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·*Original Article*·

Protective effects of estrogens and caloric restriction during aging on various rat testis parameters

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Abstract

Aim: To investigate the effects of 17β-estradiol (E2), *Peganum harmala* extract (PHE) and caloric restriction (CR) on various testis parameters during aging. **Methods:** Twelve-month-old male rats were treated for 6 months with either E2 or PHE, or submitted to CR (40%). **Results:** Our results show that estrogens and CR are able to protect the male gonad by preventing the decrease of testosterone and E2 levels as well as the decrease of aromatase and estrogen receptor gene expressions. Indeed, E2, PHE and CR treatments induced an increase in the superoxide dismutase activities and decreased the activity of testicular enzymes: gamma-glutamyl transferase, alkaline phosphatase, lactate deshydrogenase as well as the aspartate and lactate transaminases in aged animals. In addition, the testicular catalase and gluthatione peroxidase activities were enhanced in E2, PHE and CR-treated rats compared to untreated animals at 18 months of age. Moreover, the positive effects of estradiol, PHE and CR were further supported by a lower level of lipid peroxidation. Recovery of spermatogenesis was recorded in treated rats. **Conclusion:** Besides a low caloric diet which is beneficial for spermatogenesis, a protective antioxydant role of estrogens is suggested. Estrogens delay testicular cell damage, which leads to functional senescence and, therefore, estrogens are helpful in protecting the reproductive functions from the adverse effects exerted by reactive oxygen species (ROS) produced in large quantities in the aged testis. *(Asian J Androl 2008 Nov; 10: 837–845)*

Keywords: aging; (phyto)estrogens; 17β-estradiol; *Peganum harmala*; caloric restriction; rat testis; antioxidant enzymes

1 Introduction

Aging in men is accompanied by a reproductive senescence associated with a chronic state of oxidative stress following a functional deficit in Leydig, Sertoli and germ cells [1–3]. The decrease of steroidogenesis associated with the imbalance between prooxidant and antioxidant activities leads to oxidative damage of various cellular processes [4]. The risk of oxidative damage and the lipid peroxidation is especially high in steroid synthesizing tissues, because, in addition to oxidative phosphorylation, they use molecular oxygen for steroid synthesis [5, 6]. Indeed, it has been shown that free radicals inhibit steroidogenesis by interfering with cholesterol transport to the mitochondria and/or the catalytic function of P450 enzymes [7, 8], which leads (i) to an

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increase of lipid peroxidation [9]; (ii) to an enhancement of the toxicity indexes such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), gamma-glutamyl transferase (GGT) and alkaline phosphatase (PAL) activities [10]; and (iii) to the decline of the antioxidant barrier. To protect against the adverse effects of reactive oxygen species (ROS), mammalian cells are equipped with various enzymatic and non-enzymatic antioxidant scavengers. The major enzymatic antioxidants are superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) [11]. Among the non-enzymatic antioxidants are chemicals, such as estrogens [12, 13], growth factors [14] or vitamin E [15], and flavonoids (e.g. quercetin and herbacetin), found in many plants, such as *Peganum harmala* [16, 17]. These bio-molecules are known by their capacity to scavenge free radicals and to modulate the expression of genes encoding antioxidant enzymes.

Caloric restriction (CR) is also known to slow aging and to delay the appearance of age-associated physiopathological changes [18, 19] and, therefore, to induce some benefits on the longevity of rats [20]. The mechanisms underlying the robust protective effects of CR remain to be identified; however, it has been suggested that the most significant effect of CR on aging is the associated reduction in oxidative stress at the cellular level. Indeed, CR suppresses the age-related oxidative damage of lipids, DNA and proteins, and increases the resistances of cells to oxidative stress [21] and/or induces changes in the expression of stress-response genes in testicular cells of aged rats [22, 23]. We have reported that the rat testicular cells, including germ cells, are able to synthesize estrogens, and these cells are also equipped with estrogen receptors, therefore suggesting a putative role for these female hormones in spermatogenesis [24]. Because aromatase and antioxidant strategies are concerned with the control of male fertility, our working hypothesis was to check the long-term effect of treatment with either pure estrogens (or phytoestrogens) or caloric restriction during aging on aromatase and estrogen receptor gene expression in rat testis. In addition, some parameters of oxidative stress, such as lipid peroxydation (thiobarbituric acid reactive substance) and antioxidant enzymatic activities (CAT, SOD and GPx), as well as other enzyme activities involved in the toxicity index (AST, ALT, LDH, GGT and PAL), have been measured in testes of rats at the end of the treatment.

2 Materials and methods

2.1 Animals and treatments

Male Wistar rats aged 12 months were used. Animals were maintained in the animal house facility (Faculty of Sciences, University of Sfax, Sfax, Tunisia) at a constant temperature of 25 ± 3ºC, under 12 h : 12 h light: dark cycle. The animals (six per group) were fed with standard chow and were given access to tap water *ad libitum*. The 12-month-old rats were divided into four groups and treated for 6 months: they were either fed *ad libitum* (control) or received 17β-estradiol (1 μg/kg body weight, daily) [25] or *Peganum harmala* extract (PHE) (50 mg/kg body weight, daily) [26] by gastric gavage, or submitted to caloric restriction (CR) diet. In the CR group, rats were fed with 60% of the quantity of food given to the control animals (equivalent to 1 726 KJ/w daily). The sham-control rats were gavaged with water. The other six intact male rats aged 4 months were used as a reference group. The handling of the animals was approved by the local Ethical Committee for the care and use of laboratory animals. At the age of 18 months, the animals were weighed, then killed by decapitation, and the trunk blood was collected. The serum was prepared by centrifugation (1 500 × *g*, 15 min, 4ºC) and the testes were removed, cleaned of fat and weighted; all these samples were stored at –80ºC until used.

2.2 RNA extraction and quantification of transcripts

Total RNAs from testes were extracted using the guanidium thiocyanate–phenol-chlorofom method of Chomczynski and Sacchi (1987) [27]. Briefly, testes were homogenized in 600 μL of lysis buffer (1 mol/L Tris, 4 mol/L guanidium thiocyanate, 0.5% sarcosyl, 1% β-mercaptoethanol), then 0.1 volume of 2 mol/L sodium acetate, 1 volume of phenol and 0.2 volume of isoamylic chloroform-alcohol (v/v : 49/1) were added to the preparation. After 15 min of incubation in a cold bath, the samples were centrifuged at 10 000 × *g* at 4ºC for 15 min. RNAs were precipitated at –80ºC by adding 1 volume of isopropanol. After centrifugation, the pellets were washed with 75% ethanol, dried and dissolved in 50 μL of diethylpyrocarbonate treated water. They were stored at –80ºC until used. The quantity of total RNA was determined by the measurement of the optical density at 260 nm. The purity was evaluated by the ratio 260/280 nm and the integrity was controlled by electrophoresis on a 1.5% agarose gel.

For the semi quantitative reverse transcription-polymerase chain reaction (RT-PCR), 2 μg of total RNAs were reverse transcribed into cDNA as follows: 1 h at 42ºC with 200 IU Moloney murine leukemia virus reverse transcriptase (M-MLV-RT), 0.5 mmol/L dNTP, 0.2 μg oligo-dT and 20 IU RNasin in a final volume of 40 μL. Then, cDNAs coding for aromatase, estrogen receptor alpha (ERα), estrogen receptor beta (ERβ) and ribosomal protein L19 were amplified by PCR using specific primers (Table 1) as reprorted elsewhere [28]. We have chosen L19 transcripts which did not vary among the samples in order to correct the difference in the quantities of total RNA used for reverse transcription [29]. The amplified products were run on a 2%-agarose-gel stained with ethidium bromide, visualized under UV transillumination and analyzed with NIH software (http://rsb.info.nih.gov/nih-image).

2.3 Steroid determinations and measurements of antioxidant enzymatic activities

After homogenization of testes in a phosphate buffer (1 g/2 mL), steroids were extracted by diethylether according to our reported method [30]. The estradiol and testosterone levels were then measured by RIA using highly specific antibodies from P.A.R.I.S (Compiègne, France). The intra- and inter-assay coefficients of variation were 8% and 5% for estradiol and 4.6% and 7.5% for testosterone. The sensitivities were 6 pg/tube and 12 pg/tube respectively for estradiol and testosterone.

The lipid peroxidation was determined in the homogenates from control and treated rat testes by quantification of the thiobarbituric acid reactive substances (TBARS) using the method of Buege and Aust [31]. The SOD activity was assayed by the spectrophotometric method of Marklund & Marklund [32]. The activities of GPx and CAT were measured by the method of Pagila and Valentine [33] and Aebi [34] respectively. The protein level was determined by the method of Lowry *et al.* [35]. The testicular LDH, AST and ALT, GGT, and PAL activities, were determined using commercial kits from Sigma (Munich, Germany) and Boehringer (Mannheim, Germany).

For histological studies, pieces of testes were fixed in a Bouin's solution for 24 h, then embedded in paraffin. Sections of 5-μm thickness were stained with hematoxylin-eosin and examined under Olympus CX41 light microscope (Olympus Industrial America Inc., Orangeburg, NY, USA).

Data are presented as means ± SEM. The determinations were performed from six animals per group and the differences were examined by the one-way analysis of variance (ANOVA) followed by the Fisher test, and the significance was accepted at $P < 0.05$ (StatView; SAS Institute, Cary, NC, USA).

3 Results

3.1 Aromatase, ERα and ERβ gene expression in testis of control and treated animals

We performed a semi-quantitative RT-PCR to determine whether the amounts of aromatase and estrogen receptors (ERs) transcripts in testes were affected by

Table 1. Sequences of primers. Size of fragments amplified and number of cycles used for amplification. Genbank accession number are indicated in parenthesis. S, sense primer; R, reverse primer; PCR, polymerase chain reaction. L19, a stable control (control gene which their expression not changed in pathological conditions).

Gene	Primer	Sequence	Orientation and position	Size of PCR product (bp)	No. of cycle
Aromatase	5'-ARO	GCTTCTCATCGCAGAGTATCCGG	S (1555–1577)	290	33
(M33986)	3'-ARO	CAAGGGTAAATTCATTGGGCTTGG	R (1821–1844)		
ERα	5'-ERα	AATTCTGACAATCGACGCCAG	S (545–565)	345	29
(X61098)	3'-ERα	GTGCTTCAACATTCTCCCTCCTC	R (867–889)		
ERβ	5'-ERβ	GAAGCTGAACCACCCAATGT	S (1100–1120)	211	40
(U57439)	3'-ERβ	CAGTCCCACCATTAGCACCT	R (1291–1310)		
L19	5'-L19	GAAATCGCCAATGCCAACTC	S (119–138)	290	24
(NM_031103)	3'-L19	ACCTTCAGGTACAGGCTGTG	R (389–408)		

aging and the various treatments. In the 18-month untreated rats, the amount of aromatase transcripts was significantly lower compared to that of 4-month-old animals. Conversely, in the E2, PHE and CR groups the level of aromatase mRNA was increased by 62%, 44% and 65%, respectively, compared to 18-month-old control rats and was significantly higher than that in 12-month-old rats (Figure 1A).

The amount of ERα mRNA was decreased of 56% between 4 and 18 months in control rats (Figure 2A). Conversely, in the three other groups, the levels of transcripts were significantly higher (66%, 60% and 58%, respectively, in E2, PHE and CR-treated rats) compared to control rats aged 18 months. The levels of ERβ transcripts were significantly decreased by 85% in control rats between 4 and 18 months (Figure 3A). However, in 18-month-old rats the treatments induced a significant ($P < 0.01$) increase of the level of ERβ transcripts: 87%, 37% and 77%, respectively, in E2, PHE and CR animals compared to controls of the same age.

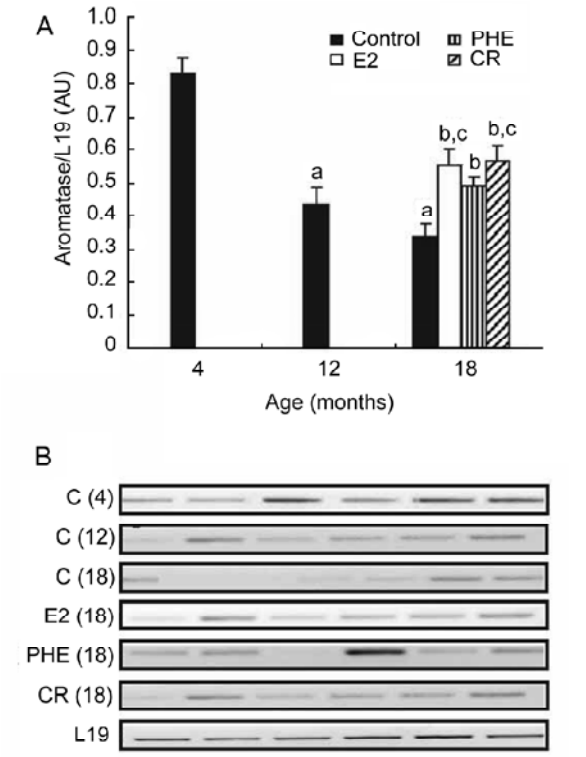

Figure 1. (A): Expression of aromatase, in testis of rats aged 4, 12 and 18 months, either fed *ad libitum*, treated with 17β-estradiol (E2), *Peganum harmala* extract (PHE), or submitted to caloric restriction (CR). The data are expressed as mean ± SEM ($n = 6$). AU: arbitrary unit. (a): Control rats significantly different from 4-month-old rats ($P < 0.05$). (b): E2, PHE and CR treated rats significantly different from 12-month-old animals ($P < 0.05$). (c): E2, PHE and CR treated rats significantly different from the same age control rats ($P < 0.05$). (B): Representative signal of the amplification of aromatase gene obtained by polymerase chain reaction (PCR) using total RNA from testes of rats aged of 4 months (C [4]), 1 year (C [12]), 18 months (C [18]), and in 18-month-old animals treated with E2, PHE or CR. L19, a stable control (control gene which their expression does not changed in pathological conditions).

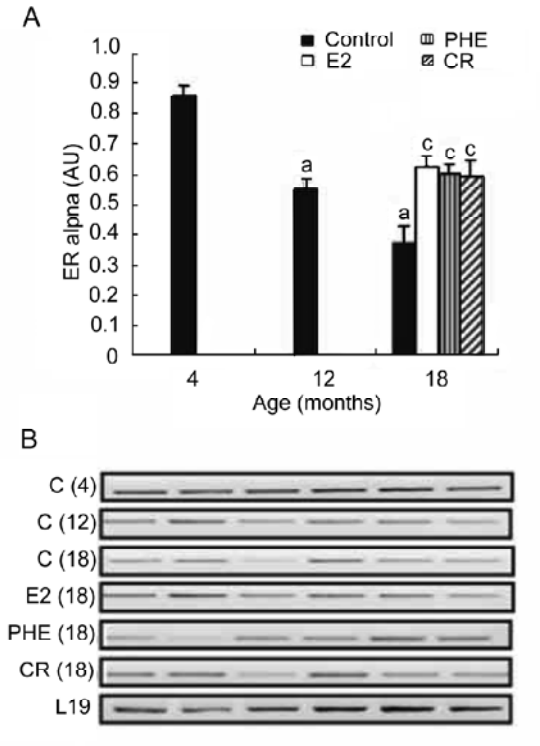

Figure 2. (A): Expression of ERα gene in testis of rats either fed *ad libitum* (12- and 18-month-old controls), or treated with 17β-estradiol (E2), *Peganum harmala* extract (PHE) or submitted to caloric restriction (CR). The data are expressed as mean ± SEM ($n = 6$). (a): Control rats significantly different from 4-month-old rats ($P < 0.05$). (b): E2, PHE and CR treated rats significantly different from 12-month-old animals ($P < 0.05$). (c): E2, PHE and CR treated rats significantly different from the same age control rats ($P < 0.05$). (B): Representative signal of the amplification of ERα gene obtained by polymerase chain reaction (PCR) using total RNA from testes of rats aged of 4 months (C [4]), 1 year (C [12]), 18 months (C [18]), and in 18-month-old animals treated with E2, PHE or CR. L19, a stable control (control gene which their expression does not changed in pathological conditions).

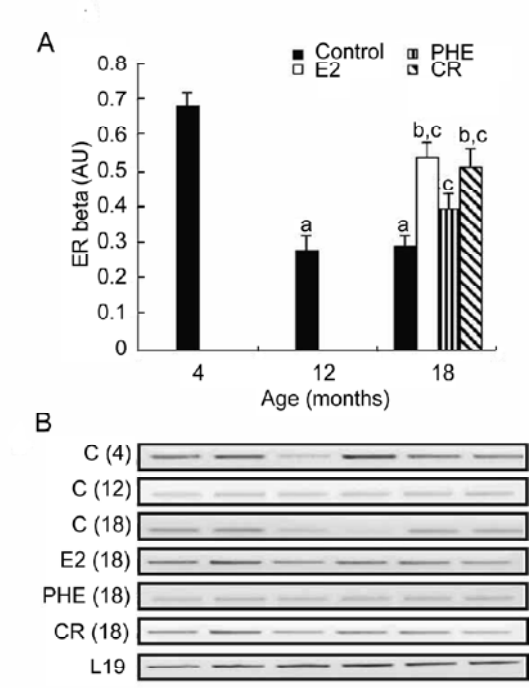

Figure 3. (A): Expression of ERβ gene in testis of rats either fed *ad libitum* (12- and 18-month-old controls), or treated with 17β-estradiol (E2) or *Peganum harmala* extract (PHE) or submitted to caloric restriction (CR). The data are expressed as mean ± SEM (n = 6). (a): Control rats significantly different from 4-month-old rats ($P < 0.05$). (b): E2, PHE and CR treated rats significantly different from 12-month-old animals ($P < 0.05$). (c): E2, PHE and CR treated rats significantly different from the same age control rats ($P < 0.05$). (B): Representative signal of the amplification of ERβ gene obtained by polymerase chain reaction using total RNA from testes of rats aged 4 months (C [4]), 1 year (C [12]), 18 months (C [18]), and in 18-month-old animals treated with E2, PHE or CR. L19, a stable control (control gene which their expression does not changed in pathological conditions).

3.2 Effects of various treatments on testosterone and estradiol concentrations in testis

The levels of testosterone were significantly decreased ($P < 0.001$) in 18-month-old rats compared to animals aged of 4 months (Figure 4C). After treatment with either E2 or PHE and CR, an increase of the endogenous testosterone concentrations (54%, 49% and 42% respectively, compared to control rats) was recorded and the levels were identical to that of 12-month-old rats.

The testicular level of estradiol was diminished by 33% between the age of 4 and 18 months (Figure 4B). However, in E2, PHE and CR-treated rats the estradiol levels were enhanced by 103%, 87% and 18%,

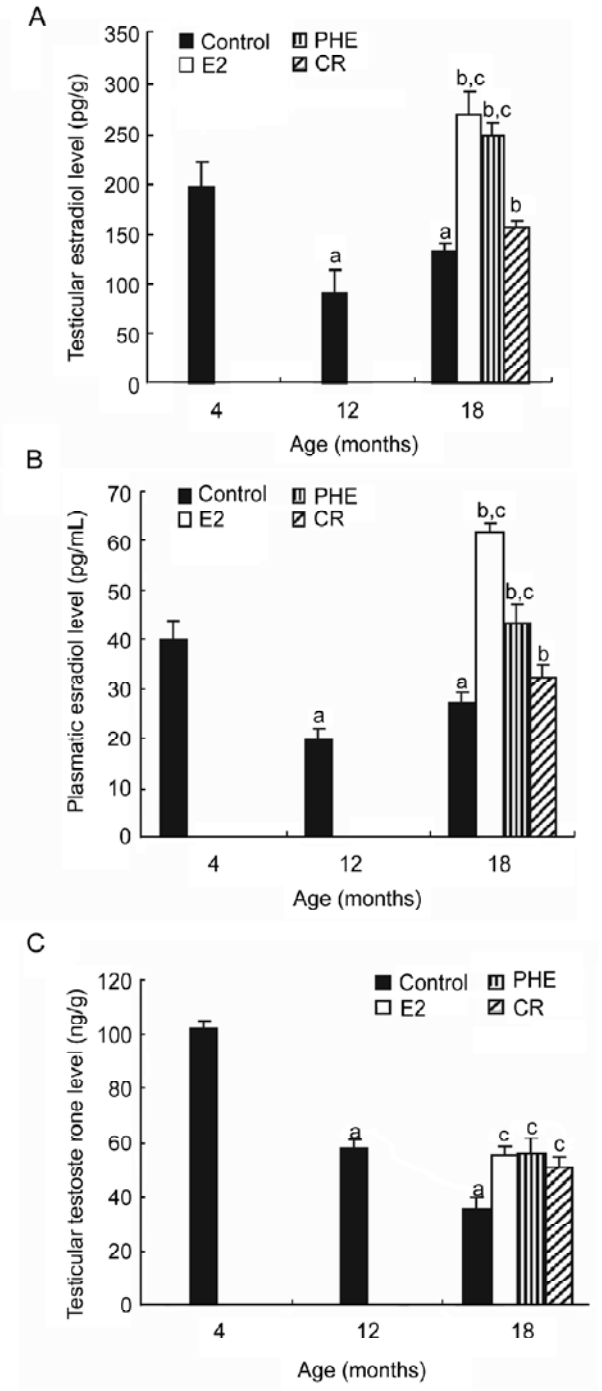

Figure 4. Testicular concentrations of testosterone (A) and 17β-estradiol (E2) (B), and serum levels of estradiol (C), in control and rats treated with E2 or *Peganum harmala* extract (PHE) or submitted to caloric restriction (CR). The data are expressed as mean ± SEM (n = 6). (a): Control rats significantly different from 4-month-old rats ($P < 0.05$). (b): E2, PHE and CR treated rats significantly different from 12-month-old animals ($P < 0.05$). (c): E2, PHE and CR treated rats significantly different from the same age control rats ($P < 0.05$).

respectively, compared to the control rats (Figure 5B). As far as the blood estradiol levels were concerned, significant ($P > 0.05$) variations were observed in control rats as well as in CR animals; conversely, and as expected in E2 and PHE-treated rats, the levels of estradiol were increased by 127% and 60% (Figure 4C).

3.3 SOD, CAT, GPx activities and TBARs levels in testes of control and treated rats (Table 2)

After either estradiol, plant extract or caloric restriction treatment, a significant ($P < 0.05$) increase in SOD activities (49%, 66% and 33%, respectively) was recorded compared to the control rats. The CAT activity was enhanced by 19%, 21% and 27%, respectively, in E2, PHE and CR animals compared to untreated 18-month-old rats. It is of note that these enzyme activities were higher than those measured in 1-year-old rats. The GPx activity was increased in the three groups of treated rats (23%, 39% and 18%, respectively), but not significantly ($P > 0.05$) compared to the control animals. The testicular TBARs levels were significantly increased in the control rats aged 18 months compared to 1-year-old animals. When rats were treated either with E2 or PHE, or submitted to CR, a significant ($P < 0.05$) decrease in TBARs levels by 20%, 16% and 26% was observed compared to the control animals of the same age.

3.4 LDH, AST, ALT, GGT and PAL activities in rat testes (Table 2)

In control rats aged 12 and 18 months a significant increase in the activity of testicular LDH, GGT, PAL, AST and ALT was observed when compared to 4-month-old animals. In 18-month-old E2, PHE and CR-treated rats, a significant decrease in all these enzyme activities compared to the untreated rats of the same age was recorded.

3.5 Histological changes in testes of control and treated rats

Because a positive effect of E2, PHE and CR was observed on the relative testicular weight, we performed histological analyses of spermatogenesis (Figure 5). Although not evaluated quantitatively, a qualitative evaluation of spermatogenesis was performed: a depletion of germ cells at various stages of development was observed in old-untreated rat (Figure 5C), when compared to 4-month-old rats (Figure 5A). In E2 (Figure 5D), PHE (Figure 5E) and CR (Figure 5F)-treated rats, spermatogenesis proceeded normally and was similar to that of 12-month-old rats (Figure 5B).

4 Discussion

Our results indicate that in male rats aging is accompanied by an increase in the production of free radicals.

Table 2. Testicular antioxidant enzyme activities: super oxidase dimutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), thiobarbituric acid reactive substances (TBARs) level and toxicity indexes aspartate transaminase (AST), alanine transaminase (ALT), gamma-glutamyl transferase (GGT), phenylalanine ammonia lyase (PAL) and lactic dehydrogenase (LDH) activities in 4-, 12- and 18-month-old rats either fed *ad libitum* (control), or treated with 17β-estradiol (E2), *Peganum harmala* extract (PHE), or submitted to caloric restriction (CR) for 6 months starting at the age of 1 year. SOD, U/mg protein; CAT, mmoles H_2O_2 utilized/min/mg protein; GPx, mmoles GSS utilized/min/mg protein; TBARS, nmoles/mg protein. AST, ALT, GGT, PAL, LDH, IU/mg protein. The data are expressed as mean ± SEM ($n = 6$). (a): Control rats significantly different from 4-month-old rats ($P < 0.05$). (b): E2, PHE and CR treated rats significantly different from 12-month-old animals ($P < 0.05$). (c): E2, PHE and CR treated rats significantly different from the same age control rats ($P < 0.05$).

Enzymes	4 months	12 months	18 months			
			Control	E2	PHE	CR
SOD	5.15 ± 0.41	2.54 ± 0.28[a]	2.63 ± 0.46[a]	3.76 ± 0.14[a,b,c]	4.22 ± 0.31[a,b,c]	3.38 ± 0.31[a,b,c]
CAT	374 ± 22	251 ± 22[a]	255 ± 10[a]	316 ± 23[a,b,c]	342 ± 12[a,b,c]	325 ± 11[a,b,c]
GPX	37.3 ± 4.0	28.1 ± 1.3	28.8 ± 2.1	34.3 ± 2.0	35.0 ± 2.6	34.1 ± 1.8
TBARS	0.54 ± 0.06	0.88 ± 0.08[a]	1.03 ± 0.02[a]	0.81 ± 0.02[a,c]	0.87 ± 0.08[a,c]	0.76 ± 0.04[a,c]
AST	6.2 ± 1.3	10.9 ± 2.2[a]	34.0 ± 1.4[a]	13.6 ± 0.9[b,c]	25.7 ± 0.8[a,b,c]	19.6 ± 0.7[a,b,c]
ALT	11.0 ± 1.3	19.3 ± 2.3[a]	41.0 ± 2.2[a]	16.3 ± 1.0[c]	30.0 ± 3.0[a,b,c]	23.8 ± 1.0[a,c]
GGT	0.40 ± 0.12	1.68 ± 0.45[a]	2.25 ± 0.30[a]	1.00 ± 0.18[a]	0.75 ± 0.08[b,c]	0.65 ± 0.11[b,c]
PAL	3.4 ± 1.5	12.0 ± 1.0[a]	47.0 ± 1.8[a]	11.5 ± 1.8[a,c]	34.7 ± 1.3[a,b,c]	30.1 ± 51.0[a,b,c]
LDH	5.1 ± 1.4	17.0 ± 5.0[a]	57.0 ± 6.9[a]	32.0 ± 4.0[a,c]	23.0 ± 4.1[a,c]	37.7 ± 9.0[a,c]

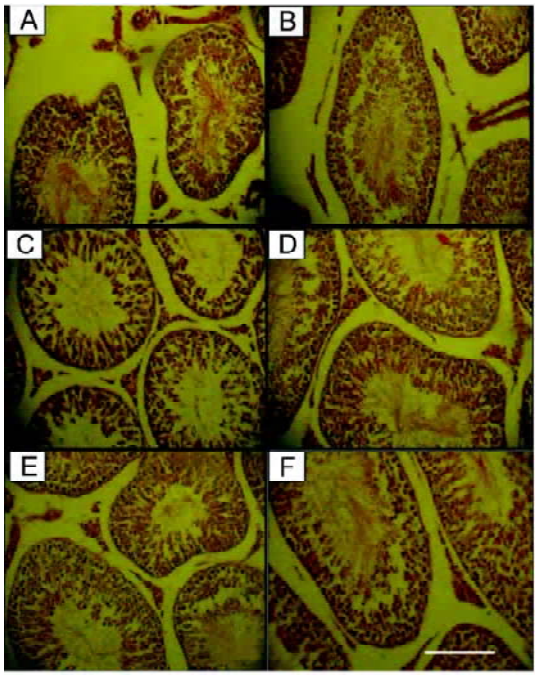

Figure 5. Effect of aging (C), 17β-estradiol (E2) (D), *Peganum harmala* extract (PHE) (E) and caloric restriction (CR) (F) on the histological morphology of rat testes (magnification: × 100). Control rat aged 4 months (A) showing a regular course of spermatogenesis. Control rat aged 12 months (B) showing a normal development of spermatogenesis. Control rat aged 18 months (C) with alterations of spermatogenesis. In E2 (D), PHE (E) and CR (F) testes of 18-month-old rats, a positive effect was observed with well-developed germ cells. Bar = 140 μm.

These changes are likely the consequence of a lower number of Leydig, Sertoli and germ cells in old rats compared to younger animals, as reported by Chen *et al*. [22]. These decreases in the numbers of testicular cells might lead to lower levels of aromatase and ERα/β transcripts and, therefore, to a diminution of testosterone and estrogen syntheses. The low steroids output synthesized mainly by the Leydig cells could be also related to the production of free radicals, which induce damages to the cell macromolecules content, as shown by the increase of the toxicity indexes, such as AST, ALT, PAL, GGT and LDH activities and the lipid peroxidation in testes [9, 10]; in parallel, the testicular SOD, CAT and GPx contents are diminished [22]. All these changes can alter the testicular cells, including spermatozoa and, therefore, the sperm production leading to an alteration of the male fertility. Although we did not perform quantitative analyses of the germ cells, it was clear that the spermatogenesis was altered in the untreated 18-month-old rat testes, which likely accounts for the decrease in testicular weight reported by Henkel *et al*. [36] and in the agreement with our previous study in which a diminution of the number of spermatozoa has been observed in aged rats [37].

However, in rats treated with either estrogens or plant extract over 6 months, the activities of the above enzymes were back to their normal control levels, suggesting an antioxidant protective role of estrogens [38]. Moreover, caloric restriction could slow aging and induce some benefits on the longevity of rats [20]. Because we have demonstrated that most of the rat testicular cells express aromatase and are equipped with estrogens receptors [39] we have herein analyzed the effects of either estrogens or CR over a period of 6 months (12–18 months), corresponding to aging in the male rat. The various treatments induce a positive and a significant effect on aromatase and ERs genes expression in aged rat testes; moreover, the classical parameter of oxidative stress (TBARs) and antioxidant enzymatic activities (catalase, superoxide dismutase and glutathione peroxydase) have been affected, particularly by the estrogen treatment. Therefore, we could speculate that an increase in free radicals formation consecutive to a diminution of the aromatase and ERs genes expression associated with a lower level of testosterone in testis of 18-month-old rats (compared to younger animals), likely accounts for the observed alterations of spermatogenesis in aged animals, as reported by Chen *et al*. [22]. The mechanisms concerned by the protective effect of estrogens treatment are not clearly understood up to now. However, estrogens enhance the antioxidant enzyme activities, such as SOD, CAT and GPx [12] and, therefore, could protect the testicular cells against damage and death produced by free radicals. Moreover, this suggests that the beneficial effect of a caloric restriction diet could be in part mediated by estrogens via a parallel increase of ERs expression, as reported elsewhere [37]. Indeed, lower estrogen levels are responsible for enhanced-free radical generation [40, 41], leading to an increase in lipid peroxidation, and a concommittant reduction of the antioxidant barrier activity, especially in the testis [12]. Estrogens can exert an antioxidant role by scavenging free radicals [42] and, therefore, they may prevent any damage induced by these free radicals on cell protein and DNA contents [38]. Consequently, E2, PHE and CR treatments appear to be an adaptive strategy to preserve

testicular integrity and function during aging in male rats. The diminution of the mitochondrial ROS production could also be evoked as a putative target for estrogens and caloric restriction [43]. As reported by Gancarczynk *et al*. [44] in the bank vole, additionnal studies are necessary to elucidate the mechanisms of actions of estrogens in the testicular cells and, in particular, at the mitochondrial level.

In conclusion, according to the localization of aromatase in Leydig cells as well as in germ cells of rodents [45], and taking into account the widespread distribution of ERs in the testicular cells [46], an antioxidant role of estrogens is possible during aging. According to our observations in aged rats, the testicular expression of aromatase and ERs in estrogen and PHE-treated rats, as well as in animals submitted to CR, are higher than in control animals. That suggests a protective, physiologically relevant effect of estrogens, especially in lowering oxidative stress via the increase of the cellular antioxidant defense system, as suggested by Borras *et al.* [12] and Nam *et al.* [47]. In our preliminary study [37], a decrease in testicular estradiol levels associated with diminutions in the number and motility of epididymal spermatozoa were recorded during aging. In addition, caloric restriction was beneficial to support full spermatogenesis in old rats. Therefore, a low caloric diet might improve the protection of the cells against ROS via an improvement of the cellular antioxidant defense system in which estrogens are probably concerned, as demonstrated by the effectiveness of the estradiol treatments.

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