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PROTECTIVE ROLE OF POMEGRANATE PEEL AND *PIPER LONGUM* FRUIT ON THE TESTICULAR FUNCTION OF THIOACETAMIDE-INDUCED REPRODUCTIVE TOXICITY OF MALE ALBINO RATS

Sarwar Nawzad Jafar* and Kalthum Asaf Mawlood

*Department of Biology, College of Education, Salahaddin University-Erbil, Erbil, Iraq.

Abstract

The present study was aimed to investigate protective effects of the extract of *piper longum* fruit, pomegranate peel, silymarin and vitamins C and E on reproductive toxicity induced by thioacetamide in male rat by evaluating the oxidative biomarkers including, malondialdehyde (MDA), catalase (CAT), superoxide dismutase (SOD) and reduced glutathione (GSH) in testes tissue and plasma level of testosterone and sperm parameters in adult male rats. Reproductive toxicity was induced by thioacetamide (200 mg/kg BW) and treatment with herbal and vitamin supplements were orally administered by gavage for 8 weeks. At the end of the study, oxidative biomarkers, testosterone and sperm parameters including concentration, motility, viability, and abnormality in adult male rats were measured. The results of this study indicated that thioacetamide significantly increased MDA, and decreased testosterone and antioxidant enzyme activity and impaired sperm parameters ($p<0.05$). While, treatment with PPE200, PPE400, PL200, PL400, S, E, and C had protective effects on toxicity induced by thioacetamide and could significantly improve the above parameters ($p<0.05$). The results also indicated that PPE and PL acted in a dose-dependent manner. Given the antioxidant and anti-inflammatory role of their compounds, it seems reasonable that higher doses have a greater potential for treatment. It can be concluded that thioacetamide causes oxidative damage and impaired reproductive function and pomegranate peel and PL fruit extract, and vitamins E and C can improve reproductive toxicity by thioacetamide in male rats. Therefore, using herbal supplements and vitamins can be helpful in improving sexual function.

Key words: Thioacetamide, *Piper Longum*, Pomegranate, Reproductive Toxicity, Oxidative Stress

Introduction

Infertility is a major clinical problem in the realms of medicine and psychiatry. According to the World Health Organization (WHO), infertility is "a disease of the reproductive system defined by the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse (and there is no other reason, such as breastfeeding or postpartum amenorrhea) (Vander Borght and Wyns, 2018). It is estimated that 5 to 15 percent of couples are infertile and male sexual dysfunction plays an important role in its occurrence (Akbari and Jelodar, 2013). Male sexual dysfunction is caused by a disorder in the production of sperm or male sex hormones. It is also caused by some diseases and factors such as infections, cryptorchidism, varicocele, obstructive lesions, trauma and oxidative stress as a new important cause that has received a lot of attention in recent years (Agarwal *et al.*, 2005). Oxidative stress is defined as an imbalance between the productions of reactive oxygen species (ROS) and antioxidant defense mechanisms. Other factors such as alcohol, drugs, and smoking as well as exposure to certain toxic chemicals can also cause infertility (Khaki *et al.*, 2011). It is now known that continuous exposure to thioacetamide can cause acute and irreversible liver damage, and oxidative stress is known to be the leading cause of this phenomenon. In addition to the damage to the liver tissue, water also damages the tissues of the kidneys, testicles, and spleen (Al Bader *et al.*, 1999; Abul *et al.*, 2002; al-Bader *et al.*, 2000). Thioacetamide is a thiono-sulfurcompound inducing chronic liver injury through thioacetamide-S-oxide, a free radical, which covalently binds to hepatic macromolecules, leading to necrosis of hepatocytes (Muriel *et al.*, 2017). Thioacetamide-S-oxide resulted from the metabolization of thioacetamide by

***Author for correspondence :** E-mail : sarwar.jaaffar@su.edu.krd

cytochrome P450 enzymes of liver microsomal and is converted to a toxic intermediate due to the oxidation process (Hajovsky *et al.*, 2012). The liver plays an essential role in the production of plasma transporter proteins and polyunsaturated fatty acids (PUFA). Moreover, it was reported that liver damage caused by risk factors such as alcohol, thioacetamide, and other toxic chemicals can affect plasma levels of elements and hormones (Abul *et al.*, 2002; Akbari *et al.*, 2017; Akbari *et al.*, 2019). It has been reported that thioacetamide-induced liver cirrhosis can change the serum content of essential amino acids and PUFA (Fontana *et al.*, 1996). Although high levels of PUFA make spermatozoa prone to damage by ROS, it is essential for its membrane to form. In addition, liver damage by thioacetamide results in decreased levels of antioxidant enzymes and an increase in the amount of ROS that is itself one of the main factors in infertility.

The use of medicinal plant and herbal compounds containing phenols is increasing nowadays due to their potential role in the treatment and prevention of some diseases and can play an important role in enhancing the sexual ability and improving reproductive performance (Nikseresht *et al.*, 2015; Akbari *et al.*, 2017).

The pomegranate (*Punica granatum*) is a fruit-bearing deciduous shrub in the family Lythraceae, subfamily *Punicoideae*. The pomegranate originated in the region extending from modern-day Iran to northern India and has been cultivated since ancient times throughout the Mediterranean region (Morton, 1987). Pomegranate has been acclaimed for its medical advantages; a long time ago pomegranate was grown and used up as fresh fruit or in beverage form particularly in the Mediterranean locale. Pomegranate fruit includes seed and peel that have strong antioxidant potential (Kaur *et al.*, 2006). Comparing to the seeds the peel contains almost 75% of overall phenolic content which includes tannins, gallocatechins, catechins and prodelphinidins (Singh *et al.*, 2002b). These antioxidant molecules have been demonstrated that the anticarcinogenic, antimicrobial and anti-inflammatory and antioxidant properties (Derakhshan *et al.*, 2018). *Piper longum* (PL) is another important herbal medicine that acts as a nutrient and as a good remedy due to its phytochemical compounds (Sharma and Sahu, 2016; Kumar *et al.*, 2011); phenols, flavonoids, alkaloids, glycosides, sterols, and tannins (Trivedi *et al.*, 2011) which represent anti-inflammation, hepatoprotective and antioxidant activities are most of its phytochemicals (Cheong *et al.*, 2018; Salehi *et al.*, 2019; Kumar *et al.*, 2009). Silymarin contains phytochemical compounds such as phenols and flavonoids and has hepatoprotective, anti-inflammatory and antioxidant effects(Sajedianfard *et al.*, 2013). Therefore, the aim of present study was to investigate protective effects of the extract of PL fruit, pomegranate peel, silymarin and vitamins C and E on reproductive toxicity induced by thioacetamide in male rat by evaluating the oxidative biomarkers including, catalase (CAT), superoxide dismutase (SOD) and reduced glutathione (GSH), plasma level of testosterone and sperm parameters in adult male rats.

Plant Materials and Chemicals

The full ripe pomegranate fruits (*Punica granatum* L.) were washed two times once with tap water, the second time with distilled water. Then, peel and the seeds were separated, and the peels were cut into smaller parts before being air-dried. mechanical blender was used to ground the peel into a fine powder and sieves used to separate the finest powder 40-mesh size and kept in the refrigerator prior to extraction. Methanol was used for the extraction instead of ethanol because the previous finding showed that using methanol gives a better yield in pomegranate extraction (Singh *et al.*, 2002a). And based on Singh *et al*., (2002) sample was extracted. 25 g peel powder was extracted by 100 ml of methanol using a magnetic stirrer at 30°C for 2 hours. For the removal of peel particles, the extract was filtered using Whatman No. 41 filter paper. The remaining residue was extracted again with methanol for 2 hours. The methanolic extracts were collected and distilled using the rotary evaporator under reduced pressure and stored at 4°C until assay.

Ethanol alcohol was used for the extraction of plant *Piper longum* (long pepper) fruit and the method was based on the protocol used by Christina *et al*., (Christina *et al.*, 2006). Ethanolic extract was prepared using 100g/ L of ethanol ratio, 100g of *Piper longum* dried fruit powder extracted with 1 L of absolute ethanol at 55–65°C for 8 hours using Soxhlet extractor. Then the extract was distilled & dried using a rotary evaporator at 45°C for 30 min. The dried extract stored at 4°C until the start of the experiment.

Thioacetamide and all other chemicals purchased from Sigma-Aldrich (Sigma-Aldrich, Switzerland), vitamin E and C were purchased from Solar (Solar bio co., China). The dose concentration of thioacetamide and silymarin was fixed based on the previous study carried out by (Aydýn *et al.*, 2010) and (Ahmad *et al.*, 2002), respectively.

Ferric Reducing Antioxidant Power (FRAP) assay

The FRAP assay measured the antioxidant capacity,

and it is based on the change in absorbance at 593 nm, because of the formation of blue-tinted Fe^{++} tripyridyltriazine solution from the colorless oxidized Fe^{+++} form, by the effect of electron-donating antioxidants. In order to determine the antioxidant capacity of the vitamins and plant extracts ferric reduction activity based on the protocol implemented by (Benzie and Strain, 1996) was used. The reagent for the FRAP assay is constituted with mixing of 2, 4, 6-tripyridyltriazine (TPTZ) 10 mM/L, FeCl3(20 mM/L) and HCl (40 mM/L). 100 µl of the plant extracts and vitamins (Conc. 1 mg/ml) and 2ml of the FRAP solution add in a test tube, then mixtures were shaken continuously and left in dark for half an hour. After 30 mins had passed absorbance at 593 nm was recorded. The standard curve for ascorbic acid was prepared to utilize ascending concentrations for the purpose of comparison, as shown in Fig. 1. The FRAP value calculated by the following equation for each compound.

Animals

Healthy adult male Wistar rats, *Rattus norvegicus*, were procured from the Laboratory Animal Center (College of Science, University of Zakho, Duhok Province, Iraq). The rats were acclimated to the animal housing for two weeks before the start of the study. Rats were fed on a balanced rat chow, and water *ad libitum*.

Ethical guidelines were followed for handling and performing experiments on animals. The rats were housed in plastic cages (50cm × 30cm × 10cm). The room temperature was maintained at 23± 2°C, as well as light/dark cycle of 12:12 h/day.

Experimental Design

The experiment was conducted on forty-five rats of 8 weeks old (weighing 200 ± 25g). and the rats were acclimatized to the lab condition one week prior to the experiment, rats were divided into 9 groups each consists of 6 rats. Rats of Group I was treated with physiological saline and considered as a negative control group. Rats of Group II were treated with thioacetamide (TAA) (200 mg/kg BW) intraperitoneal twice a week for 8 weeks (Guerra[1] *et al*., 2010). Rats of Group III were treated with pomegranate peel extract (PPE) (200 mg/Kg BW) orally along with TAA (200 mg/kg BW) (therapeutic group low dose). Rats of Group IV were treated with PPE (400 mg/Kg BW) orally along with TAA (200 mg/kg) (therapeutic group high dose). Rats of Group V were treated with *Piper longum* extract (PL) (200 mg/Kg BW) orally along with TAA (200 mg/kg BW) (therapeutic group low dose). Rats of Group VI were treated with PL (400 mg/Kg BW) orally along with TAA (200 mg/kg) (therapeutic group high dose). Rats of Group VII were treated with Silymarin (50 mg/Kg BW) orally along with TAA (200 mg/kg). Rats of groups VIII and IX were given vitamin E and C each 50 mg/kg of BW orally with TAA (200 mg/kg). Animals were weighed weekly. All rats were sacrificed 24 hours after overnight fasting. Blood samples were collected; serum was separated for assay of the testis biomarker. The testis was harvested, washed in normal saline, blotted with filter paper, and weighed. The left testis tissue was kept in -20°C, which homogenized later for determination of oxidative stress parameters.

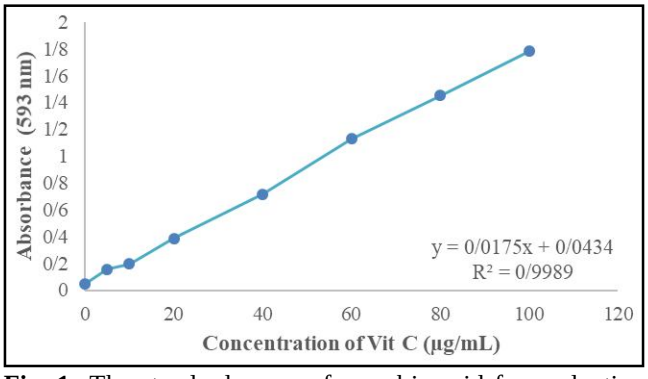

Fig. 1: The standard curve of ascorbic acid for evaluating FRAP test.

Biochemical tests

The collected blood samples were left to clot then they were separated at 3000 rpm for 10 minutes. Serum was used for the assay of C-reactive protein (CRP), albumin, total serum protein (TSP) and total testosterone were spectrophotometrically measured by Autoanalyzer (Autoanalyzer 3 HR., Seal Analytical, UK).

Sperm Quality assessment

After the rats were sacrificed, epididymis was removed instantly. Pairs of fine forceps were used to squeeze out the contents of cauda epididymis then it was subjected to sperm quality analysis. Sperm quality was determined by these parameters: sperm concentration,

Table 1: Anti-oxidant activity of the *Punica granatum*, *piper longum*, silymarin, vitamin E and ascorbic acid measured by FRAP assay*.

Comp-ounds	Absor-bance	FRAP value (IC50)		
		µM	µM/mg	µM/g
PPE	2.803	157	1570	15700
Vitamin C	2.194	122.891	1228.91	12289.1
Vitamin E	1.86	104.182	1041.82	10418.2
S	2.207	123.619	1236.19	12361.9
PL	2.314	129.612	1296.12	12961.2

* PPE: Pomegranate Peel Extract, PL: *Piper longum*. Extract, S: Silymarin

motility, viability, and abnormality. Sperm concentration was analyzed using the hemocytometer (WHO, 2010). Sperm suspensions from the caudal epididymis were diluted 1:20 with phosphate-buffered saline (PBS, pH 7.4) solution by using the pipette with a white bead. The diluted samples were put into the counting chamber and the number of sperms was counted using a hemocytometer with improved Neubauer chamber under a light microscope. The hemocytometer is allowed to stand for 5 minutes for sedimentation, then sperms were counted in large eight squares of 1mm2 each area except the central erythrocyte counting area of Neubauer 's chamber was performed and multiplied by 5×10^4 factor (Narayana *et al.*, 2005) to calculate the total count of spermatozoa/ epididymis (million/epididymis). Sperm motility was analyzed and averaged by counting the motile and non-motile spermatozoa and expressed as the percent motility. After incubation and diffusion time, 10 µL of the suspension was placed on a warmed microscope slide and covered by a warmed cover slip, then the percentage of motile sperm was counted under Nikon binocular microscope with a warmed stage (observed at 400-fold magnification), being 200 sperms per rat counted throughout at least 10 fields. The assessment of sperm motility was done consonant to the WHO protocol (WHO, 2010). Sperm viability was analyzed by the eosin-nigrosin staining method. The nonviable spermatozoa, which were stained red, and the viable ones, which were unstained, were counted under the light microscope. The viability of sperm was expressed as the percent of viable spermatozoa. Morphology of spermatozoa was assessed following staining of the smear with eosin-nigrosine stain. Then slides were viewed under a light microscope with oil immersion objective at 100x magnification. A total of 200 spermatozoa were examined on each slide for each animal, and total sperm abnormality (tail and head) rates were expressed as a percentage (Türk *et al.*, 2007).

Data Presentation and Statistical Analysis

Data of the experiment were presented as Mean ± Standard Error of Mean (SEM). Analysis of variance (one-way ANOVA) applied to compare between groups accompanied by "Dunnett's Multiple comparison test" to find statistical significance. P-value of less and equal to 0.05 was regarded to be significant. For calculation of ANOVA and Dunnett's multiple comparison test, GraphPad Prism 7.0 software was used.

Results

FRAP Reducing Power

The antioxidant activity of the P*unica granatum, Piper longum*, silymarin, vitamins E and C was measured by FRAP method and has been presented in Table 1. It was shown that electron-donating compounds have a higher reducing power. PPE indicates that they are most effective electron donor and can reduce the oxidized intermediates highly reactive molecules like free radicals and reactive oxygen species of peroxidation processes and the FRAP value 15700 µM/g while the *Piper longum* extract has lower antioxidant activity (FRAP value = 8441 µM/g) when compared with both PPE and ascorbic acid. Vitamins E and C usually used as a standard for preparing the standard curve and has high antioxidant activity (FRAP value = 12289.1 µM/g and 10418.2 µM/g, respectively) which almost identical to the activity of silymarin.

Thioacetamide decreased the weight of the body, GIS and RCE

Our results showed that the thioacetamide group (TAA) gained the least weight during the experiment period (31.6±12.33) compared to the other groups, while treatment with different doses of PPE, s, and vitamins E and C significantly gained more weight compared to TAA group($p<0.05$). Although treatment with PL200 and PL400

Table 2: weight gain ratio and gonadosomatic index (GSI) changes in experimental groups*

	Initial W.(g)	Final W. (g)	Weight gain (g)	GSI (%)	RCE (%)
Control	210.6 ± 4.611	307.8 ± 9.754 a	97.2± 9.77 a	0.403± 0.022 a	0.279 ± 0.015 a
TAA	221 ± 8.159	252.8 ± 14.58 b	31.6±12.33 b	0.526 ±0.03 b	0.19 ± 0.016 b
PP200	208 ± 3.886	266 ± 5.736 c	58± 3.391 c	0.493 ± 0.02 c	0.262 ± 0.016 a
PP400	211.2 ± 4.341	267.8 ± 8.787 c	56.55± 12.14 c	0.460± 0.02d	0.247 ± 0.011c
PL200	223 ± 6.458	272 ± 9.022 c	39± 7.855 d	0.473±0.029d	0.216 ± 0.14 d
PL400	218 ± 4.29	260 ± 8.276 c	43 ± 6.1 d	0.469 ± 0.023d	0.225 ± 0.022d
S	204.8 ± 3.292	277.8 ± 10.66 d	81.1 ± 3.723 e	0.471 ± 0.021d	0.26 ± 0.009 a
E	232.4 ± 5.026	301 ± 12.43 a	68.6 ± 7.67 f	0.418 ± 0.07 a	0.214 ± 0.023d
C	221 ± 7.362	277.4 ± 15.31d	56.4 ± 10.98 c	0.445 ± 0.01e	0.226 ± 0.014d

*Results indicate as mean ±standard error of mean. Different lowercase letters show significant differences between groups ($p<0.05$, n=5) RCE: right cauda epididymis, GSI: gonadosomatic index PPE: pomegranate peel extract; PL: *Piper longum*. Extract. TAA: Thioacetamide, S: silymarin, E: vitamin E, C: Vitamin C.

as same as pomegranate significantly gained weight compared to the TAA group, they showed the least improvement effect compared to the other groups($p<0.05$). Treatment with silymarin and vitamin E showed the highest improvement effect compared to the other groups. Gonadosomatic index (GSI) in the TAA group was significantly lower than that in the control group, and treatment with PL200, PL400, PPE200, PPE400, S, E and C significantly improved it compared to TAA group. The weight of right cauda epididymis (RCE) in the TAA group significantly was lower than the other groups, and treatment with PL200, PL400, PPE200, PPE400, S, E, and C significantly improved it compared to TAA group($p<0.05$).

Table 3: The mean value (±SEM) of albumin, total serum protein (TSP) and C-reactive protein (CRP) in different groups*.

Parameters groups	Albumin(g/dL)	TSP(g/dL)	CRP(ìg/mL)	Testosterone (mg/dL)
control	4.162 ± 0.11**	7.38 ± 0.156*	0.168 ± 0.014a	3.35 ± 0.38 a
TAA	3.664 ± 0.083##	6.24 ± 0.067#	0.3458 ± 0.01b	0.91 ± 0.66 b
PPE200	4.22 ± 0.041***	7.246 ± 0.182*	0.208 ± 0.02c	2.89 ± 0.43 a
PPE400	4.146 ± 0.084**	7.125 ± 0.132*	0.2 ± 0.013c	2.39 ± 0.18 c
PL200	3.94 ± 0.055	6.74 ± 0.12	0.214 ± 0.033c	1.71 ± 0.31 d
PL400	4.058 ± 0.14*	6.985 ± 0.105*	0.204 ± 0.015c	2.63 ±0.21e
S	4.1 ± 0.11**	6.754 ± 0.075*	0.17 ± 0.014a	2.75 ± 0.21 e
E	4.016 ± 0.042*	6.798 ± 0.058*	0.102 ± 0.011d	2.03 ± 0.22f
C	4.014 ± 0.035*	6.804 ± 0.088**	0.266 ± 0.023e	2.52 ± 0.18 e

*Results indicate as mean ±standard error of mean. Different lowercase letters show significant differences between groups ($p<0.05$, n=5) RCE: right cauda epididymis, GSI: gonadosomatic index PPE: pomegranate peel extract; PL: *Piper longum*. Extract. TAA: Thioacetamide, S: silymarin, E: vitamin E, C: Vitamin C.

Table 4: Mean + SEM values of MDA, GSH levels, CAT, and SOD activities in different treatment groups*.

Parameters groups	MDA(nmol/ mg protein)	GSH(nmol/ mg protein)	CAT(nmol/ mg protein)	SOD(nmol/ mg protein)
control	31.71 ±8.1 [a]	186.8 ±10.66 [a]	52.33 ± 1.91[a]	50.4 ± 1.51 [a]
TAA	85.66 ± 8.8[b]	47.2 ±7.18 [b]	14.69 ± 2.18 [b]	30.45 ± 1.33 [b]
PPE200	37.22 ± 5.41 [c]	102.8 ± 4.87 [c]	47.2 ±3.40 [a]	41.35 ± 0.9 [c]
PPE400	36.31 ± 3.641 [c]	126 ± 5.1 [d]	54.53 ± 2.14 [a]	47.53 ± 2.23 [a]
PL200	33.31 ± 4.15 [a]	183.6 ± 20.35 [a]	146.9 ±23.85[c]	36.97 ± 1.61 [d]
PL400	32.92 ± 7.813 [a]	164.4 ± 7.53 [a]	40.92 ±2.59 [d]	37.07 ± 1.321 [d]
S	53.03 ± 1.1 [d]	163.6 ± 8 [a]	73.97 ± 6.91 [e]	40.85 ± 1.1 [c]
E	36.66 ± 5.1 [c]	145.2 ± 13.42 [d]	46.59 ± 4. [a]	40.82 ± 1.21 [c]
C	26.01 ± 8.99 [e]	164.7 ± 3.77 [a]	66.08 ± 9.53 [f]	39.15 ± 0.62 [c]

*Results indicate as mean ±standard error of mean. Different lowercase letters show significant differences between groups ($p<0.05$, n=5). MDA: malondialdehyde; GSH: reduced glutathione; CAT: catalase; PPE: pomegranate peel extract; PL: *Piper longum* Extract. TAA: Thioacetamide, S: silymarin, E vitamin E, C: Vitamin C

Serum proteins

The mean value (±SEM) of albumin, total serum protein (TSP) and C-reactive protein (CRP) in different groups were showed in Table 3. The results showed that the level of TSP, CRP, and albumin significantly increased in the TAA group compared to other groups ($p<0.05$). Treatment with PP200, PP400, PL200, PL 400, S, E and C in TAA rats significantly decreased the levels of these proteins compared to the TAA group. However, they had no significant difference in PP200, PP400, PL200, PL 400, S, E and C groups compared to the control group ($p>0.05$).

The levels testosterone (0.91 ± 0.66) in TAA groups was the lowest of concentration in studied groups, and the highest level of it was observed in the control group (3.35 ± 0.38). Treatment with PPE200, PPE 400. PP400, PL400, S, E, and C significantly increased the level of it ($p<0.05$).

Thioacetamide induced oxidative stress in testes

Table 3 shows that the activities of antioxidant enzymes and GSH decreased, and the level of MDA increased in thioacetamide group compared to control group, while treatment with different doses of PPE and PL, silymarin and vitamins E and C significantly improve these parameters compared to thioacetamide group ($p<0.05$). The thioacetamide group had the highest level of MDA when compared to other groups. Comparing to the control with treatment groups showed a non-significant difference ($p>0.05$). The thioacetamide group showed significantly lower GSH levels compared to control and the rest groups. The activities of catalase and SOD enzyme in the thioacetamide group significantly decreased compared to the others, and treatment with different doses of PPE and PL, silymarin and vitamins E and C significantly improve these parameters compared to thioacetamide group($p<0.05$).

Thioacetamide decreased the level of testosterone and sperm quality

The total abnormality (tail and head) and motility of sperm significantly increased and decreased in the TAA group, respectively, compared to other groups, while treatment with PPE200, PPE 400. PP400, PL400, S, E, and C significantly

improved it ($p<0.05$). The highest sperm motility was observed in the control group and the lowest was in the TAA group ($p<0.05$). The results showed that the highest sperm count was observed in the control group and the lowest was in the TAA group. The level of sperm counts significantly decreased in the TAA group compared to other groups, while treatment with PPE200, PPE 400. PP400, PL400, S, E and C significantly improved it ($p<0.05$).

Discussion

The results of this study showed that thioacetamide could decrease GSI and testosterone level, increase the percentage of abnormal sperm and decrease motility and sperm count, and while treatment with PPE200. PP400, PL200 PL400, S and vitamins C and E could improve all of them. In agreement with our findings, Abul *et al.*, (2002) showed that thioacetamide can cause reproductive toxicity and disruption of sperm parameters(Abul *et al.*, 2002). They showed that thioacetamide can decrease testicular antioxidant enzyme levels and impair testicular function. They also measured the levels of some of the trace elements such as copper, manganese, zinc and selenium, and showed that the testicular levels of these elements which are associated with antioxidant enzymes activity significantly decreased by treatment with thioacetamide(Abul *et al.*, 2002). It is clear that changes in the levels of these elements can cause oxidative damage because they have antioxidant activity and act as a cofactor for antioxidant enzymes. In a study Akbari et al., showed that alcohol-induced liver injury can disrupt the homeostasis of these elements and impair the activity of antioxidant enzymes (Akbari *et al.*, 2019). In addition, they showed that impaired homeostasis of these elements can lead to oxidative damage to testicular tissue and lower testosterone levels (Akbari *et al.*, 2017). Although we did not measure the levels of these elements in this study, these studies agree that liver damage caused by chemical compounds such as ethanol or thioacetamide can impair the homeostasis of these elements (Abul *et al.*, 2002; Akbari *et al.*, 2017; Akbari *et al.*, 2019). The liver makes albumin and SHBG and they are responsible for the transfer of testosterone into the bloodstream. In addition, the researchers showed that hepatic injury in addition to impaired homeostasis of essential elements can alter plasma levels of testosterone transporter plasma proteins such as sex hormone-binding globulin (SHBG) (Akbari *et al.*, 2017; Akbari *et al.*, 2019). Moreover, the results also showed that plasma protein levels such as albumin decreased significantly as a result of thioacetamide. By lowering the amount of plasma level of SHBG and albumin, the free levels of this hormone increase and, as a result, its clearance from the bloodstream increases, which could be a good reason for lowering testosterone levels in our study (Laurent *et al.*, 2016). Based on the results of these studies, it seems reasonable to conclude that liver damage can cause reproductive disorders.

Our results also showed that thioacetamide can damage the reproductive system so that testosterone levels decreased and sperm parameters changed in the thioacetamide group, while treatment with PPE200. PP400, PL200 PL400, S and vitamins C and E could improve all of them. Abul *et al.*, (2002) showed that damage to the reproductive system by thioacetamide could be due to a decrease in antioxidant enzymes and an increase in the amount of free radicals (Abul *et al.*, 2002). The results were in line with our findings. In our study decrease in the levels of antioxidant enzymes can be due to the elimination of free radicals or disruption of their activity due to changes in the levels of the trace elements (Abul *et al.*, 2002; Akbari *et al.*, 2017; Walczak–Jedrzejowska *et al.*, 2013). It is believed that oxidative stress is the main cause of impaired reproductive function in many pathophysiological conditions (Agarwal *et al.*, 2005; Walczak–Jedrzejowska *et al.*, 2013). Increasing the levels of free radicals or decreasing the activity of the oxidative system in the testes and other sex glands can cause male infertility. It has been shown that increased levels of free radicals in semen can cause male infertility(Sharma and Agarwal, 1996; John Aitken *et al.*, 1989). Increased levels of free radicals in the testis can cause structural damage to the testicular tissue by activating cellular apoptosis pathways (Rahimi Anbarkeh *et al.*, 2019). These changes can lead to reducing seminiferous tubules diameter (Suleiman *et al.*, 1996). It should be noted that the antioxidant defense in spermatozoa is not strong, because sperm require a minimum level of ROS to maturate, specifically, this

Table 5: Mean + SEM values of sperm parameters (%) and serum testosterone (mg/dL) in the different treatment group in testis homogenate.

Parameters groups	Motility (%)	Sperm Count (%)	Abnormality (%)
Control	78.2 ±7.18 [a]	70± 2.53 [a]	6.2 ± 0.97 [a]
TAA	29.6 ± 5.97 [b]	32.2 ± 2.08 [b]	18.6 ± 1.75 [b]
PPE200	61.6 ± 3.14 [c]	57.4 ± 3.08 [c]	8.6 ± 1.47 [a]
PPE400	64.6 ± 3.8 [c]	63.3 ± 2.64 [c]	9 ± 1.7 [a]
PL200	52.6 ± 3.1 [d]	53.2± 3.44 [c]	7.8 ± 1.02 [a]
PL400	68.1 ± 8.0 [c]	66.25 ± 5.6 [c]	9.5 ± 1.11 [c]
S	59± 1.81 [d]	67 ± 8.02 [d]	9.5 ±2.37 [c]
E	60±1 [c]	54 ± 2.92 [c]	6.5 ± 0.35 [a]
C	52.3±3.95 [d]	53.2 ± 3.33 [c]	10 ± 1.92 [c]

*Results indicate as mean ±standard error of mean. Different lowercase letters show significant differences between groups ($p<0.05$, n=5). PPE: pomegranate peel extract; PL, *Piper longum* extract. TAA: Thioacetamide, S: silymarin, E vitamin E, C vitamin C.

process involves acrosome reaction and capacitation (Adewoyin *et al.*, 2017; de Lamirande and Gagnon, 1993). So, spermatozoa are always susceptible to oxidative damage. Therefore, the use of antioxidant-containing compounds is suggested to support spermatozoa function and viability. Spermatozoa and seminal plasma have active antioxidant systems that prevent cellular damage induced by free radicals. The endogenous antioxidant defense systems include enzymatic such as SOD, GPx and CAT, and non-enzymatic such as vitamins C and E and glutathione. These systems can be supported by pharmacological and herbal supplements that have (exogenous) antioxidant roles. Accordingly, the use of herbal supplements to enhance sexual arousal has been of interest to date. Our results showed that treatment with PPE200. PP400, PL200 PL400, S, and vitamins C and E could improve the level of MDA and the activities of the antioxidant enzymes. Many studies have shown that pomegranates and *Piper longum* contain phenols, flavonoids, alkaloids, glycosides, sterols, catechins, gallocatechins and prodelphinidins that represent the high anti-inflammation and antioxidative activity (Cheong *et al.*, 2018; Salehi *et al.*, 2019; Kumar *et al.*, 2009). Our results also showed that high levels of PPE and PL showed better performance. This result indicates that these two herbal compounds acted in a dose-dependent manner. Given the antioxidant and anti-inflammatory role of their compounds, it seems reasonable that higher doses have a greater potential for treatment. The main mechanism for neutralizing free radicals is the exchange of electrons from free radicals to antioxidant molecules or vice versa. Compounds such as phenols, flavonoids, and vitamin C and E have always been known to work well in neutralizing free radicals. These compounds act as electron donors to free radicals and neutralize them. Given the presence of these compounds and their potential different activities, it seems reasonable that increasing their levels would improve antioxidant defense and enhance the reproductive system. However, it should be noted that thioacetamide alters the homeostasis of essential elements, which in turn can be a risk factor in impaired reproductive function(Abul *et al.*, 2002), and herbal compounds may play a role in ameliorating this disorder, need to be investigated.

It can be concluded that thioacetamide causes oxidative damage and impaired reproductive function and pomegranate peel and PL fruit extract, and vitamins E and C can improve reproductive toxicity by thioacetamide in male rats. Therefore, using herbal supplements and vitamins can be helpful to enhance reproductive function.

Conflict of Interest

Authors have no conflict of interest to declare.

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