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Antioxidant activity of *punica granatum* L against the reproductive toxicity of an organic solvent toluene in male wistar rat

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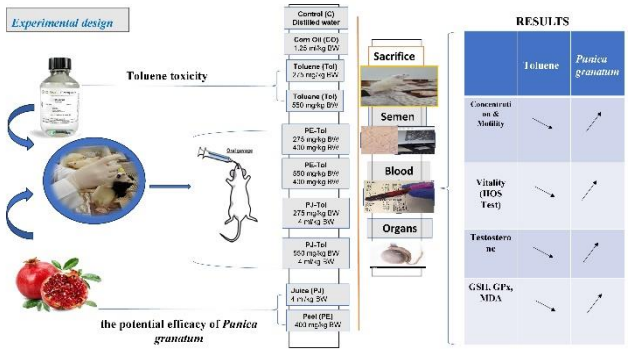
Graphical abstract



Abstract

The purpose of our study was to assess the adverse outcomes of exposure to toluene (Tol) on the male reproductive system and to investigate the potential efficacy of *Punica granatum* juice (PJ) and peel aqueous extract (PAE) against these effects. Rats were divided into the following groups: Control (C), positive controls (CO: 1.25 mL/kg BW, PJ: 4 mL/kg BW, PE: 400 mg/kg BW), Tol1: 275mg/kg BW, Tol2: 550 mg/kg BW, and the four mixtures (PJ-Tol1, PJ-Tol2, PE-Tol1, PE-Tol2). After 6 weeks of oral treatment, sperm parameters were assessed using the CASA system, along with measurements of testosterone concentration, and testicular malondialdehyde (MDA), glutathione (GSH) levels, GSH-peroxidase (GSH-Px) activity. The results indicated that toluene-induced reproductive toxicity is dose-dependent, which was evidenced by significant reductions in sperm concentration, motility, vitality, and plasma testosterone levels ($P \leq 0.05$) with a marked elevation in dead and immotile sperm. Additionally, toluene reprotoxicity resulted in increased lipid peroxidation and decreased anti-oxidants capacity. However, the status of most parameters was markedly restored in the group treated with PAE combined with Tol compared to PJ and Tol group. Based on this evidence, we conclude that subchronic exposure to toluene can induce significant testicular dysfunction and oxidative damage in male rats ($P \leq 0.05$). PAE demonstrated greater efficacy in to mitigating Tol reprotoxicity compared to PJ by enhancing antioxidant defences.

Key words: Casa system, oxidative damage, biomarkers, sperm parameters, toluene

1. Introduction

Lifestyle, along with various environmental and occupational agents, may impair male fertility, leading to a variety of adverse clinical outcomes that affect reproductive efficiency (Leisegang and Dutta, 2020; Kahalerras *et al*., 2021). According to statistics, nearly half of infertility cases in couples are due to male factors (Aryanpur *et al*., 2011). Common causes of male infertility include substances abuse and drugs, which have become a major concern for society, particularly among children and adolescents in both developed and developing countries (Taha *et al*., 2020).

Toluene is one of the most abused substances, with potentially dramatic health repercussions. Similar to many other substances of abuse, such as alcohol and other central nervous system depressants, toluene shares the same expected pharmacological effects and mechanisms of action (Bowen 2006). Being a lipophilic substance, toluene has a great affinity for lipids and can easily diffuse into tissues (Tas *et al*., 2011).

Exposure to toluene is considered a predisposing factor for the occurrence of toxic effects, mainly on the central nervous system. Beyond its impact on the CNS; an excessive toluene intake, can also cause damage to the liver, kidney, gastrointestinal tract, cardiovascular, and

reproductive systems (Grandjean and Landrigan, 2006; Tas *et al*., 2011; Violante-Soria *et al*., 2019).

various types of organic solvents have been shown to exert toxic effects on reproductive parameters, there is limited research addressing how toluene exposure contributes to damage in the reproductive male system, both in humans and animals compared to other systems.

Although the precise mechanism by which toluene induces testicular toxicity is poorly understood, some evidence indicates that oxidative stress is a key. Toluene disrupts tissue redox balance, resulting in damage to cell membranes, lipids, proteins and DNA, and ultimately activate cell death signaling pathways (Kamel and Shehata 2008; Moro *et al*. 2010; Wang *et al*. 2013). (**Figure 1**)

As a result of the oxidative stress adverse pathologies, several studies largely focused on plant-bases substances as essential elements to mitigate the side effects of modern synthetic treatments (Bone and Mills 2013).

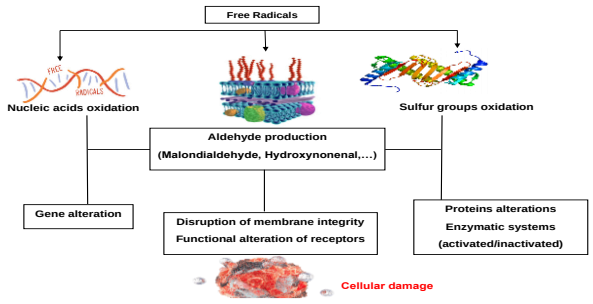


Figure 1. Oxidative Stress Consequences

Substances derived from plant extracts, fractions, essential oils, seed and peel powders, are integral to traditional medicine and are increasingly being utilized to treat many clinical disorders emerging from exposure to different chemical agents (Kiełczykowska and Musik, 2020).

Punica granatum L. commonly known as Pomegranate belongs to the Punicacea family recently described as nature's power fruit (Mphahlele *et al*. 2014; Thangavelu *et al*. 2017). It has been cultivated and consumed in different cultures, particularly in the Middle East, both as a fresh fruit and as a juice (Dkhil *et al*. 2013). Both pomegranate juice and peel are believed to have strong free-radical scavenging potential, due to this plant rich array of active nutrients (Mphahlele *et al*. 2014). These compounds exhibit potent anti-oxidant activities, which put pomegranate in a higher-grade capacity (Tapias *et al*. 2014). This fruit is also associated with a broad range of important physiological properties such as anti-lipoperoxidative, anti-inflammatory, cardio-preventive, antidiarrheal, anticancer properties, as well as DNA repair activities (Aviram *et al*. 2002; Hong *et al*. 2008; Dkhil *et al*. 2013; Thangavelu *et al*. 2017).

Based on the facts outlined above, the present study was undertaken to investigate the effects of oral administered toluene on the testicular function. Moreover, we tried to see whether the effects of toluene could be mitigated by treatment with pomegranate juice and the aqueous extract of its peel.

2. Materials and methods

2.1. Chemical

Toluene with a purity of 99.5 % was purchased from Sigma-Aldrich Chemicals Co., St. Louis, MO, USA). All other chemicals used in this experiment were analytical grade.

2.2. Plant

Pomegranates were harvested in November from Sidi Djemil Farm in Besbes, El-taref province, Algeria. After collection, the fruits were hand washed with tap water to remove dust, and then manually peeled without separating the seeds. Pomegranate juice was prepared daily using a commercial centrifugal blender (Sinbo, Turkey). The fresh peels were dried, crushed and then macerated in distilled water for 72 hour at room temperature (20° C), after which the mixture was filtered through cotton gauze.

2.3. Animals

One hundred adult male Wistar rats (220±30 g) were procured from Pasteur Institute, Algiers, Algeria. The animals were housed in polyethylene cages under standard conditions in the A

animal house at the department of biology. They were provided tap water *ad libitum* and fed a standard diet containing all the necessary elements (corn, soy, minerals, and vitamins) purchased from the ONAB agro-food complex in Bejaia, Algeria.

All experimental procedures were carried out according to the National Academies Press (NAP) Guide for the care and use of laboratory animals and approved by the Animal Sciences Ethical Committee of our institution.

2.4. Experimental design

Rats were divided into ten groups, and then treated daily per gavage for 6 weeks as follows: Control (C): received distilled water.

(CO): received 1.25 mL/kg b.w of corn oil.

(PJ): given 4mL/kg bw of pomegranate fresh juice.

(PAE): treated with 400 mg/kg b.w of pomegranate fresh peel aqueous extract.

(Tol1): treated with 275 mg/kg b.w of toluene dissolved in corn oil.

(Tol2): treated with 550 mg/kg b.w of toluene dissolved in corn oil.

(PJ-Tol1): treated by both toluene (275 mg/kg b.w) and pomegranate juice (4 mL/kg b.w).

(PJ-Tol2): treated by both toluene (550 mg/kg b.w) and pomegranate juice (4 mL/kg b.w).

(PAE-Tol1): treated by both toluene (275 mg/kg b.w) and pomegranate peel aqueous extract (400 mg/kg b.w).

(PAE-Tol2): treated by both toluene (550 mg/kg b.w) and pomegranate peel aqueous extract (400 mg/kg b.w).

2.5. Sample collection

After 6 weeks of treatment, all rats were sacrificed, and blood samples were collected in heparin tubes. Plasma

was immediately obtained by centrifuging blood at 3000 g for 10 min. reproductive organs (testis and epididymis) from each animal were carefully removed, rinsed with 0.9% NaCl weighed and stored at −20 °C for the determination of oxidative stress parameters.

2.6. Measurement of organs weights

Testis and epididymis weights were measured immediately after decapitation with a precision balance (KERN PRS 320-3, Balingen-Germany).

2.7. Sperm parameter evaluation

Sperm Class Analysis (SCA®, Microptic, Barcelona, spain) was used to assess the sperm quality characteristics. After decapitation, semen was obtained from the cauda epididymis and the analysed immediately after dilution in 0.9% NaCl physiological solution. A drop of dilute semen was transferred with a micropipette to a GoldCyto 20-µm counting chamber. This preparation was examined under a microscope set to 37° C (Nikon-eclipse E200, Barcelona, Spain) with (×4) negative-phase contrast, combined with a phase contrast condenser, and integrated into a computer.

2.8. Hypo-Osmotic Swelling (HOS) test

Sperm vitality was evaluated using Hypo-osmotic Swelling Test, developed by Jeyendran et al. (1984). This test is based on semi-permeability of intact sperm membrane. Approximately 1.0 µL of semen was added to 500 µL of a hypo-osmotic solutions prepared by dissolving sodium citrate and fructose in distilled water, and then incubated at 37° C for about 30 min. After that, a drop of diluted semen was pipetted onto a glass slide, allowed to dry, and then examined under an optical microscope at (×40) (Leica DMLB, Wetzlar, Germany).

2.9. Determination of testosterone

Plasma testosterone levels were estimated by using a chemiluminescence immunoassay-based commercial kit (Access testosterone 33560) and an Access immunoassay analyzer (Beckman CoulterAccess2, California, USA).

2.10. Determination of oxidative stress parameters

Lipid peroxidation levels were measured in testis homogenate as malondialdehyde (MDA) using the method described by Ohkawa *et al*. (1979), which is based on determination of thiobarbituric acid (TBA) reaction. The reduced glutathione level (GSH) was assessed spectrophotometrically following the method of Weckbecker and Cory (1988), observing yellow color development compared to a blank reagent. Glutathione peroxidase enzymatic activity (GPx) was carried out using the method of Flohé and Gunzler (1984), which is based on the reduction of hydrogen peroxide (H_2O_2) in the presence of reduced glutathione (GSH).

2.11. Determination of total protein

Protein concentration was determined according to the method of Bradford (1976) using bovine serum albumin as a standard.

2.12. Statistical analysis

Data were entered and expressed Mean±SEM. Statical analysis were performed using the statistical package SPSS (version 26.0) for Windows (IBM Corporation, Armonk, NY, USA). Differences between mean values among experimental groups were evaluated using One-Way Analysis of Variance (ANOVA), followed by Tukey's post hoc test. A p-value of less than 0.05 was considered statically significant

3. Results

3.1. Organs weights

Data of reproductive organs weight (testis and epididymis) are illustrated in table 1. The testicular absolute weight was significantly decreased in the Tol2 group compared to the control (C) and positive control groups (PJ and PAE). Nonetheless, no significant increase in the group treated with pomegranate juice and peel aqueous supplementation compared to the rats of (Tol2) group was observed. No statistically significant change in epididymis absolute weight was revealed between the toluene-exposed groups and control groups.

Table 1. Effect of toluene and its combination with pomegranate juice and peel aqueous extract on testis and epididymis absolute weight (means ± SEM)

Groups	Testis weight (g)	Epididymis weight (g)
C	1.69± 0.02	0.64± 0.01
CO	1.69±0.01	0.64± 0.01
PJ	1.72± 0.05	0.67± 0.01
PAE	1.77± 0.02	0.70± 0.008
D1	1.66± 0.06	0.64± 0.01
D2	1.54 ± 0.03[acd]	0.59± 0.03
PJD1	1.64± 0.02	0.64± 0.007
PJD2	1.60 ± 0.03	0.63± 0.02
PAED1	1.69 ± 0.02	0.66± 0.01
PAED2	1.66 ± 0.02	0.63± 0.005

[a]Significant difference compared to control (C),

[bcd]Significant difference compared to positive control (CO, PJ, PAE),

[e]Significant difference compared to (D1),

[f]Significant difference compared to (D2),

3.2. Sperm concentration and vitality

Table 2 represents sperm concentration and vitality results, showing a significant decrease in sperm concentration in rats treated with high dose of toluene only (Tol2) compared to the control (C) and positive control groups (CO, PJ and PAE). Notably, sperm concentration increased significantly after treatment with pomegranate peel aqueous extract (PAE-Tol2) supplementation compared to the Tol2 group.

The percentage of live sperm in the Tol2 group was significantly reduced; meanwhile the percentage of death sperm was significantly elevated than the corresponding control and positive controls. However, there was a marked significant increase in sperm vitality with a significant decrease in death sperm in the groups of the toluene-pomegranate juice and toluene-peel aqueous extract combination compared to the toluene groups.

Table 2: Effect of toluene and its combination with pomegranate juice and peel aqueous extract on sperm concentration and vitality (means ± SEM)

Groups	Sperm concentration (10^6/ mL)	Sperm vitality	
		(Alive sperm) (%)	(Dead sperm) (%)
C	88.6 ±4.7	90.0± 0.4	9.9± 0.4
CO	84.7±4.3	90.4± 0.3	9.4± 1.2
PJ	92.4 ±5.7	91.7± 1.01	8.2± 1.01
PAE	96.1±8.1	91.7± 1.3	8.2± 1.3
D1	82.9 ±10.2	81.8± 0.4^{abcd}	$19.7±2.05^{abcd}$
D2	$51.3±1.8^{abcde}$	$29.2±1.1^{abcde}$	$70.7±1.1^{abcde}$
PJD1	84±2.6	85.5± 4.5	14.5± 0.5
PJD2	69,2 ±4.3	57.8± 4.3^{cf}	42.1± $4,3^{cf}$
PAED1	99.4 ±1.6	88.6± 1.8	11.0± 0.1^{e}
PAED2	79.9± 2.1^{f}	86.6± 0.7^{f}	11.8 ± 0.2^{f}

[a]Significant difference compared to control (C),

[bcd]Significant difference compared to positive control (CO, PJ, PAE),

[e]Significant difference compared to (D1),

[f]Significant difference compared to (D2),

Table 3: Effect of toluene and its combination with pomegranate juice and peel aqueous extract on sperm motility (means ± SEM)

Groups	Total motility (%)	Progressive (%)	Non-progressive (%)	Immotile (%)
C	62.8± 5.5	6.1± 1.4	56.6± 3.7	37.1± 5.1
CO	67.3± 5.4	3.8± 0.4	63.4± 4.6	32.6± 4.7
PJ	66.7± 6.2	8.3± 0.4^{b}	58.4± 5.8	33.2± 5.8
PAE	67.1± 0.6	9.4± 0.8^{b}	57.6± 1.2	32.8± 0.3
D1	59.3± 0.6	4.2± 2.7^{d}	42.1± 2.7^{b}	53.6± 5.7
D2	23.4± 1.4^{abcd}	0.7± 0.2^{acd}	$22.6±1.6^{abcde}$	76.5± 1.4^{abcde}
PJD1	59.3± 0,6	3.3± 0.2^{c}	55.9± 2.6	40.6± 2.4
PJD2	50.2± 3.3^{f}	2.8± 0.3^{c}	47.3± 0.4^{f}	49.8± 3.3^{f}
PAED1	63.8± 4.1	7.1± 0.5	56.66 ± 0.9	36.1± 0.3
PAED2	57.2 ± 7.2^{f}	3.2 ± 0.04^{d}	54.03 ± 5.2^{f}	42.7± 7.2^{f}

[a]Significant difference compared to control (C),

[bcd]Significant difference compared to positive control (CO, PJ, PAE),

[e]Significant difference compared to (D1),

[f]Significant difference compared to (D2),

3.3. Sperm motility

As indicated in **table 3**, sperm motility results revealed a significant reduction in the percentages of total, progressive and non-progressive motility, whereas the percentage of immotile sperm was significantly increased in rats of Tol2 group compared to the control and positive control groups (CO, PJ and PAE). Conversely, toluene treatment combined with pomegranate juice (PJ-Tol2) and with peel aqueous extract (PAE-Tol2) significantly enhanced sperm motility and decreased the percentage of immotile sperm compared to toluene Tol2 group.

Our results also revealed a significant decrease in parameters reflecting velocity indicators including amplitude of lateral head displacement (ALH), beat cross frequency (BCF), curvilinear velocity (VCL), straight line velocity (VSL), average path velocity (VAP), and linearity (LIN) in the Tol2 group. On the other hand, there was no significant difference in VCL in the Tol1 and Tol2 groups compared to the control and positive control groups (**Figure 2** (A, B, C, D)).

3.4. Testosterone levels

As shown in figure 3, exposure to toluene (Tol2) has significantly reduced testosterone concentration compared to the control and positive control groups (CO, PJ and PAE). Testosterone levels in the group supplemented with peel aqueous extract (PAE-Tol2) were clearly higher than in the group treated with toluene only. Pomegranate juice supplementation also increased testosterone levels, though this elevation was non-significant compared to the Tol2 group.

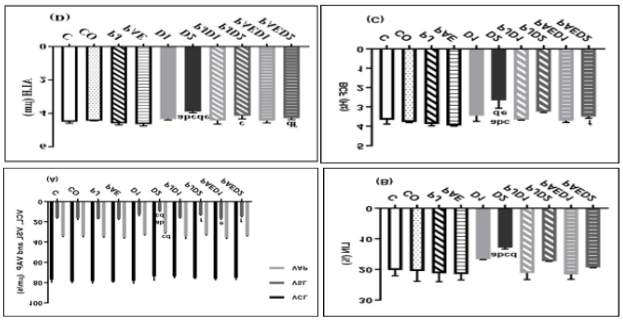


Figure 2. Effect of toluene and its combination with pomegranate juice and peel aqueous extracton kinematics parameters. (A): Sperm velocity; (B): Sperm linearity; (C): Beat cross frequency; (D): Amplitude of lateral head displacement

[a]Significant difference compared to control (C),

[bcd]Significant difference compared to positive control (CO, PJ, PAE),

[e]Significant difference compared to (D1),

[f]Significant difference compared to (D2),

3.5. Lipid peroxidation

The results obtained for MDA concentration in testicular tissue (Table 4) showed a significant increase in the Tol2 group compared to the control and positive control groups (CO, PJ and PAE). Supplementation with pomegranate peel aqueous extract PAE-Tol2 significantly reduced MDA levels compared to the Tol2 group. In contrast, supplementation with pomegranate juice did not show any significant differences in comparison to the Tol2 group. In the low dose toluene treated group Tol1, MDA levels, were not significantly different compared to the control and positive control groups.

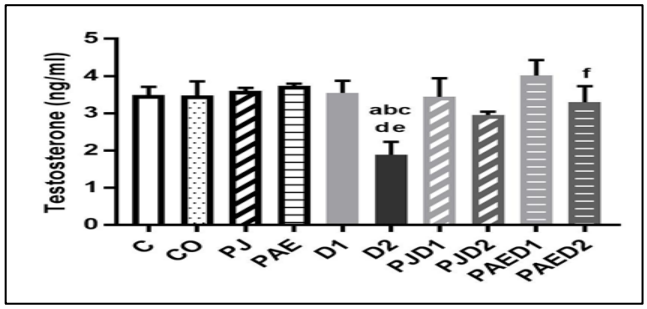


Figure 3. Effect of toluene and its combination with pomegranate juice and peel aqueous extract on testosterone levels (means ± SEM)

[a]Significant difference compared to control (C),

[bcd]Significant difference compared to positive control (CO, PJ, PAE),

[e]Significant difference compared to (D1),

[f]Significant difference compared to (D2),

3.6. Glutathione and glutathione peroxidase activity

The antioxidant profile (**Table 4**) revealed that high-dose of toluene Tol2 significantly decreased testicular GSH levels and GPx activity relative to the control and positive controls (CO, PJ and PAE), while combined treatment with pomegranate peel aqueous extract significantly increased these parameters compared to the Tol2 group. There was no significant increase in GSH levels and GPx activity in the combination of pomegranate juice and toluene (PJ-Tol2) group compared to the Tol2 group. Moreover, these parameters showed no significant differences in the Tol1 group compared to the controls.

Table 4: Effect of toluene and its combination with pomegranate juice and peel aqueous extract on levels of testicular GSH, MDA and GPx enzymatic activity (means ± SEM)

Groups	MDA (nmoL/mg tissue)	GSH (nmoL/mg prts)	GPx (nmoL GSH/mg prts)
C	0.27± 0.01	25.8± 1.04	0.34± 0.005
CO	0.27± 0.01	25.8±0.5	0.34± 0.0007
PJ	0.26± 0.01	27.9± 0.5	0.37± 0.009
PAE	0.24± 0.01	28.7± 0.3	0.4± 0.01
D1	0.31± 0.02	25.0± 0.8^{d}	0.32± 0.01^{d}
D2	0.5±0.01^{abcde}	22.0±0.2^{abcd}	0.22±0.004^{abcde}
PJD1	0.28± 0.01	26,0± 0,4	0.34± 0.01
PJD2	0.36± 0.01^{cf}	24.4± 0.6^{c}	0.24± 0.008^{c}
PAED1	0.27± 0.01	27.0± 0.6	0.36± 0.02
PAED2	0.29± 0.015^{f}	25.6± 0.4^{df}	0.36 ± 0.028^{d}

[a]Significant difference compared to control (C),

[bcd]Significant difference compared to positive control (CO, PJ, PAE),

[e]Significant difference compared to (D1),

[f]Significant difference compared to (D2),

4. Discussion

Although the frequent conception that reproductive issues are commonly woman's concern, infertility occurs in equal amounts for females and males. Data indicates that substances and drug abuse create a high incidence in the global decline in male fertility, emerging as a critical health issue (Taha *et al*., 2020). This experimental study was conducted to evaluate the effects of toluene both alone and in combination with *Punica granatum* juice and peel aqueous extract on reproductive indices and antioxidant status in adult male rats.

In the present study, our findings demonstrated that toluene has led to a momentous reduction in testicular absolute weight. These results corroborate with previous

study reported by Djemil et al. (2015) and disaccorded with another one by Nakai et al. (2003). Testicular weight basically depends on the mass of the differentiated spermatogenic cells. Accordingly, a decreased number of germ cells, lower steroidogenic enzyme activity, and inhibited spermatogenesis may be the causes of testis weight's reduction (Takahashi and Oishi 2001). The reduction in testicular weight observed in this study is a pointer to toluene-induced alteration on the germ cells differentiation and gonadotropins release (Nakai *et al*., 2003), where the gonads weight loss is an indication of spermatogenic cell arrest and steroid biosynthesis suppression in Leydig cells (Goldman *et al*., 1989; Ihsan *et al*., 2011).

Sperm parameters including concentration and vitality are important functional indicators to assess sperm fertilizing capacity. Epididymal sperm concentration was notably dropped in toluene treated animals, according to our findings. Furthermore, within the same group, the hypo-osmotic swelling test indicated a clear decline in sperm vitality. Such results are consistent with those who reported a reduction sperm concentration and vitality in male rats after 150 ppm toluene oral administration (Djemil *et al*., 2015). Previously it was established that toluene provokes disturbance in the meiotic process of spermatogenesis, which may ultimately elicit sperm concentration lacking. Other authors have postulated that alteration of testicular germ epithelium DNA could be the origin of sperm concentration regression (Schardein, 1993; Murata *et al*., 1999). Regarding the drop witnessed in sperm vitality the cause perhaps was associated to a decrease in the Follicle Stimulating Hormone (FSH) concentration that is a mediator in the activation of growth factors and production of nutrients by Sertoli cells, which maintain the maturation and normalization of the micro-environment around sperm and germ cells (Svensson *et al*., 1992; Menegazzo *et al*., 2011). Apparently, polyunsaturated lipids oxidation of sperm can also alter sperm vitality by changing membranes' fluidity and permeability (Çeribaşi *et al*., 2010).

Consistently, toluene provokes a decline in sperm motility and other kinematic parameters (VSL, VAP, LIN, ALH and BCF). Spermatic motility with all its parameters such as VCL, VAP, VSL, BCF, LIN, and STR was reported to be essential elements to estimate fertility rate (Moore and Akhondi, 1996). Thus, changes in these indicators play a main role in the pathogenesis of toluene reproductive toxicity. Proper ionic balance plays a critical role in regulating sperm flagellar motility. In fact, there is abundant experimental evidence stating that the psychoactive drugs were demonstrated to alter sperm motility through a variety of mechanisms including regulation of calcium signalling (Srivastava and Coutinho, 2010). It is equally known that exposure to toluene interact with a variety of ion channels as it can cause voltage-sensitive calcium channels inhibition and produces noticeable changes in different systems (Tillar *et al*., 2002). Therefore, it can be speculated that sperm motility diminution returns to voltage-dependent calcium channel arrest ''CatSper'' located in the mid and principal piece of the flagellum, as a result, obstruction of calcic flux, which is the principal mediator engaged during sperm motility (Darszon *et al*., 2005). In the same vein, high-level toluene brings an inhibition to the function of potassium channels (BK and GirK) and the voltage-sensitive sodium channels (Na_v) (Del Re *et al*., 2006; Scior *et al*., 2009), the important regulators for cellular processes including sperm regulation function such as maturation, motility and viability (Publicover *et al*., 2008; Yi *et al*., 2011; Cejudo-Roman *et al*., 2013). Thus, it is hypothesized that the observed deterioration in sperm quality under these circumstances suggests that toluene can deeply disrupt ion channels in sperm, thereby affecting male fertility.

In terms of investigating the role of *Punica granatum*, the pomegranate peel aqueous extract appears to be more effective than pomegranate juice in moderating toluene's effect on spermatic parameters. In parallel, pomegranate peel extract has been demonstrated to boost sperm count, motility and viability, along with increasing seminiferous tubule diameter and epithelium thickness due to the antioxidant activity of its phenolic compounds (Zeweil *et al*. 2013; Tapias *et al*. 2014; Utomo *et al*. 2019). It has been documented that metabolites such catechin and quercetin exhibit the capacity to improve sperm quality and reduce lipid peroxidation (Taepongsorat *et al*. 2008; Boonsorn *et al*. 2010). Moreover, Flavonols such as gallocatechin, are potent antioxidants proven twice as effective as the vitamin E (Plumb *et al*. 2002), which, at daily dose of 300 mg, significantly elevated sperm motility in infertile men (Suleiman *et al*. 1996). Furtermore, the anthocyanin in pomegranate has been suggested to stimulate reproductive hormone regulation by modulating the development and the function of the testicular germ line and sertoli cells (Utomo *et al*. 2019).

Steroid hormones are believed to be a crucial upstream factor in the stimulation and control of the maintenance and development of male reproductive functions. In this experiment, similar to Nakai et al. (2003) toluene exhibited a remarkable drop in plasma testosterone concentration in male rats administrated higher toluene dose. In this case, toluene may have impaired Gonadotropin-Releasing Hormone (GnRH) neurons in the hypothalamus-hypophyseal-gonadal axis resulting in the reduction of luteinizing hormone (LH) and testosterone secretion, which induce the signals for the synthesis of testosterone (Yilmaz *et al*., 2001). An inhibition of these signals results in a reduction in serum testosterone levels, as has been observed in men exposed to low concentrations of toluene where their LH level was affected (Luderer *et al*., 1999).

As well, it has been suggested that reactive oxygen species (ROS) generated by toluene through biotransformation processes can increase the expression of Cyclooxygenase (COX-II) (Kamel and Shehata, 2008), which might impact androgen synthesis via the production of a large amount of inflammatory prostaglandins (Fouad and Jresat, 2014).

So far, according to the above findings, it is clear that poor sperm quality was attributed to oxidative testicular injuries. The administration of toluene elicited a weakening in the antioxidant defence system by reducing the ratio of GSH and GPx activity accompanied by a rise in MDA levels. Several clinical trials and records from the literature confirmed that organic solvents, bringing about intense oxidative stress that may cause an imbalance in cells production of enzymatic and non-enzymatic antioxidants (Bayil *et al*. 2008; Moro *et al*. 2010). It also concurs with Kamel and Shehata (2008) who documented a clear diminution GSH and GPx testis levels reported that

The reduction in the antioxidants activity is an indication of the override generation of ROS (Ochsendorf 1999). GSH diminution could be also caused by an elevation in the amount of nitric oxide (NO), therefore increasing the susceptibility to oxidative stress to impair the testicular function (Ekici *et al*. 2012). It has been equally proposed that H_2O_2 augmentation might correlate with GPx activity depletion (Kamel and Shehata 2008).

In the actual study, toluene caused an elevation in MDA level, which is in accordance with the work of Moro *et al*. (2010), who suggested a correlation between MDA concentration and blood toluene levels. In this circumstance, the higher concentration of MDA is a direct evidence of toxic effect of toluene that generates free radicals and alters cell lipid membranes (Bayil *et al*. 2008).There was a relationship between MDA level and DNA damage in case of toluene exposure (Moro *et al*. 2012), especially in testis and sperm membranes of mammals including humans, where a high amount of polyunsaturated fatty acids are found, which make them sensitive to oxidative stress that might produce loss of membrane integrity and subsequently could impair cell function (Wathes *et al*. 2007).

Contrariwise, most parameters were close to normal in rats supplemented with the combined treatments of pomegranate juice and peel aqueous extract. The present findings are similar to the results of Dkhil et al. (2013), who demonstrated that pomegranate juice and peel methanolic extract were able to stimulate androgens levels and to provide a remarkable increase in antioxidant enzymatic activities and GSH concentration, with lower MDA, COX-II, and NO level. Punicalagin was found in pomegranate, has been reported to perform an important role in activating Nrf2 and *PI3K-Akt* expression, which are extensive factors that neutralize and regulate oxidative damages by stimulating the cellular antioxidant system and inhibiting inflammatory pathways (Xu *et al*. 2015; Thangavelu *et al*. 2017). Ellagic acid, another component in pomegranate, provides a significant reduction on both OH and $O^{\cdot}{}_2^-$levels and hence suppresses oxidative DNA damage (Türk *et al*, 2008b). Indeed, these compounds, in addition to quercetin are stronger compared to other known substances like Vitamins C and E, coenzyme Q-10, and α-lipoic acid (Aviram *et al*. 2002), which give to pomegranate a property to reduce ROS by scavenging free radicals, chelating divalent cations, and stimulating C-glutamyl cysteine synthetase production, a critical enzyme in GSH biosynthesis (Bishayee *et al*. 2011). In the same way, pomegranate could preserve GSH concentration by stimulating C-glutamyl cysteine synthetase production, a critical enzyme in GSH biosynthesis (Moskaug *et al*. 2005).

Finally, our findings indicated that pomegranate peel aqueous extract has a higher ability to weaken reprotoxic injuries induced by toluene more than pulp juice. Accordingly, pomegranate peels have exerted an antioxidant activity against free radical damage higher than that of seeds and pulps (Qu *et al*. 2010). Moreover, pomegranate peel was reported to have the highest antioxidant activity compared to other species of fruits peels (Li *et al*. 2006).

5. Conclusion

The present study has shown that the adverse effects of toluene on rats after 6 weeks exposure was dose-dependent and appeared to be linked to several disorders of male fertility, confirmed by impaired sperm concentration, vitality, and motility indexes, as well as to an alternation in testosterone synthesis. Likewise, toluene altered the balance of the redox system by decreasing the GSH levels and GPx enzymatic activity, while it increased MDA concentration. Despite the demonstrated toxic effect of toluene, the mechanisms of action have not been fully understood.

On the other hand, pomegranate juice and especially peel extract attenuated toluene-induced reprotoxicty almost completely, which is certainly related to the various components represented mainly by phenolic compounds.

Declarations

The authors report no conflicts of interest related to this study.

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