/kg/day) during 12 weeks showed a testosterone increase only when exposed to the higher dosage (75 mg/kg) [27]. Thus, we hypothesized that the chrysin dose employed in this study (25 mg/kg/day) was not sufficient to cause testosterone boosting and consequent prostate development in male and female pubertal gerbils.

On the other hand, interestingly, the pubertal gerbils exposed to chrysin showed upregulation of AR (males and females) and ERα (females). This increase in the frequency of AR and ERα immunostaining appears to be permanent, since males and females of adult gerbils that were exposed to chrysin during prepubertal development also had a higher frequency of AR- and ERα-positive cells. The interaction of chrysin with hormone receptors is poorly understood, but some *in vitro* studies have demonstrated the effects of flavonoids on androgenic and estrogenic receptors [28,29,30]. Studies with other flavonoid types (quercetin, apigenin, naringenin) have demonstrated that these compounds may act as an antagonist of AR, through direct competition with endogenous androgens [30,31]. In addition, several studies have shown that chrysin can bind to both forms of estrogen receptors (ERα and ERβ), triggering estrogenic or anti-estrogenic responses [28,29], [32]. In this study, the upregulation of AR and ERα in pubertal gerbils exposed to chrysin did not promote immediate morphofunctional changes in the prostate, since these glands had histology similar to the control group. However, the persistence of high AR- and ERα-positive cell rates during adult life promoted a significant alteration in gerbil prostate morphophysiology. Thus, adult males and females exposed to chrysin showed tissue changes similar to those observed in animals treated with testosterone. These changes include increased cell proliferation, epithelial hyperplastic development and increased secretory capacity. In this context, we believe that the prostate overstimulation has resulted from the interaction of chrysin with AR and ERα during prepuberal development.

Although the health benefits of the polyphenols are widely demonstrated, the most abundant flavonoids in the human diet are not necessarily the most bioactive [1]. Gambelunghe and colleagues showed that the intake of chrysin-rich foods (propolis and honey) for 21 days does not alter the testosterone levels in adult men [33]. Since the bioavailability of chrysin from plant foods is low, the intake of flavonoid-rich supplements has grown greatly in recent years, mainly due to its anticancer, antioxidant and anti-inflammatory properties [4,10]. However, many studies suggest that excessive chrysin intake can be harmful to health, particularly in specific populations, such as pregnant women, newborns and patients with hormone-dependent tumors, since chrysin can interfere with biological responses evoked by estrogens [1,27]. In rodents, postnatal development and maturation of the prostate extends to the end of puberty end (~3 months old) [34]. During this process, several genes are expressed at specific sites and times, under the precise regulation of androgens and estrogens [12,15]. Thus, any disruption of the endogenous hormonal environment during this period may cause irreversible changes in prostate morphology. In this context, the results of this study demonstrate that chrysin exposure during prepubertal development permanently alters the regulation of androgenic and estrogenic receptors in the male and female prostate, allowing the formation of proliferative and super activated adult glands. These findings suggest that chrysin consumption should be carried out with caution, especially at critical stages of the development of hormone-dependent organs.

5. Conclusion

In conclusion, chrysin exposure during prepubertal development disrupted the regulation of androgenic and estrogen receptors in the prostate, potentiating the response of this gland to the biological effects of endogenous steroids. As a result, the prostates, in adult animals, have become more proliferative and developed. Thus, excessive phytoestrogen consumption during critical development stages should be considered with caution, since chrysin may permanently alter the morphophysiology of hormone-dependent organs.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgements

This paper was supported by a grant from the Brazilian agency CNPq (Brazilian National Research and Development Council, Procs. Nrs. 475148/2012-6, 471129/2013-5 and 306251/2016-7, FAPEG (Goiás Research Foundation, Procs. Nr. 05/2012) and FAPESP (São Paulo Research Foundation, Procs. Nr. 2014/18330-0).

References

[1] C. Ly, J. Yockell-Lelièvre, Z.M. Ferraro, J.T. Arnason, J. Ferrier, A. Gruslin, The effects of dietary polyphenols on reproductive health and early development, Hum. Reprod. Update 21 (2) (2015) 228–248.

[2] K. Guo, Z. Liang, L. Liu, F. Li, H. Wang, Flavonoids intake and risk of prostate cancer: a meta-analysis of observational studies, Andrologia 48 (10) (2016) 1175–1182.

[3] O. Ciftci, I. Ozdemir, M. Aydin, A. Beytur, Beneficial effects of chrysin on the reproductive system of adult male rats, Andrologia 44 (3) (2012) 181–186.

[4] K.L. Ivey, M.K. Jensen, J.M. Hodgson, A.H. Eliassen, A. Cassidy, E.B. Rimm, Association of flavonoid-rich foods and flavonoids with risk of all-cause mortality, Br. J. Nutr. 117 (10) (2017) 1470–1477.

[5] K. Jana, X. Yin, R.B. Schiffer, J.J. Chen, A.K. Pandey, D.M. Stocco, P. Grammas, X. Wang, Chrysin, a natural flavonoid enhances steroidogenesis and steroidogenic acute regulatory protein gene expression in mouse Leydig cells, J. Endocrinol. 197 (2) (2008) 315–323.

[6] H.A. Darwish, H.H. Arab, R.M. Abdelsalam, Chrysin alleviates testicular dysfunction in adjuvant arthritic rats via suppression of inflammation and apoptosis: comparison with celecoxib, Toxicol. Appl. Pharmacol. 279 (2) (2014) 129–140.
[7] J.C. Le Bail, T. Laroche, F. Marre-Fournier, G. Habrioux, Aromatase and 17 beta-hydroxysteroid dehydrogenase inhibition by flavonoids, Cancer Lett. 133 (1) (1998) 101–106.
[8] N. Ta, T. Walle, Aromatase inhibition by bioavailable methylated flavones, J. Steroid Biochem. Mol. Biol. 107 (1–2) (2007) 127–129.
[9] K. Dhawan, S. Kumar, A. Sharma, Beneficial effects of chrysin and benzoflavone on virility in 2-year-old male rats, J. Med. Food 5 (1) (2002) 43–48.
[10] G. Séralini, S. Moslemi, Aromatase inhibitors: past, present and future, Mol. Cell. Endocrinol. 178 (1–2) (2001) 117–131.
[11] B.Y. Khoo, S.L. Chua, P. Balaram, Apoptotic effects of chrysin in human cancer cell lines, Int. J. Mol. Sci. 11 (5) (2010) 2188–2199.
[12] P.C. Marker, A.A. Donjacour, R. Dahiya, G.R. Cunha, Hormonal, cellular, and molecular control of prostatic development, Dev. Biol. 253 (2) (2003) 165–174.
[13] M. Zaviacic, R.J. Ablin, The female prostate and prostate-specific antigen. Immunohistochemical localization, implications of this prostate marker in women and reasons for using the term "prostate" in the human female, Histol. Histopathol. 15 (1) (2000) 131–142.
[14] M.F. Biancardi, F.C.A. Dos Santos, H.F. de Carvalho, B.D.A. Sanches, S.R. Taboga, Female prostate: historical, developmental, and morphological perspectives, Cell Biol. Int. (2017) (Epub ahead of print).
[15] G.R. Cunha, W. Ricke, A. Thomson, P.C. Marker, G. Risbridger, S.W. Hayward, Y.Z. Wang, A.A. Donjacour, T. Kurita, Hormonal, cellular, and molecular regulation of normal and neoplastic prostatic development, J. Steroid Biochem. Mol. Biol. 92 (4) (2004) 221–236.
[16] P. Ramesh, C.S. Reddy, K.S. Babu, P.M. Reddy, V.S. Rao, T. Parthasarathy, Synthesis, characterization and molecular docking studies of novel 2-amino 3-cyano pyrano [2,3*H*] chrysin derivatives as potential antimicrobial agents, Med. Chem. Res. 24 (2015) 3696–3709.
[17] T.G. Passalacqua, L.A. Dutra, L. Almeida, A.M. Velasquez, F.A. Torres, P.R. Yamazaki, M.B. Bastos, L.O. Regasini, P.A. Michels, V.S. Bolzani, M.A. Graminha, Synthesis and evaluation of novel prenylatedchalcone derivatives as anti-leishmanial and anti-trypanosomal compounds, Bioorg. Med. Chem. Lett. 25 (2015) 3342–3345.
[18] M.L. Zeraik, V.F. Ximenes, L.O. Regasini, L.A. Dutra, D.H. Silva, L.M. Fonseca, D. Coelho, S.A. Machado, V.S. Bolzani, 4′-Aminochalcones as novel inhibitors of the chlorinating activity of myeloperoxidase, Curr. Med. Chem. 19 (2012) 5405–5413.
[19] B.D. Sanches, M.F. Biancardi, F.C. Dos Santos, R.M. Góes, P.S. Vilamaior, S.R. Taboga, Budding process during the organogenesis of the ventral prostatic lobe in Mongolian gerbil, Microsc. Res. Tech. 77 (2014) 458–466.
[20] M.E. Pinto-Fochi, A.C. Negrin, W.R. Scarano, S.R. Taboga, R.M. Góes, Sexual maturation of the Mongolian gerbil (*Meriones unguiculatus*): a histological, hormonal and spermatic evaluation, Reprod. Fertil. Dev. 28 (6) (2016) 815–823.
[21] E.R. Weibel, Principles and methods for the morphometric study of the lung and other organs, Lab. Investig. 12 (1963) 131–155.
[22] M.F. Biancardi, A.P. Perez, C.R. Caires, R.M. Góes, P.S. Vilamaior, F.C. Santos, S.R. Taboga, Prenatal exposure to testosterone masculinises the female gerbil and promotes the development of lesions in the prostate (Skene's gland), Reprod. Fertil. Dev. 27 (7) (2015) 1000–1011.
[23] M.F. Biancardi, A.P. Perez, C.R. Caires, L.R. Falleiros, R.M. Góes, P.S. Vilamaior, D.R. Freitas, F.C. Santos, S.R. Taboga, Prenatal and pubertal testosterone exposure imprint permanent modifications in the prostate that predispose to the development of lesions in old Mongolian gerbils, Asian J. Androl. 19 (2) (2017) 160–167.
[24] F.C. Santos, R.P. Leite, A.M. Custódio, K.P. Carvalho, L.H. Monteiro-Leal, A.B. Santos, R.M. Góes, H.F. Carvalho, S.R. Taboga, Testosterone stimulates growth and secretory activity of the female prostate in the adult gerbil (*Meriones unguiculatus*), Biol. Reprod. 75 (3) (2006) 370–379.
[25] W.R. Scarano, P.S. Vilamaior, S.R. Taboga, Tissue evidence of the testosterone role on the abnormal growth and aging effects reversion in the gerbil (*Meriones unguiculatus*) prostate, Anat. Rec. A: Discov. Mol. Cell. Evol. Biol. 288 (11) (2006) 1190–1200.
[26] N. Ali, S. Rashid, S. Nafees, S.K. Hasan, S. Sultana, Beneficial effects of chrysin against methotrexate-induced hepatotoxicity via attenuation of oxidative stress and apoptosis, Mol. Cell. Biochem. 385 (1–2) (2014) 215–223.
[27] A.S. Amin Altawash, A.Z. Shahneh, H. Moravej, M. Ansari, Chrysin-induced sperm parameters and fatty acid profile changes improve reproductive performance of roosters, Theriogenology 104 (2017) 72–79.
[28] G.G. Kuiper, J.G. Lemmen, B. Carlsson, J.C. Corton, S.H. Safe, P.T. van der Saag, B. van der Burg, J.A. Gustafsson, Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta, Endocrinology 139 (10) (1998) 4252–4263.
[29] F.A. Resende, A.P. de Oliveira, M.S. de Camargo, W. Vilegas, E.A. Varanda, Evaluation of estrogenic potential of flavonoids using a recombinant yeast strain and MCF7/BUS cell proliferation assay, PLoS One 8 (10) (2013) e74881.
[30] T. Boam, Anti-androgenic effects of flavonols in prostate cancer, Ecancermedicalscience 9 (2015) 585.
[31] Y. Wu, J.A. Doering, Z. Ma, S. Tang, H. Liu, X. Zhang, X. Wang, H. Yu, Identification of androgen receptor antagonists: in vitro investigation and classification methodology for flavonoid, Chemosphere 158 (2016) 72–79.
[32] G. Leclercq, Y. Jacquot, Interactions of isoflavones and other plant derived estrogens with estrogen receptors for prevention and treatment of breast cancer-considerations concerning related efficacy and safety, J. Steroid Biochem. Mol. Biol. 139 (2014) 237–244.
[33] C. Gambelunghe, R. Rossi, M. Sommavilla, C. Ferranti, R. Rossi, C. Ciculi, S. Gizzi, A. Micheletti, S. Rufini, Effects of chrysin on urinary testosterone levels in human males, J. Med. Food 6 (4) (2003) 387–390.
[34] G.S. Prins, O. Putz, Molecular signaling pathways that regulate prostate gland development, Differentiation 76 (6) (2008) 641–659.