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ORIGINAL ARTICLE

The effects of *Permethrin* and antioxidant properties of *Allium cepa* (onion) on testicles parameters of male rats

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Abstract

The aim of this study was to evaluate the effects of *Permethrin* on the testes of adult Wistar rats, and evaluate the efficacy of *Onion* juice supplementation. The male rats were divided into five groups: I: control; II: DMSO (0/5 ml); III: *Permethrin* (35 mg/kg) in 0/5 ml DMSO; IV: *Permethrin* (35 mg/kg) + *Onion* (3 ml); V: onion (3 ml). After 60 days, male rats in all groups prepared to evaluate genital organs weights, the Leydig cells culturing and measurement of testosterone hormone level. *Permethrin* have negative impacts on Sperm parameters, the Leydig cells count and reduced testosterone serum level in groups 3 and 4. However, in group 4, *ONION* with antioxidant properties can modify these effects. *Onion* showed a significant remedy effect on sperm parameters and also leads to increases in the number of Leydig cells. These findings indicated that *Permethrin* have the negative effects on the reproductive system and fertility.

Keywords

Permethrin, testicles parameters, antioxidants, *Allium cepa* (onion)

History

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Introduction

Most environmental chemicals are endocrine disrupting that targets the reproductive disorders. These chemicals impair testis function by altering the pro-oxidant/antioxidant equilibrium of cells, therefore caused the initiation of cell death programming. The utilization of chemicals in recent agriculture methods has remarkably increased fertility problems (Heba El-Sayed et al., 2016). These safety effects are distinct via degree and the type of exposure to them. These chemicals have toxicities properties such as hormonal or mutagenic activity and they are of specific concern when considered in the background of acute and susceptible gate of development. The preferable concern of the scientific document is about very small exposures to some endocrine disrupting chemicals, particularly at critical phases of fetal development, which may affect detrimental reproductive and other consequences. Pyrethroids are insecticides that derived from natural compounds known as Pyrethrins from the *Chrysanthemum* genus of plants (Casida, 1980). *Permethrin* (type 1 pyrethroids) is one of the most broadly used insecticides in food/feed yields, livestock and livestock housing, methods of forwarding, buildings, and for inhabitable area. As well as, *Permethrin* is available for treatment application and inhibition of head lice and scabies. According to most studies, *Permethrin* has low toxic activity to mammalian and weakly absorbed via the skin. Actually, the direct usage of *Permethrin* to control earthy pests can result in remnant on soil and vegetables, and lead to exposure to mammalians and rodents. Since, *in vivo* study demonstrated that absorption of *Permethrin* based on repulsion of urinary metabolites is minimal (Vander Rhee et al., 1989). Diffusion of *Permethrin* with highest level accumulated in fat and the brain, which this may be due to the lipophilic property of the *Permethrin* molecule. In addition, metabolism of it occurs mostly in the liver and where through oxidation process (by the cytochrome p450 system also hydrolysis) break down into metabolites (Vander Rhee et al., 1989). The large duration exposure of *Permethrin* in humans may be caused due to some neural-dependent disorders like muscle weakness, nausea, shortness of breath, headache, excessive salivation and seizures. *Permethrin* has no significant immunotoxicity or genotoxicity effects in humans, but Environmental Protection Agency (EPA) classified it as a maybe human carcinogen, that is based upon the studies on mice and rat fed *Permethrin* with liver and lung tumors (Ishmael & Litchfield, 1988). During spermatogenesis, undifferentiated germ cells proliferate and transformed to spermatozoa. The tight junctions between the sertoli cells build an impressible blood–testis barrier, which controls the circulation of nutrients and growth factors that are needed for the development of germ cells. So, each stress that is adequate to impair this barrier resulted in failure of the spermatogenesis. Increased levels of ROS can be noxious to testis function (Chargui et al., 2011), so to prevail it, the testis is equipped with strong antioxidant system to support it from the adverse effects of ROS. The use of natural antioxidants to overcome the negative consequences affecting fertility is required. These compounds may be produced in the body or received

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from daily food and fruit intake. Therapy methods to decline seminal oxidative stress levels may increase actual vision and the result of assisted reproductive technologies. Onion (*Allium cepa*) is a generic food plant in the treatment and inhibition of some diseases. There are some *onion* types, in both color and flavor, that possess various concentrations of phenolic compounds and flavonoids. The flavonoids observed in onion are quercetin aglycone, glucosides and in some species include isorhamnetin monoglycoside or kaempferol monoglycoside. Extracts of *onion* due to phenolic compounds display antidiarrhoeal activity, anticancer activities, antimutagenic properties, antiulcer possess, antispasmodic and anti-proliferative activity (Lee et al., 2008). The aim of this study was to examine of antioxidant properties of *onion* juice against *Permethrin*-induced damages in the testis, appendix organs of genital tract and especially the Leydig cells producing testosterone hormone. In addition, we measured testosterone levels to confirm the activities of Leydig cells in all groups.

Materials and methods

Animals and treatment

Permethrin and dimethyl sulfoxide (DMSO) were purchased from Sigma Aldrich (St. Louis, MO), and *Onions* were taken from urban centers in Tabriz city of Iran. *Onion* juice was taken from fresh onions and it gavaged to rats every day. Male rats were prepared from animal care center of medical faculty of Tabriz of Iran. 30 male rats were randomly selected and divided into five groups ($n = 6$). Certain period of study was two months which during this period, group 1 was considered as a Control group. Group 2 received daily 0/5 ml *DMSO* via intra-peritoneal injection. *DMSO* was used for the solution of *Permethrin*. Male rats of group 3 received 35 mg/kg *Permethrin* in 0/5 ml *DMSO* via intra-peritoneal injection. 1/40 of the LD_{50} value of *Permethrin* was chosen based on the ''no observed adverse effect level'' (NOAEL) for PM of 25 mg/kg (Vadhana et al., 2013). The rats in group 4, in addition to *Permethrin* (at the same dose) received 3 ml *onion* juice through oral gavage. And the rats in group 5 were treated daily with 3 ml *onion* juice in the same way (Khaki et al., 2012).

Measurement of testis and appendixes organs weights

Over the same period, each rat was anesthetized by ketamin-xylene and to determine the weight changes, reproductive organs (testicles, seminal vesicles, epididymis and prostate gland) were immediately removed. After weighing organs, testicles and epididymis were used to isolate the Leydig cells and Sperm parameters, respectively.

Biochemical analysis of sperm cells

For this purpose, left and right epididymis of each rat was poured into tubes containing prepared RPMI medium. The samples were transferred into the petri dish containing culture medium and were split into smaller pieces. Broken pieces moved into 24-hole plate containing medium culture plus 4 mg/ml BSA and incubated at 37 °c for 2 min. Tissue pieces were removed from culture medium and the sperm suspension were transferred to an incubator. 1/100 dilution of suspension prepared and a drop was placed on a Neubauer chamber cell counting to Sperms numeration (Chen et al., 2010). Finally, sperm motility of rats in each group was evaluated by a Olympus microscope. We also studied the sperm morphology based on head and tail of them under a microscope. The sperm morphology was observed in four cases out of which pin head and normal tail considered as normal for rat sperm.

The separation Leydig cell of adult male rat and the cells count

Ingredients for the isolation of Leydig cells were prepared as follows: DPBS (Sigma Aldrich, St. Louis, MO), NBCS (DIBCO, Thermo Fisher Scientific, Waltham, MA), Collagenase type I (*Clostridium histolyticum* Sigma), Trypsin inhibitor (Glycine max (soyben) Sigma), Percoll® (cell culture tested Sigma), DMEM Medium, High glocuse (Biosera, South Korea), Neutral Red dye powder (Bio Reagent Sigma Chemical Co., Germany). After male rats were anesthetized by ketamin- xylene, left and right testicles were removed and then taken in a petri dish containing DPBS. Testicles tissue was washed three times in DPBS supplemented with 0/1% NBCS. Then knitted pieces were transferred into a 1 ml tube containing 0/04% collagenase type 1 and 10 μg/ml trypsin inhibitor. Next, the tube was incubated in a water bath at 34°C for 40 min. The resulting cell suspension was diluted to 4 times in DPBS. The supernatant was removed and centrifuged for 3 min in 200*g*. The cell pellet was washed 2 times with DPBS and diluted by 0/5 ml of it. In the next stage, suspension was placed within 21, 26, 40 and 60% concentration of Percoll®. The suspension was centrifuged for 10 min at 1500*g*. The cell pellet was washed 4 times with DPBS and DMEM medium supplemented by 10% NBCS. The solution was centrifuged again for 3 min at 200*g*. Then the cell pellet was diluted with 0/5 ml DMEM medium and finally 10λ from the cell suspension and Neutral red removed and the Leydig cell concentration was measured by Neubauer chamber count cell (Antonio et al., 2001; Ekayanti et al., 2013).

Serum testosterone levels: To evaluate testosterone hormone, kits from Immunotech Bekman Company (Fullerton, CA) were used. The kits assay sensitivity was 0/025 ng/ml with double-antibody immunoassay.

Results

Testis and appendix organs weights

As can be seen in Table 1, the weight testes of rats in groups 3 and 4 with the other groups showed a significant difference. This remarkable difference is also seen in the weight of the epididymis, prostate and seminal vesicles (p values <0.001). Therefore, *Permethrin* through different ways induces negative effects in the reproductive structures. About weight loss in the male genital organs, it may be said that *Permethrin* exposure resulted to these outcomes via cell damages, increased inflammation or disruption in the blood flow. However in group 4, *onion* juice moderated the negative effects of *Permethrin* and compensated the weight loss of

Table 1. The weight of reproduction organs after treatment by *PER* and *onion*.

Groups weight (g)	C (mean ± SD)	DMSO (mean ± SD)	PER (mean ± SD)	PER + AC (mean ± SD)	AC (mean ± SD)
Left epididymis	0.63 ± 0.031	0.59 ± 0.04	0.2 ± 0.03*	0.34 ± 0.031**	0.69 ± 0.03
Right epididymis	0.62 ± 0.02	0.59 ± 0.04	0.2 ± 0.03*	0.31 ± 0.03**	0.67 ± 0.02
Left testis	1.64 ± 0.04	1.58 ± 0.05	1.09 ± 0.06*	1.27 ± 0.03**	1.71 ± 0.03
Right testis	1.62 ± 0.05	1.56 ± 0.04	1.03 ± 0.03*	1.24 ± 0.04**	1.7 ± 0.04
Prostate	0.58 ± 0.02	0.54 ± 0.02	0.345 ± 0.031*	0.45 ± 0.03**	0.64 ± 0.03
Left seminal vesicle	1.71 ± 0.03	1.69 ± 0.03	1.27 ± 0.03*	1.39 ± 0.04**	1.8 ± 0.03
Right seminal vesicle	1.69 ± 0.03	1.69 ± 0.03	1.23 ± 0.03*	1.38 ± 0.02**	1.78 ± 0.04

Values are expressed as means ± SD of 30 rats.
*, **With ANOVA statistically was observed a significant difference between all groups (p values <0.001).
C: Control; PER: *Permethrin*; DMSO: Dimethyl sulfoxide; AC: *Allium cepa* (onion).

Table 2. The counts of sperm and Leydig cells after treated by *PER* and *onion*.

Groups	C (mean ± SD)	DMSO (mean ± SD)	PER (mean ± SD)	PER + AC (mean ± SD)	AC (mean ± SD)
Live Leydig cell counts $\times 10^4$/g	45.66 ± 4.63	41.66 ± 6.59	22.16 ± 4.26*	28.33 ± 4.92**	**47.5 ± 3.39**
Live sperm cell counts $\times 10^6$/g	59.33 ± 6.65	55.33 ± 6.8	32.17 ± 3.76*	45 ± 4.38**	**70.67 ± 3.61**

The value is mean ± SD of six rats in each group.
* and **Mann–Whitney U-test was used for comparison between means (p value < 0.001).
C: Control; PER: *Permethrin*; DMSO: Dimethyl sulfoxide; AC: *Allium cepa* (onion).

Table 3. The sperm cells morphology after treated by *PER* and *onion*.

Groups	C	DMSO	PER	PER + AC	AC
Pin head (%)	80%	75%	40%*	52%**	82%
Round head (%)	10%	15%	25%*	20%**	5%
Long head (%)	5%	5%	20%*	15%**	5%
Deformed head (%)	5%	5%	15%*	13%	8%
Normal tail (%)	85%	83%	50%*	55%**	88%
Short tail (%)	5%	7%	20%*	15%**	3%
Long tail (%)	5%	5%	15%*	10%**	4%
Cut off tail (%)	5%	5%	15%*	10%**	3%

* and **Statistical significance is p value <0.05.
C: Control; PER: *Permethrin*; DMSO: Dimethyl sulfoxide; AC: *Allium cepa* (onion).

genital organs. In group 5, which only gavages by *onion* juice even compared to the control group increases organ weight, which reflects the antioxidants property of *onion*. In group 2, *DMSO* did not show adverse effects on organ weights.

Biochemical results in sperm cells

It has observed that, in terms of sperm count, *Permethrin* caused to dramatic reduction in group 3 and 4 compared to other groups, but onion juice improved it (p values <0.001) (Table 2). Here, *DMSO* has no effect on this parameter. In men, it can be traced to natural antioxidants like *onion,* which has been noted in the previous findings. In this study, the Sperm morphology was evaluated based on their head and tail. As can be seen in Table 3, pin head and normal tail were considered as natural indicators of Sperms. In this case, *Permethrin* imposed its interactions on normal sperm morphology (p value <0.05) (Table 3; Figure 1). So, one of the effects of *Permethrin* reduces the number of normal Sperm that can be considered for several reasons. For example, *Permethrin* may be affect germ cell line of the spermatogenesis before it directly induces damage to spermatozoa. Naturally, the fertilization of sperm will be disrupted by reducing normal and matured among them. By reducing abnormal sperms and increasing normal sperms, *onion* juice indicates its positive impact. The sperm motility affected by *Permethrin* shows a significant reduction compared to other groups (p vvlue <0.05) (Table 4). We studied the motility of sperms from four perspectives and we observed that straight moving, which is essential to fertility of sperms, impaired in the *Permethrin* group, but in group 3, *onion* juice partly eliminates the effect of it. *DMSO* has no remarkable activity on the sperm motility. In those rats, which consumed only by *onion* juice, we observed that not only it has significant differences with rest of the groups but also improves the sperm motility, especially straight moving. This shows that *onions* can increase sperm fertilization potency.

The number of Leydig cells

The number of Leydig cells alive with Neutral red staining (Figure 2A, B) shows that *Permethrin* caused reduction in their counts (p value < 0.001) (Table 2). Perhaps, one of the most important causes of reduced fertility is the Leydig cell death induced by toxicity of *Permethrin* (Figure 2C, D) which leads to a decrease in Testosterone synthesis. In this case, *Permethrin* may act as a disruptor and inhibits adequate nutrition with blood supply to cells or with activate macrophages and inflammatory conditions. But, *onion* group shows an increase in the number of these cells, which indicates that unlike *Permethrin*, *onion* juice will provide favorable conditions for growth and function of Leydig cells.

Testosterone serum level

As can be seen in Table 5, *Permethrin* can reduce testosterone serum level (p value < 0.001). Perhaps, according to the above results, this process is done through induction of changes in the Leydig cells that disrupts testosterone synthesis.

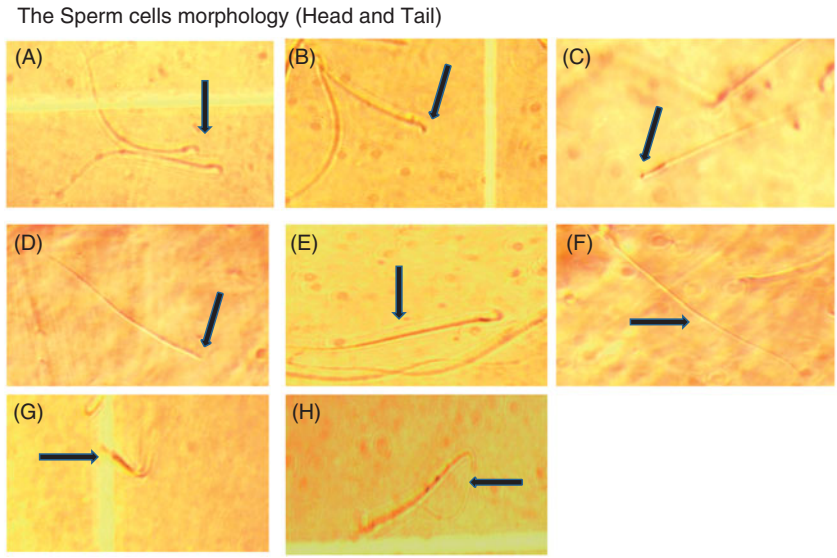

Figure 1. The sperm head and tail morphology, A (Pin head); B (Round head); C (long head); D (Deformed head); E (Short tail); F (long tail); G (Cut off) and H (abnormal tail) Olympus microscopic (40×).

Table 4. The types of sperm motility after treated by *PER* and *onion* juice.

Groups	C	DMSO	PER	PER + AC	AC
Straight moving (%)	70%	65%	30%*	40%**	72%
Zig-zag moving (%)	15%	18%	25%*	28%**	12%
Vibrating (%)	10%	10%	20%*	20%**	10%
Non-motile (%)	3%	7%	25%*	12%**	6%

* and ** Statistical significance is p value <0.05.
C: Control; PER: *Permethrin*; DMSO: Dimethyl sulfoxide; AC: *Allium cepa* (onion).

Somewhat in group 4, *onion* juice has moderated this trend which could come back to its positive role.

Discussion

It is confirmed that insecticides can affect male and female reproductive system (Heba El-Sayed et al., 2016; Tugba & Nazan, 2015). Most studies have shown that pyrethroid can disrupt male genital function, which in this case, that several theories have been reported. For example, pyrethroids may reach the testicles and act on the membrane of seminiferous tubules (Ahmad et al., 2011). Another presumption about pyrethroids effects on male reproductive could be because of a neuro-endocrine-mediated phenomenon or a hormone-disrupting feature (Yousef et al., 2003). It is reported that pyethroids can affect anti androgenic activity or changes in mitochondrial action causing a delay in motility and eventually leading to cell death (Sun et al., 2007). It is also shown that chronic exposure to pyrethroids may cause derangement in endocrine functions through androgen activity where they have a strong tendency to link with androgen receptors and sex hormone binding globulin that led to noxious effects on semen quality (Charles & Bruce, 1990; Meeker et al., 2009). Therefore, there are different views on the effects of pesticides on male spermatogenesis. It was reported that these chemicals have mutagenic risk on male germ cells and chromosomal aberrations (Giri & Sharma, 2003; Shukla & Taneja, 2002). Exposure to some insecticides (*Permethrin*, butylate, coumaphos Chlorpyrifos, fonofos and phorate) showed remarkable relationship with raised prostate cancer rates, in which case these compounds represent gene interactions. These pesticides may be imposing effects by inhibition of the p450 hepatic enzymes function that metabolizes testosterone, estradiol and estrone hormones (Scott et al., 2010). Environmental Protection Agency (EPA) lists *Permethrin* as a suspected endocrine disrupter that in a long-term usage has shown that it reduces testes weights in mice and could affect disrupting testosterone production in the adult male mouse (Zhang et al., 2007). The data analysis from *Permethrin* treatments revealed that it caused hard testicular histopathological injury and decreases testis weight. In this study, we observed that *Permethrin* led to weight loss in the reproductive organelles. However, based on above assumptions, it cannot be stated with certainly about *Permethrin* effects. Testosterone data analysis displayed a significant reduction in the experimental groups treated by *Permethrin*. Perhaps it links to male sex hormone (androgen receptors) and also to peripheral benzodiazepine receptor (PBR), which agitates testosterone synthesis (Charles & Bruce, 1990; Ramadan et al., 1988). Researchers established that *Permethrin* had notable estrogenic capability as it prevented the binding of 17β-estradiol to the estrogen receptor. However, one of the most likely mechanisms affecting testosterone is the mitochondrial membrane impairment in the Leydig cells (Rousseau-Merck et al., 1990). We observed that the sperm and Leydig cells are reduced by *Permethrin,* which shows the cytotoxicity and hormonal disruption of it. Finding in rats have shown that *Permethrin* affects male reproductive actions by reducing the motility of mature sperm cells (Perobelli et al., 2010). The mixed use of Pyridostigmine bromide, DEET and *Permethrin*-induced apoptosis in germ cells, sertoli cells and Leydig cells of testicular rats (Abou-Donia et al., 2004). Since *Permethrin* exposure to human cell cultures shows an increase in chromosome aberrations and DNA damage (Turkez & Aydin, 2013), it is possible that in the case of sperms and Leydig cells through damage to DNA resulted in these outcomes. Antioxidants are the most serious defense versus free radical impels infertility. (Ashok & Lucky, 2010; Hemadi et al., 2013). There are many natural

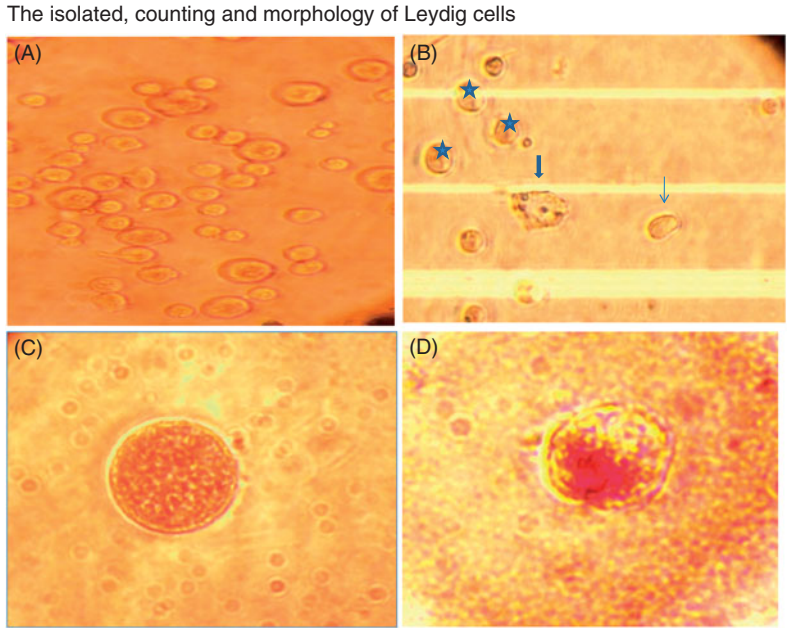

Figure 2. A (the isolated Leydig cells of male rat staining with Neutral red), B (counting the Leudig cells by Neubauer chamber (Asterisks; Adult Leydig cells, Thin arrow; Aging Leydig cell, Bold arrow; Premature Leydig-like cell) Olympus microscopic (10×), C and D (Alive and Dead adult Leydig cell stained by Neutral Red).

Table 5. Difference testosterone serum levels after treatment with *Permethrin* and *onion* juice.

Group	Testosterone
p Values control & DMSO	0.024
p Values control & *Permethrin*	<0.001*
p Values control & *Permethrin* + *Onion*	0.091
p Values control & *Onion*	0.439
p Values DMSO & *Permethrin*	<0.001*
p Values DMSO & *Permethrin* + *Onion*	0.522
p Values DMSO & *Onion*	0.011
p Values *Permethrin* & *Permethrin* + *Onion*	<0.001*
p Values *Permethrin* & *Onion*	<0.001*
p Values *Permethrin* + *Onion* & *Onion*	0.031

*Statistical significance is p value < 0.001. Mann–Whitney U-test was used to detect differences between groups.

antioxidants that have the ability to improve mammalian spermatogenesis. Although all antioxidants have a common goal to deal with free radicals and ROS, but it is worth noting that each of them in a certain way gain these properties. Many clinical examinations have investigated the potential of antioxidant compounds that give remedy to oxidative stress-induced male infertility (Ross et al., 2010). The use of *onion* juice, as antioxidants demonstrated, can be used to reduce the effects of *Permethrin* in male fertility. Quercetin of onion can disable the free radical effects, which refer to antioxidant property of it (Khaki et al., 2010). The studies on *Allium cepa* showed that it is perfect among the antioxidants like selenium and E, A, C and glutathione. Also this finding confirmed that C, E and B vitamins are beneficial in reducing the toxicant effects on testes tissue. The administration of *onion* affects recovery prostate cancer by reducing oxidative stress (Galeone et al., 2006). Kikelomo et al. (2008) have shown that *onion* juice reduces the toxic effects of cadmium on the testis and spermatogenesis. As well as this antioxidant through declined negative effects of environmental pollutions improves the testis structure and enhances the concentration of spermatozoa in epididymal ducts. *Onion* juice is effective on the sexual hormones in rats after inducing an antiepileptic drug and the results show that consuming *onion* is useful in increasing testosterone hormone and fertility (Khaki et al., 2012). In this study, *onion* juice shows a significant potential to increase the number, percentage of viability and motility of sperm. On the other hand, through unknown pathway, reduced damage to the Leydig cells was an improvement in testosterone synthesis. According to the results of this study, it is recommended that to reduce the adverse effects of *Permethrin*, *onion* can be used as an antioxidant supplement to improve male fertility. And certainly, reduced use of pesticides in agricultural industry and in homes will provide the way to solving these problems.

Conclusions

According to our results, *Permethrin* has a negative impact on testis parameters in adult rats. *Onion* juice due to antioxidant properties reduced these effects. So we can say that to eliminate potential hazards of insecticides on male fertility, it is better to consume these chemical compounds that has been reduced, along with the natural antioxidants.

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