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Antioxidant Properties of Allium cepa (Onion) Against Permethrin-Induced Toxicity on *LHCGR* and *SF1* Genes in Male Rats

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Abstract

Objectives: The use of pyrethroids (PYRs) due to availability and less toxicity to mammals is wide spread. Permethrin (PER) is type I pyrethroid which is usually used in agriculture industry and homes. High dose of these chemicals has destructive effects on biological systems mainly in spermatogenesis cycle. The aim of this study was to investigate the effects of PER on key genes of SF1 (steroidogenic factor 1) and LHCGR (luteinizing hormone/choriogonadotropin receptor) as well as hormonal levels (Luteinizing hormone [LH] and follicle-stimulating hormone [FSH]) of testis. A natural antioxidant (onion) was used to investigate its positive impact on the negative effects of PER.

Materials and Methods: Adult male rats were divided into 5 groups. Group 1 was considered as the control group. Groups 2 and 3 received DMSO (dimethyl sulfoxide) and PER per day (35 mg/kg), respectively. Group 4 received 3 mL of onion juice in addition to PER (with the same dose). Group 5 only received 3 mL of onion juice. After 2 months, the gene expression levels were examined using real-time reverse transcriptase polymerase chain reaction (RT-PCR). Testes were processed for sexual hormones level.

Results: The results showed that exposure to PER demonstrated a significant reduction in expression of genes and hormonal levels. Onion juice had a moderate function in the expression of genes and prevented a decrease in serum levels of FSH and LH compared to normal condition.

Conclusions: This hypothesis can firmly be stated that toxicity effects of PER can reduce male fertility. Therefore, consumption of natural antioxidants like onion can lead to a decrease in these damages.

Keywords: *Allium cepa*, PER, male rat, LHCGR, SF1

Introduction

Pyrethroids (PYRs) are a group of synthetic chemicals which are a more stable form of pyrethrins (natural insecticides derived from the chrysanthemum plant). The PYRs are considerably less toxic to mammals than other classes of insecticides like organophosphates and organochlorines. The PER is a broad-spectrum PYR type I insecticide (1) which has a very low solubility in water and binds tightly to soil and sediment particles. It is used in manufacturing household insect, agriculture, and livestock products and also mosquito decrease products. The PER exists in Cis and Trans features (2). Toxicity relies on the relation of cis-trans isomers in the formulation. It may be easily absorbed from the gastrointestinal and skin tract and through inhalation of dust and spray mist. Metabolism of PER like other PYRs occurs mainly in the liver where the molecule undergoes oxidation by the cytochrome p450 and also hydrolysis, into metabolites 3-PBA that is the major metabolite of pyrethroid including cypermethrin and PER (3). The great quantity of PER is collected in fat and the brain (4). This can be demonstrated by the lipophilic nature of the PER molecule. In this study, the researchers intended to measure the effects of PER on testicular tissue. One of the main cells that control the function of the testes in a mixed ways includes the Leydig cells. The adult Leydig cells are dependent on LH hormone for proper actions. The *LHCGR* gene is responsible for regulating the expression of LH receptors that are stimulated by LH hormone (5,6). Mutations in this gene cause inactivity LH receptors and finally result in the synthesis of testosterone. The SF-1 (also known as Ad4BP or NR5A1) is an essential orphan nuclear receptor for adrenal gland and gonadal systems development that is expressed in the adrenal cortex and in the Sertoli and Leydig cells of the testis (7). Two of these targets encode proteins required for testosterone production: StAR protein which regulates cholesterol uptake into the mitochondria and cholesterol side chain cleavage enzyme

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which catalyzes the first cleavage reaction in steroid biosynthesis. The selection of these two genes is based on the hypothesis of PER effects on their expression. In addition, in this study, the hormonal changes of testes were explored after treatment by PER. Since many studies on recent research have been conducted regarding the effects of the pollutants, *Allium cepa* (natural antioxidants) was used to deal with PER. *A. cepa* (onion) is a generic food plant in the treatment and inhibition of some diseases. The flavonoids observed in onion are quercetin aglycone, glucosides, and in some species include isorhamnetin monoglycoside or kaempferol monoglycoside. Onion was selected due to its availability and improving effects on men fertility.

Objectives

While the complete avoidance of PER effects is not possible in everyday life, consumption of natural antioxidants can play an important role in counteracting the insecticides.

Materials and Methods

Experimental Animals

Thirty adult male Wistar rats with an approximate weigh of 250 ± 10 g, were randomly selected and maintained in the Breading Animal House of the Faculty of Medicine in Tabriz University. The rats were housed in plastic cages in a controlled temperature (23-1°C) room, (humidity, 55%-5%) and free from any sources of chemical contamination.

Experimental Protocol

The PER and DMSO were purchased from Sigma –Aldrich Company. Onions were bought from urban supermarkets of Tabriz as well. The rats were divided into 5 experimental groups. Group 1 (control group) received normal food and water. Group 2 (DMSO group) received 0.5 mL of DMSO by gavages. The DMSO was used as a vehicle to dissolve PER. Group 3 (PER group) received 35 mg/kg b.w./d in 0.5 ml DMSO. The dose of PER corresponded to 1/40 of the LD50 value. (8). Group 4 (PER and onion) received 3 mL of onion juice by gavages in addition to PER (with the same dose). Group 5 (onion) was treated with 3 mL of onion juice by gavages. On day 60, according to animal moral law, the rats were anesthetized with ketamine and then were killed by cervical dislocation.

Real-Time Polymerase Chain Reaction Examination

The right testicle was removed from the rat for evaluation expression rate of *SF1* and the left testicular for *LHCGR* expression level. The testis was put inside liquid nitrogen immediately after the removal and then transferred into -196°C until the RL-PCR examination. To isolate total RNA from peripheral proteins and cDNA extracted from Leydig cells these steps were followed: 1 mL RNXTM-PLUS (Cinnagen Company) for every 10^6 cells was added to micro tube and then was incubated for 5 minutes at room temperature. Then, 200 µL chloroform was added to each micro tube and was shaken vigorously by hand for 15 sec. It was then incubated at 4°C for 5 minutes. Next, it was centrifuged for 15 minutes at 4°C and 15000 g. Carefully and without shaking the tube, the supernatant phase containing RNA was isolated and transferred to another tube and equal volume of cold isopropanol was added to isolated solution and incubated for 15 minutes at 4°C. Afterwards, the micro tube was centrifuged for 15 minutes at 4°C and 14000 rpm, the supernatant extrapolated, and 1 mL ethanol of 75% was added to the micro tube. Micro tube was immediately centrifuged for 15 minutes at 4°C and 14000 rpm, then, supernatant was removed and ethanol was carefully emptied and the alcohol was allowed to evaporate for 20 minutes. The 20 µL DEPC-treated eater was added to each micro tube and the process was continued and maintained at-70°C.

The cDNA synthesis: to synthesize cDNA, Revert AID TM Firs Standard cDNA synthesis (Fermentas) kit was used according to manufactures instructions.

Primers: Primer Designing Tools, Primer 3 Plus

SF1 Gene Primers

Forward Primer: GTTTCTGCGCACCCACAGTC; plus
Reverse Primer: GTGGTAGCCGGACACCTTGT; minus

LHCGR Gene Primers

Forward Primer: CACAGGGCCGAAAACCTTTTATTC; plus
Reverse Primer: AGCATCTGGTTCAGGAGCACA; minus

Quantification of beta-actin gene expression using SYBR-green Real Time RT-PCR: For this purpose, SYBR® Premix Ex Taq kit (Takara Company of Japan) was applied. The 1/2, 1/1, and 1/3 concentrations of cDNA synthesis were prepared.

Quantification of target gene expression using SYBR-green Real Time RT-PCR: amplification reaction and analysis of data were performed by applied Bio system and Corbett. The SPSS (Statistical Package for the Social Sciences) software, version 17.0 was run for statistical analysis.

The following formula was used for data analysis:

ΔC_T= C_T target (SF1 or LHCGR) – C_T reference (Beta-actin)

$\Delta\Delta C_T = \Delta C_T$ test sample – ΔC_T control sample

Serum FSH and LH levels: To evaluate sexual hormones, kits from Immunotech Bekman Company were used. The kits assay sensitivity was 0.025 ng/mL with double-antibody immunoassay.

Results

The aim of this study was to investigate the gene expression of *SF1* and *LHCGR* of rat's testis after exposure to PER. Then, it was also sought to see if onion juice (as an antioxidant) would have any impact on changing the effects of PER. Two genes of *SF1* and *LHCGR* which

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are required for testosterone synthesis in Leydig cells were considered. As can be seen in Table 1, *LHCGR* gene expression in group 3 that was affected by PER compared to control and other groups, significantly down-regulated. This suggests that before the PER could directly affect LH receptors, it had negative genotoxicity impress on regulating gene of receptors. In addition, it can be observed that onion juice had remarkably modified the effects of PER on gene expression. The DMSO had no effects on the expression of this gene. One of the SF1 roles in Leydig cells is to regulate the StAR protein on the mitochondria; therefore, determining the expression of this gene has 2 important results: first, the genotoxicity of PER is determined. Second, the decrease or increase of the presence of steroidogenic acute regulatory protein (StAR) protein is identified as well. Naturally, lack or decline of this protein on the surface of mitochondria causes a reduction in entry of cholesterol for testosterone synthesis. As can be observed in Table 2, PER has reduced SF1 expression rate. Therefore, this is a strong possibility that PER with reduced expression of SF1 gene can affect StAR protein synthesis and finally reduce cholesterol concentration in the Leydig cells. Here, it can be seen that onion juice has modified the effect of PER on this gene, however, compared to other groups it shows a significant difference. In this study, it was found that PER even had a negative impact on sexual hormones (Table 3). Serum levels of FSH and LH under its effects were reduced ($P<0.001$), but onion juice had moderated this decline. Reducing the serum level of FSH hormone was the main reason for the destruction effect of PER on initiation of spermatogenesis. Besides, reducing the serum level of LH showed the preventing function of PER in stimulating LH receptors on the Leydig cell surface. As can be seen in the results obtained for group 5, onion juice had a significantly recovering effect on gene expression and serum levels of hormones (Table 3).

Discussion

Most evidence illustrated that many insecticides had become a potential fulmination to fertility and progression of humans and animals (9-11). Some reports showed that PER binds to androgen receptors in cells from human males and to the peripheral benzodiazepine receptor (PBR) which stimulates production of testosterone (12).Moreover, PER had significant estrogenic potency and inhibited from binding of estradiol to the estrogen receptor (13). However, estrogen is important for normal male fertility but increasing the estrogen induces Leydig cell hyperplasia in animal models and is associated with testicular cancer, cryptorchidism, and defaulted spermatogenesis (14). In the Leydig cell, after stimulation by LH, cholesterol was moved to the inner mitochondrial membrane by the StAR protein. This means that, it may be chemicals pollutants such as PER that can inhibit testosterone synthesis at the initial step. The *LHCGR* and

Table 1. Relative Expression Report for *LHCGR* Gene

Gene	Reaction Efficiency	Expression	Standard Error	95% CI	*P* (H1)
LHCGR.1	0.98	4.080	3.332-4.861	2.963-6.029	0.000
LHCGR.2	0.98	3.687	2.983-4.587	2.786-4.912	0.000
LHCGR.3	0.98	1.739*	1.000-2.481	.934-2.844	0.020
LHCGR.4	0.98	2.392*	1.849-2.983	1.727-3.737	0.000
LHCGR5	0.98	4.082	3.311-4.861	2.967-6.032	0.000

The values are expressed as means ± SD of n = 6 rats.
*Statistical results showed a significant difference between all groups. (*P* value = 0.000). LHCGR 1: control group; LHCGR 2: DMSO group; LHCGR 3: PER group; LHCGR 4: PER -onion group; LHCGR 5: onion group
The relative gene expression of each rat was calculated according to the following formula:
$\Delta C_T = C_T$ target (SF1 or LHCGR) – C_T reference (beta-actin)
$\Delta\Delta C_T = \Delta C_T$ test sample – ΔC_T control sample

Table 2. Relative Expression Report for *SFI* Gene

Gene	Reaction Efficiency	Expression	Standard Error	95% CI	*P* (H1)
SFI.1	0.96	11.492	8.614-15.205	7.468-19.485	0.002
SFI.2	0.96	11.237	8.370-15.205	7.468-18.385	0.001
SFI.3	0.96	3.837*	2.852-5.028	2.345-6.639	0.002
SFI.4	0.96	5.085*	3.733-7.530	2.063-9.941	0.002
SFI.5	0.96	11.496	8.622-5.211	7.471-9.487	0.002

* Statistical significance (*P* value <.01). SF1. 1: control group; SF1. 2: DMSO group; SF1. 3: PER group; SF1. 4: PER and onion group; SF1. 5: onion group
The relative gene expression of each rat was calculated according to the following formula:
$\Delta C_T = C_T$ target (SF1 or LHCGR) – C_T reference (Beta-actin)
$\Delta\Delta C_T = \Delta C_T$ test sample – ΔC_T control sample

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Table 3. Serum Level Difference of FSH and LH Among Groups

Group	FSH	LH
P value control & DMSO	0.091	0.003
P value control & permethrin	<0.001	<0.001
P value control & permethrin + onion	<0.001	<0.001
P value control & onion	0.204	0.082
P value DMSO & permethrin	<0.001	<0.001
P value DMSO & permethrin + onion	<0.001	<0.001
P value DMSO & onion	0.020	0.110
P value permethrin & permethrin +onion	0.077	0.006
P value permethrin & onion	<0.001	<0.001
P value permethrin + onion & onion	<0.001	<0.001

Mann-Whitney U test.

SF1 genes encoded LH receptors for LH stimulation and StAR proteins for mobilization of cholesterol, respectively. In this study, it was observed that PER decreased expression levels of these genes through genetic pathway. It is clear that without Sf1, differentiation of Leydig cell does not happen (15). In gonad-specific Sf1 knockout mice, expression of 2 main markers of fetal Leydig cell including Scc (cytochromeP450 side chain cleavage) and StAR was reduced (16). In human mesenchymal stem cells (hMSCs), constant expression of SF-1 and cAMP remedy could induce the expression of the StAR protein (17). Furthermore, SF1 omission had been shown to excite apoptosis in the primary gonads. It was found that PER acted as a genotoxic for Leydig cells. The level expression of *SF1* in group 3 and 4 strongly reduced as compared to other groups. As a result, our first guess about the effects of PER on the synthesis of StAR protein was closer to certainty. It can probably be stated that PER inhibited the testosterone synthesis through preventing the entry of cholesterol into the mitochondria. As a result, it was found that PER reduced testosterone synthesis in the experimental groups. In a study on primary amenorrhea in 46, XY female adolescents with low testosterone concentration showed that the reduction of testosterone reflected on the expression levels of *LHCGR* gene (18). Another report revealed that the *LHCGR* expression level was also considerably down-regulated in rats after being exposed to stress for 3–6 hours (19). In this lecture the results of which were obtained by determining the gene expression of *LHCGR*, a dramatic reduction can be observed after exposing to PER in the experimental groups. This down-regulation of *LHCGR* may explain the remarkable reduction of testosterone permeation from Leydig cells after LH stimulation. The germ cell's maturation in the spermatogenesis cycle depends on these hormones, thus, by interrupting hormone production or mutating hormone receptor expression this process is probably disrupted (20). According to previous studies, PER induced a significant decrease in the levels of plasma LH, FSH, and testosterone (21). The findings of the present study are consistent with the results of the above-mentioned studies. It is possible that formerly PER reduced the expression of genes, affecting FSH and LH secretion from hypothalamus. However, one of the most likely mechanisms affecting testosterone is the mitochondrial membrane impairment in the Leydig cells (22).

Antioxidants' effects of onion on spermatogenesis show that it can be used to reduce the environmental pollution (23,24).

Conclusions

As was observed in the result section, onion increasingly confronted with the negative effects of PER in both gene expression and hormonal changes. This shows that herbal antioxidants such as onions improves spermatogenesis cycle. Expanding the use of pesticides in agriculture and heavy reliance on them is the most important cause of infertility at different ages. Perhaps, epidemiological investigations and notifications in this regard is a suitable solution at the current situation.

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Ethical Issues

All animals received humane care in compliance with the guidelines of the medical research ethics committee of Tabriz University.

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