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AQUEOUS EXTRACT OF PECAN NUT SHELL REDUCES TESTICULAR DAMAGE AFTER HEAT SHOCK

KEY WORDS: *Carya illinoensis,* testis, spermatogenesis, oxidative stress.

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ABSTRACT

Heat can reduce male fertility and trigger testicular degeneration. The study aimed to verify whether the pecan shell, popularly used in tea form may protect the testes against the damage caused by heat. It was orally administered in adult rats 30 days before heat shock (HS), as well as 30 days before and 30 days after HS. Rats were evaluated at 15, 30 and 60 days after HS. Results showed an increase in epididymis and testis weight after treatment. Histopathological, histomorphometrical and electronic microscopy analyses showed a better recovery of spermatogenesis in animals treated. Furthermore, pecan reduced the thiobarbituric acid reactive substances and increased the levels of glutathione and vitamin C in the testes. Carbonyl groups, catalase activity, protein levels and plasma testosterone were not altered. In conclusion, pecan shell aqueous extract could aid a better recovery of spermatogenesis after the reversible testicular damage induced by heat.

INTRODUCTION

Several factors can damage the testis, such as heat, testicular trauma, exposure to cold, toxins, ischemia, nutritional deficiencies, exogenous administration of androgens, infections, autoimmune diseases, obstruction of sperm flow, cancer and ionizing radiation[1]. Depending on the severity of testicular damage and survival of spermatogonia, Sertoli cells and Leydig cells, sperm production can be reversibly restored [2-3].

Heat can interfere on male fertility and trigger testicular degeneration [4-5]. All tissues are susceptible to heat damage; however, the testes are particularly sensitive and can be damaged by exposure to temperatures typically found inside the abdomen. The normal testicular function occurs at 2 to 6°C below body temperature.[6-7] At high temperatures, the thermoregulatory physiological mechanisms are not sufficient to maintain the temperature of male gonad, resulting in testicular degeneration and reduced fertility.[8]

Many studies showed an inverse relationship between consumption of fruit, vegetables and cereals with incidence of cardiovascular diseases, cancer, inflammatory and degenerative processes. These pathologies are associated with oxidation in biological systems involving the generation of Reactive Oxygen Species (ROS). ROS are responsible for changes in cellular components, triggering chain reactions in fatty acids of membrane lipoproteins, affecting their structure and functional integrity.[9-10] Tissue damage depends on the type and amount of ROS, as well as the length of exposure, associated with extracellular factors such as temperature, oxygen tension and environment.[11]

The actions of antioxidants have been associated with their ability to scavenge free radicals, reducing oxidative damage of cellular biomolecules such as lipids, proteins and DNA.[12-13] Plant foods are sources of natural antioxidants.[14-15] Pecan *(Carya illinoensis)* is a member of the *Juglandaceae* family,[16] which originates the pecan shell, a vegetal by-product with low cost and high antioxidant potential considered a promising substance because of its high levels of total polifenols, condensed tannins, flavonoids substances and proanthocyanidins.[17-20] These substances inhibit lipid oxidation in foods and biological systems.[21] Furthermore, the high levels of total phenolic compounds in this nut show a

potential alternative use of pecan shells as a novel source of antioxidants.[19]

Considering that pecan shell is popularly consumed in tea form to treat toxicological diseases with high antioxidant capacity,[22-23] the present study aims to verify whether the aqueous extract (AE) of pecan nut shell can protect the testis against the damage caused by heat.

MATERIALS AND METHODS

Animals and experimental design

Forty-five adult rats (90 days old) were acclimated for 15 days and randomly selected to compose the experimental groups: 15 control group was only treated with water; 15 group pretreated with pecan nut shell AE for 30 days before the testicular heat shock (HS) and 15 group pre and post treated with pecan nut shell AE 30 days before HS and 30 days after HS. The rats were chronologically analyzed 15, 30 and 60 days after the testicular damage caused by HS to evaluate the effects of pecan nut shell AE on the spermatogenesis recovery.

In a previous study[24] we used a negative control group (animals not subjected to any experimental manipulation) to demonstrate that our control animals (subjected to heat shock) did not suffer interference from the experimental manipulation.

Adult male Wistar rats obtained from the biotery of Federal Rural University of Pernambuco were kept in an environment with controlled temperature (23 ± 2°C), humidity (60%) and 12-hour light/dark cycle. Standard pellet food and water were provided ad libitum.

Pecan Shell Aqueous Extract

Pecan nut shell [*Carya illinoensis* (Wangenh.) K. Koch] was obtained from the Herbarium (SMDB-11.528) in the Department of Biology of the Federal University of Santa Maria. The plant name has been checked with http://www.theplantlist.org. Pecan nut shell is currently sold in supermarkets with permission of the Ministry of Agriculture, Brazil. AE of pecan nut shells was freshly prepared by infusion (5% at 90°C) over 30 min, filtered using filter paper and cooled to room temperature. The AE was protected from light during all experiments. Pecan nut shell AE was produced and standardized (using HPLC) according to Benvegnú et al.[25]

Testicular Heat Shock

Animals were anesthetized (ketamine 80 mg/kg and xylazine 15 mg/kg) and then exposed to a single testicular heat shock according to Tenorio et al.[3] The testes were immersed in warm water at 43°C for 15 min; thereafter the animals were transferred to their boxes and kept at room temperature as described by Queiroz.[24]

Tissue Perfusion and Weight Evaluation of Androgen-dependent Organs

Fifteen, thirty and sixty days after HS, the rats were anaesthetized (ketamine 80 mg/kg and xylazine 15 mg/kg) and heparinized (sodium heparin, 125 UI/100g); then the right testis was homogenized in 10 volumes (10 mL/g tissue) of 10 mM Tris-HCl buffer (pH 7.4). The homogenates were centrifuged at 5000 rpm for 20 min and supernatants were used for the antioxidant assays. Blood was collected for testosterone analysis. Subsequently, animals were subjected to an intracardiac perfusion with a NaCl 0.9% solution plus heparin (50 IU/L) and then perfused with 4% glutaraldehyde in sodium phosphate buffer (0.01 M and pH 7.2). The testis, epididymis, seminal vesicles and prostate were removed and weighed.

Histomorfometrical and Histopathological Analysis of Testes

The fragments were dehydrated in an ascending series of alcohols and embedded in glycol methacrylate plastic resin, serially sectioned (4 µm) and were stained with toluidine blue/sodium borate (1%).[26] After that, photomicrographs were captured as described by Tenorio et al.[27] Testicular components were analyzed morphometrically and morphologically as described by Bringel et al. [28]

Transmission Electron Microscopy

Fragments of the testis were fixed overnight in a solution (2.5% glutaraldehyde and 4% paraformaldehyde in 0.1 M cacodylate buffer). The fragments were post-fixed in a solution containing 1% osmium tetroxide, 2 mM $CaCl_2$ and 0.8% potassium ferricyanide in 0.1 M cacodylate buffer (pH 7.2). The samples were dehydrated in acetone and embedded in SPIN-PON (Embed 812). Ultrathin sections were counterstained using 5% uranyl acetate and lead citrate. Transmission electron microscopy analyses were performed with a FEI Morgani 268D microscope.

Plasma Testosterone

Blood samples were centrifuged and the plasma being kept at -20°C until analysis.[27] The plasma testosterone analysis was performed by enzyme immunoassay with absorbance reading at 405 nm, as described by Brown et al.[29] ELISA was performed using a polyclonal anti-testosterone antibody (9R 156/7 1:7500 dilution, obtained from Coralie Munro at the University of California, Davis, CA, USA). All samples were analyzed twice with a lower than 10% intra and inter assay coefficient of variation.

Estimation of Antioxidants

Lipid peroxidation in the testis tissue was measured by estimating the formation of thiobarbituric acid reactive substances (TBARS), as previously described by Ohkawa.[30]

Carbonyl protein content was measured according to the method described by Levine et al.,[31] based on the detection of the reaction between 2,4-dinitrophenyl hydrazine and carbonyl proteins to form protein hydrazine. Results were expressed as nanomoles of carbonyl protein per gram of tissue using a using a 370-nm wavelength with a molar extinction coefficient of 22000 $M^{-1}cm^{-1}$.

Testicular reduced glutathione (GSH) content was measured according to Jacques-Silva et al.,[32] expressed as micromoles of GSH per gram of tissue. Vitamin C in the testis was also estimated as described by Galley et al.,[33] with some modifications expressed as milligrams of VIT C per milliter of plasma.

Catalase (CAT) activity was quantified in testis tissue according to Aebi,[34] which involves monitoring the disappearance of hydrogen peroxide (H_2O_2) in the presence of cell homogenate (pH 7.2 at 25°C). The enzymatic activity was expressed as micromoles of H_2O_2 per min per gram of tissue. Protein content in the testis was measured according to Lowry et al.[35]

The experimental protocol was approved by the Ethics Committee of the Federal Rural University of Pernambuco (CEUA-UFRPE 23082.008169/2010) in accordance with the basic principles for research using animals.

Statistical Analysis

A Shapiro-Wilks test was used to check the tendency of the data to normality. Depending on the normal trend of the results, we used a parametric or nonparametric test. For the data considered normally distributed, we used analysis of variance (ANOVA) with the Tukey–Kramer post-hoc test. If the data were not normally distributed, we used the nonparametric test of Kruskal–Wallis with Dunn's post-hoc test. Results were expressed as mean and (±) standard deviation. A p-value < 0.05 was considered statistically significant.

RESULTS

Androgen-dependent Organs Weight

Results of androgen-dependent organs weight are shown in table 1.

Table 1. Organ weights (g) of the rats pretreated or pre and post-treated with pecan shell aqueous extract (AE) evaluated 15 days after testicular heat shock (HS).

15 days after HS	Control Group	Pretreated	Pre and post-treated
Testes	0.76 ± 0.06	0.70 ± 0.08	0.69 ± 0.11
Epididymis	0.45 ± 0.03[a]	0.33 ± 0.06[a]	0.65 ± 0.13[b]
Prostate	1.14 ± 0.40	0.34 ± 0.06	0.65 ± 0.13
Seminal gland	1.26 ± 0.24	0.88 ± 0.41	1.23 ± 0.19
30 days after HS			
Testes	0.79 ± 0.11	1.11 ± 0.43	1.31 ± 0.47
Testes liquid	744.3 ± 108.3	1036.2 ± 401.0	1226.2 ± 440.4
Epididymis	0.41 ± 0.04[a]	0.49 ± 0.06[a]	0.56 ± 0.10[b]
Prostate	0.48 ± 0.03	0.50 ± 0.07	0.58 ± 0.13
Seminal gland	1.18 ± 0.20[a]	1.92 ± 0.23[b]	2.08 ± 0.38[b]
60 days after HS			
Testes	0.94 ± 0.10	1.07 ± 0.33	1.32 ± 0.31
Testes liquid	852.9 ± 76.8	995.2 ± 317.1	1236.9 ± 295.5
Epididymis	0.42 ± 0.08	0.50 ± 0.07	0.52 ± 0.21
Prostate	0.51 ± 0.12	0.54 ± 0.07	0.55 ± 0.78
Seminal gland	1.96 ± 0.57	1.77 ± 0.75	1.70 ± 0.34

Values are expressed as mean standard error of the mean.
Different letters in the same line indicate statistically significant difference from group at P<0.05.

Testicular weight did not statistically change; however, we observed a trend of 29% increase in testicular weight in the group pretreated with pecan shell AE and 40% in the group pre and post treated with pecan shell AE 15 days after heat shock (HS). In the same way, these animals showed a trend of 13% and 29% increase in testicular weight respectively 30 days after HS. Epididymis weight also increased 15 and 30 days after HS in animals treated with pecan shell AE.

Sixty days after HS, animals pretreated with pecan shell AE showed a 14% increase in testicular weight and pre and post-treated animals had their testicular weight increased by 40%. Similarly, the weight of the functional part of the testicle also called testicular liquid weight, increased by 17% in animals pretreated with pecan shell AE and by 44% in pre and post-treated animals. Intriguingly enough, due to the great tendency of dispersion in these results, they were not statistically significant. Prostate and seminal gland weights did not change after the treatment with pecan shell AE.

Testicular Histomorphometry

Testicular morphometric parameters in rats pretreated or pre and post-treated with pecan shell AE evaluated 30 and 60 days after testicular HS are shown in tables 2 and 3.

Table 2. Volume (μL) of the testicular components of rats pretreated or pre and post-treated with pecan shell aqueous extract (AE) evaluated 30 days after testicular heat shock (HS).

	Control Group	Pretreated	Pre and post-treated
Seminiferous tubule	395.0 ± 77.5[a]	795.3 ± 294.3[ab]	888.9 ± 336.2[b]
Seminiferous epithelium	373.3 ± 85.7[a]	638.0 ± 298.9[ab]	769.3 ± 297.8[b]
Tubular lumen	7.8 ± 9.8[a]	89.0 ± 42.4[b]	99.3 ± 53.8[b]
Tunica propria	13.9 ± 9.3	23.3 ± 9.4	20.3 ± 4.9
Leydig cells	10.2 ± 6.8[a]	42.6 ± 14.3[a]	67.3 ± 49.0[b]
Blood vessel	31.3 ± 7.6	20.5 ± 9.1	24.0 ± 15.5
Lymphatic space	317.1 ± 87.2	177.7 ± 125.4	245.8 ± 119.1

Values are expressed as mean standard error of the mean (n=5).
Different letters in the same line indicate statistically significant difference from group at P<0.05.

Table 3. Volume (μL) of the testicular components of rats pretreated or pre and post-treated with pecan shell aqueous extract (AE) evaluated 60 days after testicular heat shock (HS).

	Control Group	Pretreated	Pre and post-treated
Seminiferous tubule	650.7 ± 50.4[a]	840.6 ± 274.9[ab]	1043.9 ± 269.8[b]
Seminiferous epithelium	525.5 ± 38.8[a]	738.0 ± 268.8[b]	890.7 ± 221.1[b]
Tubular lumen	82.0 ± 30.0	88.7 ± 21.1	128.6 ± 56.8
Tunica propria	46.2 ± 7.8[a]	13.9 ± 3.0[b]	24.6 ± 7.4[b]
Leydig cells	39.6 ± 32.4	29.2 ± 7.7	36.7 ± 9.0
Blood vessel	46.5 ± 1.8[a]	21.4 ± 6.9[b]	27.2 ± 9.1[ab]
Lymphatic space	14.8 ± 103.6	104.0 ± 33.2	119.1 ± 22.2

Values are expressed as mean standard error of the mean (n=5).
Different letters in the same line indicate statistically significant difference from group at P<0.05.

Seminiferous tubule and seminiferous epithelium volumes showed statistically significant increase (125% and 106% respectively) in pre and post-treated animals with pecan shell AE for 30 days after HS. In the same way, seminiferous epithelium and seminiferous tubules volumes increased (70% and 60% respectively) in the group pre and post-treated with pecan shell AE for 60 days after HS.

The volume of tubular lumen increased in animals treated with pecan shell AE compared to the control group 30 days after HS. This occurred due to a lesser desquamation and death of germ cells, reinforcing the hypothesis of a better recovery of spermatogenesis after treatment with pecan. Volumes of tunica propria, blood vessels and lymphatic space showed no difference between the experimental groups 30 days after HS. Furthermore, the volume of Leydig cells increased in pre and post-treated animals with pecan shell AE 30 days after HS.

Sixty days after HS, the volumes of tunica propria and blood vessel decreased in animals treated with pecan shell AE; however, the

volumes of lymphatic space and tubular lumen did not change.

Testicular Histopathology

Testes showed pathological lesions compatible with severe testicular degeneration 30 days after HS. We observed degenerated seminiferous tubule, presence of clusters of germ cell debris in tubular lume, thickening of the tunica propria and intracytoplasmatic vacuolization in Sertoli cells (Figure 1A) in the control group. At 30 days after HS, rats treated had a better recovery of the testes, showing seminiferous tubules with immature pre-meiotic cells and mature germ cells, elongated spermatids and intertubular tissue with Leydig cells; seminiferous tubules with germ cells in different stages of seminiferous epithelium cycle and sperm formation in tubular lume are observed (Figure 1B). 30 days after HS, the testes also showed a better recovery of spermatogenesis in pre and post-treated animals with pecan shell AE compared to the control group (Figure 1C).

Sixty days after HS, the control group showed regenerated seminiferous tubules with germ cells in all stages of development: spermatocytes, round spermatids and elongated spermatids. Therefore, spermatogenesis is regenerated in control animals 60 days after heat shock, featuring the reversible testicular damage. However, minor pathological lesions such as vacuolization in the nucleus of Sertoli cells and thickening of tunica propria (Figure 1D) should be observed.

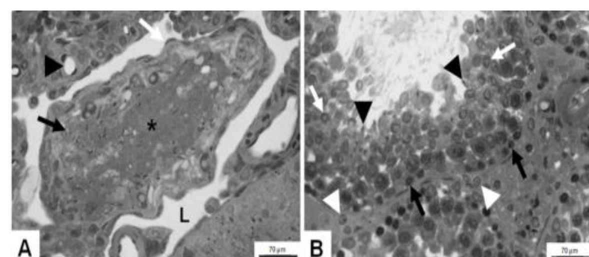
It is noteworthy that the groups treated with pecan nut shell showed spermiation in the tubules in stage VII of seminiferous epithelium cycle (Figure 1E) and the lack of pathological lesions in seminiferous tubule in stage VIII (Figure 1F) after pre and post-treatment with pecan shell AE, demonstrating a better recovery of spermatogenesis after pecan shell AE treatment.

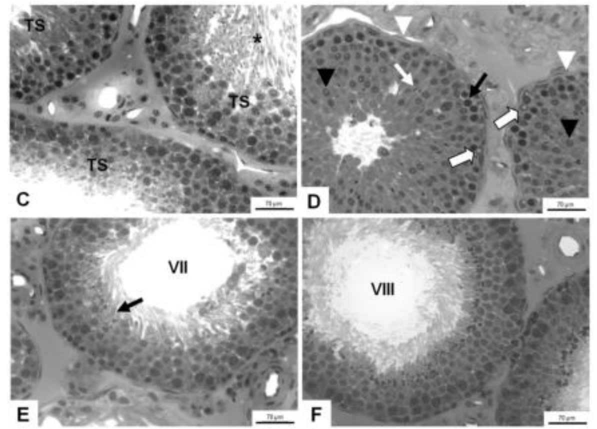
Transmission Electron Microscopy

Testis analysis using electron microscopy also showed pathological lesions compatible with testicular degeneration 30 days after HS. Several immature germ cells showed many intracytoplasmic vacuoles. Degenerated germ cells were desquamating or desquamated from seminiferous epithelium showing morphological characteristics similar to apoptosis, such as abnormal chromatin condensed near the nuclear periphery. The intertubular tissue showed Leydig cells with ultrastructural changes and collagen deposits in control animals (Figure 2A); these changes were less evident in animals treated with pecan shell AE. However, even after the pecan shell AE treatment, the germ cells still showed high electron dense mitochondria with loss of their normal organization, swelling and loss of cristae (Figure 2B). Mitochondria in germ cells treated with pecan shell AE showed less swelling and vacuolization.

Despite the reestablishment of spermatogenesis in control animals 60 days after HS (including seminiferous tubules containing spermatids), Sertoli and germ cells still showed ultrastructural changes in the testes (Figure 2C). Animals treated with pecan shell AE showed lesser severity of ultrastructural pathological lesions in testicular cells; however, these animals still showed few changes (Figure 2D).

Figure 1. Photomicrograph of testes in rats treated with pecan shell aqueous extract (AE) 30 days (control group: 1A; pretreated: 1B; pre and post-treated: 1C) and 60 days (control group: 1D; pretreated: 1E; pre and post-treated: 1F) after heat shock.



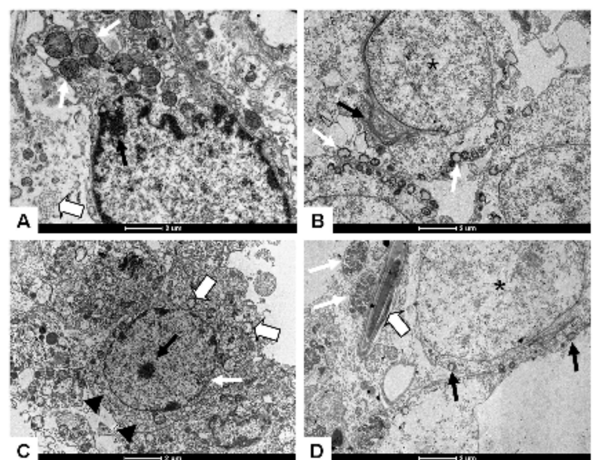


1A: degenerated seminiferous tubule (black arrow), thickening of tunica propria (white arrow), clusters of germ cell debris in tubular lume (*), blood vessels (arrow head) and lymphatic space (L). **1B**: seminiferous tubules showing immature (pre-meiotic cells: black arrow) and mature (post-meiotic cells: white arrow) germ cells, elongated spermatids (black arrow head) and intertubular tissue with Leydig cells (white arrow head) are observed. **1C**: Seminiferous tubules (TS) with germ cells in different stages of seminiferous epithelium cycle, sperm formation in tubular lume (*) is observed. **1D**: regenerated seminiferous tubules with germ cells in all stages of development: spermatocytes (black arrow), round spermatids (black arrow head) and elongated spermatids (white arrow); however, minor pathological lesions such as vacuolization in the nucleus of Sertoli cells (large arrows) and thickening of tunica propria (white arrow head) are observed. **1E**: tubule in stage VII of the seminiferous epithelium cycle, spermiation (sperm release from epithelium to lume: black arrow) is observed. **1F**: seminiferous tubule in stage VIII, the lack of pathological lesions after pre and post-treatment with pecan shell AE is observed.

Transmission Electron Microscopy

Testis analysis using electron microscopy also showed pathological lesions compatible with testicular degeneration 30 days after HS. Several immature germ cells showed many intracytoplasmic vacuoles. Degenerated germ cells were desquamating or desquamated from seminiferous epithelium showing morphological characteristics similar to apoptosis, such as abnormal chromatin condensed near the nuclear periphery. The intertubular tissue showed Leydig cells with ultrastructural changes and collagen deposits in control animals (Figure 2A).

Figure 2. Transmission electron micrograph of testis of rats treated with pecan shell aqueous extract (AE) 30 days (control group: 2A; pretreated: 2B) and 60 days (control group: 2C; pre and post-treated: 2D) after heat shock.



2A: Leydig cell in intertubular tissue showing condensed chromatin near nuclear periphery (black arrow); increased size

mitochondria (white arrow) and collagen fiber (large arrow) are observed. **2B**: Round spermatid in seminiferous epithelium (*) showing acrosome formation (black arrow) and degenerated mitochondria with loss of their normal organization and high electron-dense cristae (white arrow). **2C**: Leydig cell showing the nucleolus (black arrow) and nucleus indentation (white arrow); degenerated mitochondria (large arrow) and intracytoplasmic vacuoles (arrow head) are observed. **2D**: mature germ cells in seminiferous epithelium. Round spermatid (*) still showing ultrastructural changes such as degenerated mitochondria (black arrow). Elongated spermatid with condensed nucleus (large arrow) and tail (white arrow) is observed.

These changes were less evident in animals treated with pecan shell AE. However, even after the pecan shell AE treatment, the germ cells still showed high electron dense mitochondria with loss of their normal organization, swelling and loss of cristae (Figure 2B). Mitochondria in germ cells treated with pecan shell AE showed less swelling and vacuolization.

Despite the reestablishment of spermatogenesis in control animals 60 days after HS (including seminiferous tubules containing spermatids), Sertoli and germ cells still showed ultrastructural changes in the testes (Figure 2C).

Animals treated with pecan shell AE showed lesser severity of ultrastructural pathological lesions in testicular cells; however, these animals still showed few changes (Figure 2D).

Plasma Testosterone

No significant differences were observed in the plasma testosterone 15, 30 and 60 days after HS (Table 4).

Table 4. Plasma testosterone analysis (ng ml^{-1}) of rats pretreated or pre and post-treated with pecan shell aqueous extract (AE) evaluated 15, 30 and 60 days after testicular heat shock (HS).

Days after HS	Control Group	Pretreated	Pre and post-treated	P
15	0.68 ± 0.18	0.78 ± 0.38	1.06 ± 0.54	0.05
30	0.76 ± 0.31	0.59 ± 0.35	0.73 ± 0.17	0.62
60	1.11 ± 1.01	0.79 ± 0.18	1.14 ± 0.84	0.24

Values are expressed as mean standard error of the mean (n=5).

Antioxidants analyses

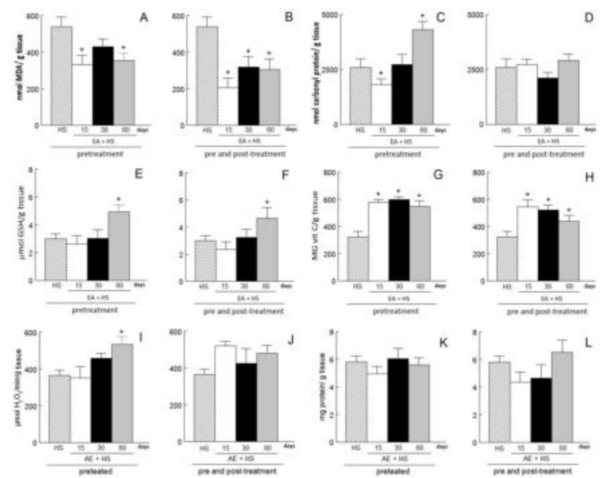
Effects of pecan shell AE treatment on lipid peroxidation levels in testes are shown in Figures 3A and 3B.

Pecan treatment significantly prevented the increase of TBARS after testicular heat shock. Carbonyl protein levels decreased 15 days after HS and increased 60 days after HS in animals pretreated with pecan shell AE (Figure 3C and D). However, in the groups of rats pre and post-treated there was no significant change in carbonyl proteins.

The effects of pecan shell and HS on reduced glutathione (GSH) levels in testes are shown in figures 3E and 3F. The GSH levels in the testis did not change 15 and 30 days after HS. However, both groups pretreated and pre and post-treated showed a significant increase in GSH levels 60 days after HS. Furthermore, vitamin C (VIT C) levels also increased significantly in all groups treated with pecan shell AE after 15, 30 and 60 days compared to the control group (Figures 3G and 3H).

Catalase (CAT) levels showed a tendency to increase in animals treated with pecan shell AE, but it showed statistical difference only 60 days after HS (Figures 3I and 3J). On the other hand, protein levels did not change within all experimental groups (Figure 3K and 3L).

Figure 3. Effects of pecan shell aqueous extract (AE) on lipid peroxidation (A and B), carbonyl protein (C and D), reduced glutathione (GSH) (E and F), vitamin C (G and H), catalase (I and J) and protein levels (K and L) in the testes of rats pretreated or pre and post-treated with pecan and evaluated 15, 30 and 60 days after testicular heat shock (HS).



+ Indicates statistically significant difference from HS (control) group at P<0.05.

DISCUSSION

Heat can reversibly interfere in male fertility and trigger testicular degeneration.[4-5] The exposure of the testis to high temperatures triggers the production of free radicals and this can cause cellular damage, leading to cell death by apoptosis.[4] High temperatures induce ROS production and reduced fertility, triggering adverse effects such as spermatogenesis damage and decreased sperm motility.[4-5;36]

Studies indicate the presence of natural bioactive molecules with antioxidant function, such as sterols, tocopherol and phenolic compounds in several nut types.[15] The pecan has molecules with ROS scavenging property and cytoprotective effects against cell damage.[37]

The results in the present study showed an increase in the weight of epididymis and testes after treatment with pecan shell AE. This increase in the weight of the organs may indicate a protective effect of pecan after exposure to high temperature. Testicular weight has a positive relationship with testicular function and spermatogenesis[38] therefore, its reduction suggests a loss of germ cells.[39]

Testes are susceptible to heat; however, the degree of damage depends on the temperature and length of heat damage. Sertoli cells, spermatogonia and Leydig cells (including testosterone production) seem to be more resistant to heat than germ cells and their survival allows for the proliferation and maturation of germ cells and consequently spermatogenesis recovery.[4;40] The heat shock (43şC for 15 min) used in this study was able to preserve spermatogonia, Sertoli and Leydig cells; as the seminiferous epithelium cycle in rats is 58 days,[2] spermatogenesis should be reestablished 60 days after heat exposure. The proper spermatogenesis recovery occurred in all animals; however, quantitative and qualitative analyses of the testes showed a better recovery of spermatogenesis in animals treated with pecan shell AE.

The better recovery of spermatogenesis observed in treated animals can be associated with the high content of antioxidant substances in *Carya illinoensis*. These antioxidant molecules, mainly polyphenols rich in hidroxyl radicals present in the pecan shell AE, have antioxidant activity and can act directly on damaged tissues as scavengers of ROS.[19;41] The AE of pecan shells produces protective effects against toxicity induced by cyclophosphamide in the heart, kidneys, liver and bladder.[25]

Several factors may induce reversible testicular damage and result

in the reduction of fertile potential.[42] Heat can induce testicular degeneration;[5] this process is characterized initially by the sloughing of germ cells in the tubular lumen and reduced height of the seminiferous epithelium, necrosis and apoptosis of germ cells and hyalinization of seminiferous tubules.[43-44]

In the present study, several seminiferous tubules exposed to heat showed a large reduction in the number of germ cells, sometimes showing only Sertoli cells and spermatogonia, which are characteristic of severe and widespread testicular damage.[39] However, the testes of animals treated with pecan shell AE showed a better recovery than those of control animals. It is noteworthy that pecan shell AE also showed a protective effect on the testis against the toxicity of cyclosphosphamide.[45]

Seminiferous tubules constitute 89% of the testicular parenchyma in rats and it has direct correlation with sperm production; furthermore, seminiferous epithelium is composed mostly by germ cells in different stages of maturation.[2;46] Our results showed an increase in the volume of seminiferous tubules and seminiferous epithelium after the treatment with pecan shell AE, which also indicated a better recovery of spermatogenesis in animals treated with pecan shell AE. Furthermore, treatment with pecan also reduced the volume of tunica propria in the seminiferous tubules, which is also an indication of better recovery of testes since the thickening of tunica propria is characteristic in testicular degeneration.[39]

The amount of seminiferous tubules may reduce in response to thermal stress, inducing the degeneration of the germ cells via apoptosis characterized by DNA fragmentation.[47-49] Daily oral supplementation with antioxidants may decrease the DNA fragmentation induced by oxidative stress.[36]

TBARS assay shows lipid peroxidation in the tissue; thus, high levels of TBARS may represent damage to cell lipids.[21;50]

Membranes in testicular cells contain a high amount of polyunsaturated fatty acids, which are more vulnerable to damage by ROS; furthermore, testicular cell membranes and sperm are more susceptible to lipid peroxidation.[51] Oral supplementation of antioxidants decreased the DNA fragmentation induced by oxidative stress in reproductive studies.[52]

Results in the present study showed a decrease in lipid peroxidation in animals exposed to heat shock and treated with pecan shell AE, which corroborates with other studies addressing the beneficial action of pecan shell AE.[25; 45; 53] This beneficial action may be due to the large amount of antioxidants such as γ-tocopherol in the pecan shell.[21; 54-55] Pecan nut also has high concentration of phenolic compounds, tannins, pro-derived flavonoids and anthocyanins with inhibitory action on substances responsible for oxidative damage.[18-19;55]

The major and first line of defense against ROS-induced organ injury is GSH and their reduction may induce tissue damage. GHS protects the cell against injury from exposure to iron ions, hyperbaric oxygen, ozone, radiation, and ultraviolet light.[57] According to our results, the levels of GSH increased in the testes of animals treated with pecan shell AE. VIT C levels also increased in the testes after ingestion of pecan shell AE. In the male reproductive system, VIT C can protect spermatogenesis and plays a great role in the integrity of semen and fertility.[11; 58]. Both GSH and VIT C results also indicate a better recovery of spermatogenesis after pecan shell AE treatment in animals exposed to heat.

Catalase works in cellular detoxification by preventing the generation of free radicals and consequently protecting cells against oxidative damage; this enzyme converts hydrogen peroxide into water and reduces the formation of hydroxyl radicals.[59-60] Catalase is found mainly in the basal membrane of testes and confers great protection against hydrogen peroxide.[61] Therefore, the increase in catalase levels due to the treatment with pecan shell AE may help in the recovery of spermatogenesis after the reversible testicular damage induced by heat.

Several studies investigated the antioxidant potential of pecan nut. Hudthagosol et al.[11] showed that the bioactive constituents of pecans are absorbable and contribute to postprandial antioxidant defense. However, further studies are needed to better verify the in vivo activity of pecans and possible toxicological effects aiming at the safe use of pecan shell infusion.[19] In the present study no signs of toxicity were observed in any animal after the treatment with pecan shell AE.

CONCLUSION

Both pretreatment or pre and post-treatment with pecan shell AE can aid a better recovery of spermatogenesis after the reversible testicular damage induced by heat. The components of by-product in pecan nut industry could reduce pathological lesions in the testicular parenchyma after tissue damage increasing antioxidant defenses and preventing oxidative injury.

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