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INVESTİGATİON OF THE EFFECT OF ROYAL JELLY ON AMOUNT OF NİTRİC OXİDE İN OVARİECTOMİZED RATS

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ABSTRACT

Aim: Epilepsy is a chronic and neurological disorder that leads to abnormal and unconscious activities in person. epilepsy can have some Risk factors such as hypoglycemia and hypoxia, head injury, CNS infection of brain tumors, oxidative stress, inflammation of the brain and increase in nitric oxide. Menopause is one of the physiological conditions that, producing cytokines such as IL-1 β, can cause to increase the brain inflammation and be the ground of neurological diseases and seizure. In recent years, the drugs reducing the inflammation of the brain have attracted attention for treating epilepsy and seizure. Royal Jelly is a slimy substance produced by worker bees. This contains a different material that has abundant biological activities such as an increase in neuronal differentiation and neurogenesis. Its most important attribute of interest is the antioxidant and anti-inflammatory activity of this substance against nitric oxide, active oxygen species and oxidative stress. The aim of this study was to investigate the effect of Royal Jelly on the nitric oxide caused by epilepsy induced by pentylenetetrazol (PTZ) in ovariectomized rats.
Material and Method: 80 rats were divided into experiment and control groups and fallen under treatment with royal jelly. 24 hours after the last injection of Royal Jelly, PTZ was injected by 80 mg/kg in intraperitoneal manner in order to make seizure and the behavior of rats was studied and evaluated for two hours and then the rats' brain was dissected and sent out of the skull. Histological cuts were done by using the microtome in CA1 region of Hippocampus and the cresyl violet coloration was used for showing the nerve cells. Measurement of nitric oxide was done by the Griess reaction with the microplate method. After the reaction and formation of color the optical absorption resulting from forming colorful matter in wave length of 540 nm was read by reader device Elisa.
Results: There was not observed any significant difference in the amount of measured nitric oxide at any of the different groups under study. While there was observed a significant difference in total amount of antioxidants and the number of neuronal cells and in all the groups under study after treatment with Royal Jelly.
Discussion: The results of this study showed that treatment with Royal Jelly has been not effective in reducing the amount of nitric oxide in the epilepsy induced by pentylenetetrazil in ovariectomized rats. This is while treatment with Royal Jelly increased the number of neuronal cells and the amount of antioxidants in rats with seizure. So it can be claimed that the Royal Jelly can be applied for reducing epilepsy effects.

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Introduction

Epilepsy is a chronic neurological disorder that is specified by a seizure in succession. In these circumstances the non-normal evacuation of electrical activities happens in the brain cells; this leads to abnormal behavior and reactions such as the involuntary movements of muscles, non-normal and unusual perception and the chaotic and irregular levels of awareness. Epilepsy in some people is created because of the brain damage, brain cancer, drug and alcohol abuse and other reasons. The

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epileptic attacks are resulted from too unusual cellular activity of cortical nerve in brain [1-3]. The most common type of attacks (60% of cases) is seizure. Two-thirds of them appear as epileptic topical attack; they turn later into the general attack, while a third appears to be a general attack. The remaining 40% are non-seizure attacks. An example of this type of absent attack that happens as a low level on consciousness and usually lasts 10 seconds long has some other physical and mental consequences including premature death, trauma and mental disorders [4, 5]. People who suffer from seizure can be classified based on the type of seizure, its agent, epilepsy syndrome and events during occurring the epilepsy. The type of seizure can be classified as the focal or generalized epilepsy [6]. The generalized seizures are classified on the basis of bodily effects; however, this disorder is the most common neurological disorder and close to 5% - 10% (approximately 60 million people) of the population of the world have experienced this disorder [7-9]. Life expectancy in these patients has been reported to be 2% - 10% less than normal people and death rate two to three times more [8, 10]. Some reasons and risk factors of epilepsy can be some perinatal problems such as hypoglycemia and hypoxia, head damage, CNS infection like genetic factors, neurocysticercosis, malnutrition, cerebral tumors [2, 4], oxidative stress, increase in nitric oxide and brain inflammation caused by increasing cytokines such as IL-1 Alpha and beta, IL-6 and the tumoral necrosis factor. Brain inflammation can be the beginning of pre-apoptotic signaling pathway and the death of neurons. So several studies have proven that brain inflammation plays an important role in the pathogenesis of epilepsy [11-13]. Menopause is one of the physiological conditions that with the production of cytokines like IL-1 β can cause brain inflammation and be the ground for neuromuscular disease and seizure. In recent years many studies have been conducted to find the connection between menopause and epilepsy [14-16]. On the one hand some changes in the amount of estrogen in menopausal time have directly a role in the aggravation of the symptoms of seizure and epilepsy. Reduction of estrogen operates as an increasing factor of seizure and brain disorder through the effects of GABA receptor. On the other hand, the estrogen operates as a regulator of nitric oxide and through reducing this hormone the amount of nitric oxide changes [17-22]. Based on some studies the changes in the amount of nitric oxide (NO) have an important and direct impact on the pathogenesis and progression of epilepsy and some diseases of the brain [23-27]. The treatment of epilepsy is at most based on the three mechanisms which involve blocking the sodium and calcium channels, upgrading and enhancing Neurotransmitter GABA and subsidiary antagonisms of glutamate receptors. But in recent years with proving the relationship of brain inflammation with creation and exacerbation of epilepsy, using the drugs reducing brain inflammation for the treatment of epilepsy and seizure has attracted the attention [11, 28]. Royal Jelly is a slimy substance produced by worker bees. This substance contains different ingredients such as proteins, carbohydrates, lipids, mineral elements and vitamins and even acetylcholine. Investigating the conducted studies, it is known that Royal Jelly has abundant biological activities such as an increase in neuronal differentiation and neurogenesis. In Iran, a study in 2014 conducted at the University of Mazandaran has shown that Royal Jelly strengthens the immune system and reduces the growth and spread of cancer cells; this can be taken into consideration as a supplement combination in the treatment of leukemia, as well as for offsetting the side effects caused by chemotherapy along with a strengthening of the immune system [29]. The most important attribute of interest of this substance in the present study is its anti-inflammatory and anti-oxidant activity against active oxygen species, nitric oxide and oxidative stress; it can be useful in reducing the cerebral effects resulting from epilepsy [30-33]. The aim of this study was to investigate the effect of Royal Jelly on the nitric oxide caused by epilepsy induced by pentylenetetrazol (PTZ) in ovariectomized rats.

Materials and methods

80 laboratory Wistar rats with a weight range of 220-250 grams were kept for a week before the start of testing in the laboratory and in animal house under the temperature of 20 ± 2 in conditions of 12:12 of lighting-dark; thus they were adapted with the weather and environmental conditions. Then the mice under study were divided into control and test groups [34]. After a week, the female mice were fallen under anesthesia with ketamine-xylazine, the ovariectomy operation and their both ovaries were removed. The control groups included: group of sham 1: cared patients with normal saline who were operated without removing ovaries; then they got the normal saline. Group of Sham 2: group of cared patients with PTZ who were operated without removing ovaries; they were exposed to seizure after surgery. Group of sham 3: cared patients treated with Royal Jelly before induction of seizure by PTZ who were undergone surgery without removing ovaries and then for fifteen days before the induction of seizure by PTZ received every day once the Royal Jelly by (mg/kg300) in the intraperitoneal manner. Group of sham 4: cared group treated with royal jelly who were exposed to surgery without removing ovaries; they received for fifteen days, every day once the Royal Jelly by (mg/kg300) in the intraperitoneal manner [35]. Group of ovariectomy 1: a group who received normal saline after ovariectomy. Group of ovariectomy 2: cared patients with PTZ who suffered a seizure after ovariectomy with PTZ. Group of ovariectomy 3: a cared group with royal jelly who received after ovariectomy for fifteen days every day a time the Royal Jelly by (mg/kg300) in the intraperitoneal manner. Group of ovariectomy 4: cared group treated with Royal Jelly before induction of seizure with PTZ; they received after ovariectomy for fifteen days before the induction of seizure with PTZ every day once the Royal Jelly in the intraperitoneal manner.

24 hours after the last injection of Royal Jelly, PTZ by 80 mg/kg was injected in intraperitoneal manner in order to make the seizure and the behavior of rats was studied and evaluated for two hours [36]. The animals were under anesthesia by intraperitoneal injection of the mixture ketamine and xylazine; by cardiac injection of saline phosphate buffer solution and then Paraformaldehyde four percent perfusion were done and the brain was removed out of the skull. Tissue fixation and processing steps were made by tissue-processing device. Histological cuts were done using the microtome in CA1 region of

hippocampus and the cresyl violet coloration was used for showing nerve cells with azure blue or purple color [37]. Measurement of nitric oxide was done by Griess reaction by the microplate method. To measure the concentration of nitrite and nitrate in an Elisa microplate, at the beginning 100 microliters were wiped clean of protein and then 100 microliters of Vanadium chloride solution were added. Then 100 microliters of mixture of Sulfonamide and NEDD in proportion of 1 to 1 were increased and incubated in 37 degrees. After the reaction and formation of color the optical absorption resulting from forming colorful matter in wave length of 540 nm was read by reader device Elisa [38].

Statistical analysis

The analysis of the data of the present study was conducted in two parts of descriptive and inferential statistics. In the descriptive statistics the central tendency and dispersion criteria were reported along with the table and the diagram. In terms of inferential statistics, the data was investigated by using the Kolmogorov-Smirnov test. With regard to normality of distribution of data ($P<0.05$) the independent sample t-test was used to compare the two groups and the variance analysis to compare more than two groups. For binary comparisons the Tukey Post Hoc test was used. For analyzing data, the SPSS software Version 18.0 (Inc., Chicago, IL, USA) was used. The significance level in this study was considered 05.0.

Results

Investigating the changes of serum nitric oxide level in the groups under study:

The results showed there was not any significant statistical difference in the amount of nitric oxide between the groups of sham ($P = 0.202$, $F (3 \& 24) = 1.659$) (table 3-1). There was no significant difference in amount of nitric oxide between the ovarectomy group ($P = 0.251$, $F (3 \& 24) = 1.458$) (table 3-1). There was no significant statistical difference in amount of nitric oxide between the groups of ovarectomy 1 and sham 1 (T-value (12) = 1.659, P = 0.787) (table 3-1).

Table 3-1: comparison of nitric oxide between groups under study

P-value†	4	3	2	1	group
0/202	4/84±2/28	10/4±04/23	12/83±1/3	9/09±1/29	sham
0/251	3/89±1/27	8/6±1/18	10/71±3/56	10/04±3/21	ovariectomy
				0/787	P-value‡

Any Mean ± SE in 7 units of laboratory rats is female. Data was compared with a one-way variance analysis tests † and ‡ t-independent.

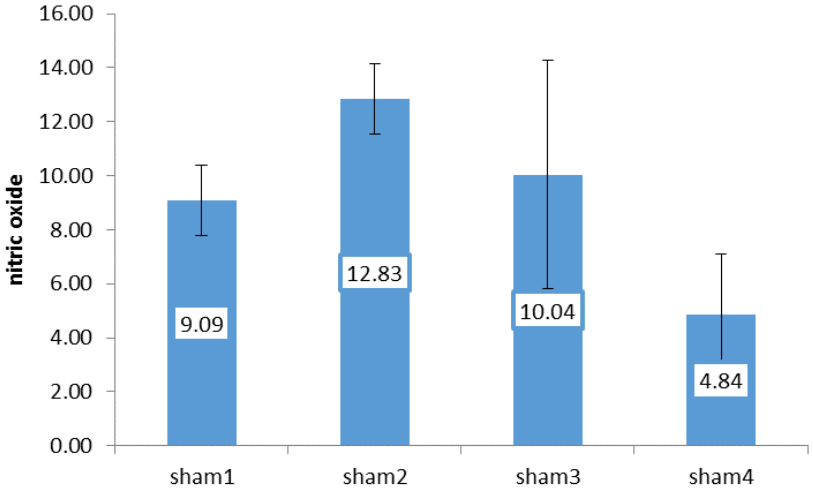

Diagram 3-1: average nitric oxide in terms of different sham groups

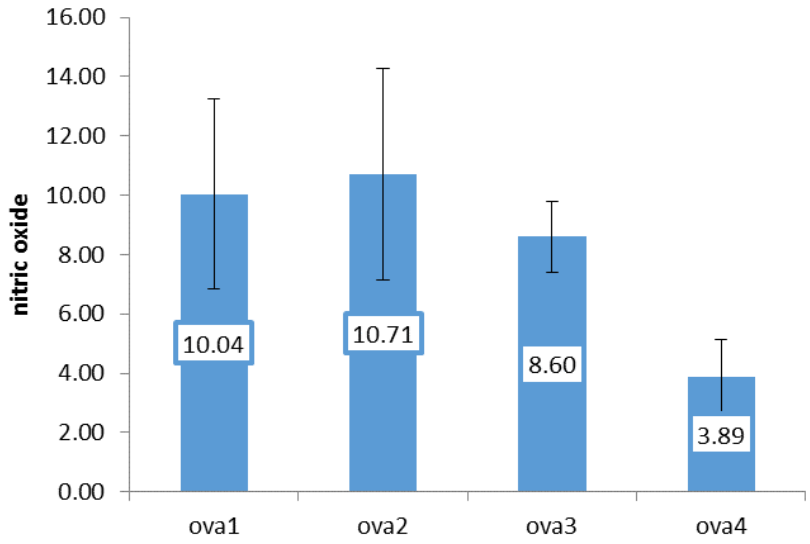

Diagram 3-1: average nitric oxide in terms of different ovariectomy groups

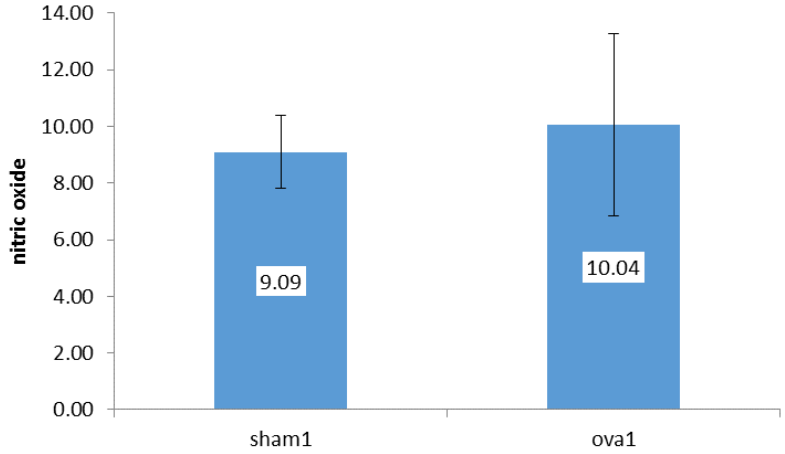

Diagram 3-1: average nitric oxide in terms of groups of sham 1 and ovariectomy 1

Investigating the total antioxidant changes in the groups under study:

According to the results there was a significant statistical difference in the total amount of antioxidants between the groups of sham (P <0.001, F (3 & 24) = 30.794), so that the total amount of antioxidants in the Group of sham 2 was less than other groups of sham (table 3-2). According to the results there was a significant statistical difference in the total amount of antioxidants between the groups of ovarectomy (P <0.001, F (3 & 24) = 63.11), so that the total amount of antioxidants in the Group of ovarectomy 2 was less than other groups of ovarectomy (table 3-2). According to the results there was a significant statistical difference in the total amount of antioxidants between the groups of sham 1 and ovarectomy 1 (P <0.004, T-value 12 =3.5), so that the average total antioxidants in the Group of ovarectomy 1 was less than group of sham 1 (table 3-2).

Table 3-2: comparison of total amount of antioxidant between groups under study

P-value†	4	3	2	1	group
<0/001	1/44^{b}±0/12	1/38^{b}±0/05	1/11^{a}±0/02	1/98^{c}±0/02	sham
<0/001	1/33^{b}±0/02	1/41^{b}±0/05	1/16^{a}±0/05	1/86^{c}±0/02	ovarectomy
				0/004	P-value‡

Every Mean ± SE in 7 units of laboratory rats is female. Data was compared with a one-way variance analysis † and ‡ t-independent. Different superscript letters indicate a significant statistical difference (P< 0.005).

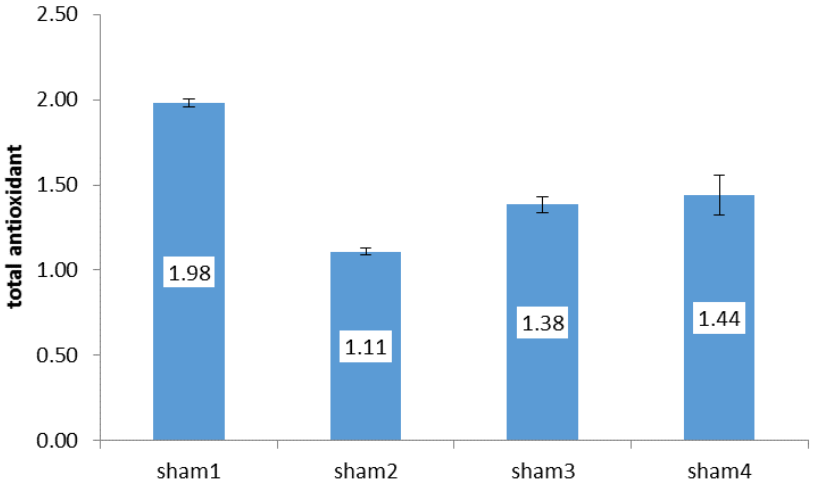

Diagram 3-4: average total antioxidant in terms of different groups of sham

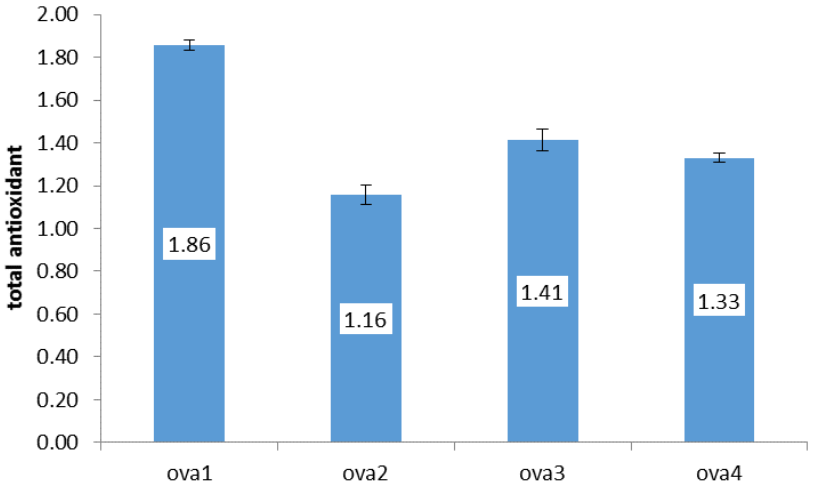

Diagram 3-5: average total antioxidant in terms of different groups of ovarectomy

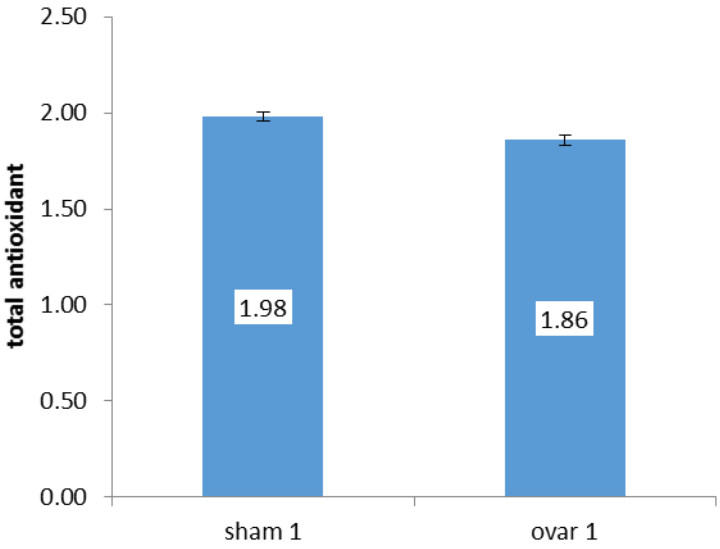

Diagram 3-6: average total antioxidant in groups of sham 1 and ovarectomy 1

Investigating the changes of number of neurons in the groups under study:
According to the results there was a significant statistical difference in the number of neurons between the groups of sham (P <0.001, F (3 & 24) = 100.677), so that the number of neurons in the Group of sham 2 and sham 3 was less than the groups of sham 1 and sham 4 (table 3-3). According to the results there was a significant statistical difference in the number of neurons between the groups of ovarectomy (P <0.001, F (3 & 24) = 32.716), so that the number of neurons in the Group of ovarectomy 2 was less than other groups of ovarectomy (table 3-3). According to the results there was a significant statistical difference in the number of neurons between the groups of sham 1 and ovarectomy 1 (T-value (12) = 2.251, P = 0.044), so that the average number of neurons in the Group of ovarectomy 1 was less than group of sham 1 (table 3-3).

Table 3-3: comparison of number of neurons between groups under study

P-value†	4	3	2	1	group
<0/001	$89/14^{b}\pm1/12$	$55/29^{a}\pm2$	$48/14^{a}\pm2/33$	$88/71^{b}\pm2/83$	sham
<0/001	$70/29^{b}\pm2/48$	$84/71^{c}\pm1/97$	$53^{a}\pm2$	$79/29^{bc}\pm3/09$	ovarectomy
-	-	-	-	0/044	P-value‡
Every Mean ± SE in 7 units of laboratory rats is female. Data was compared with a one-way variance analysis † and ‡ t-independent. Different superscript letters indicate a significant statistical difference (P< 0.005).					

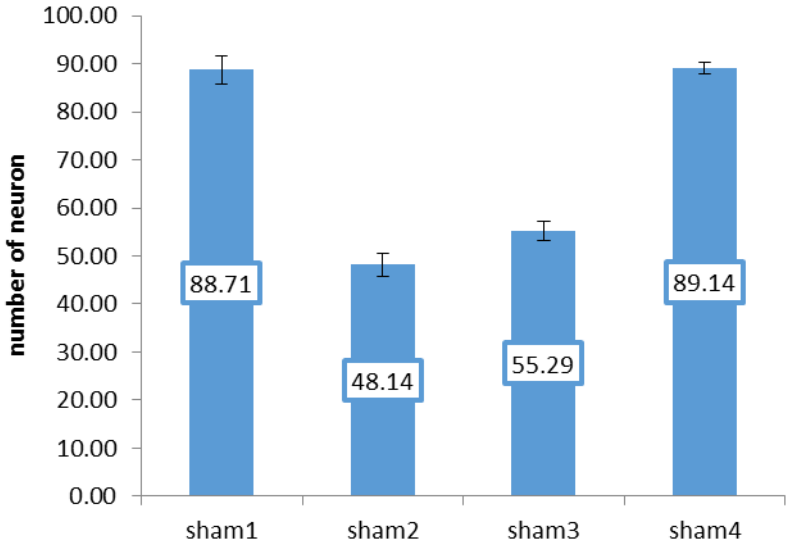

Diagram 3-7: average number of neurons in terms of groups of sham

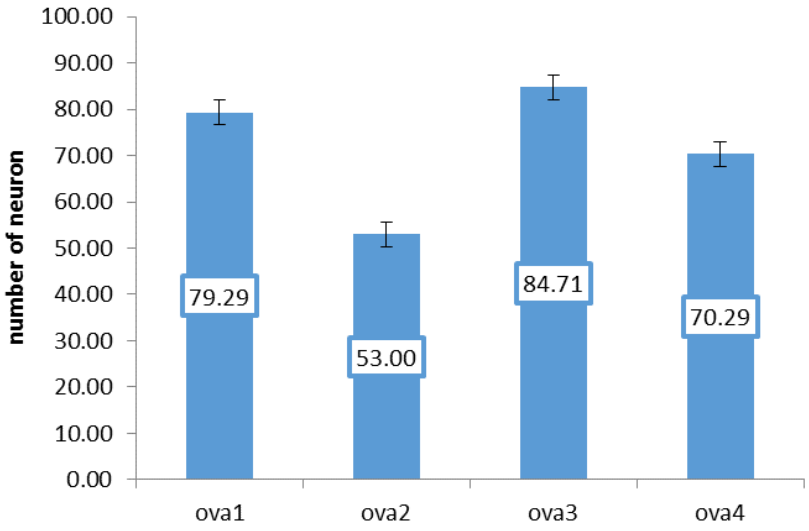

Diagram 3-8: average number of neurons in terms of groups of ovarectomy

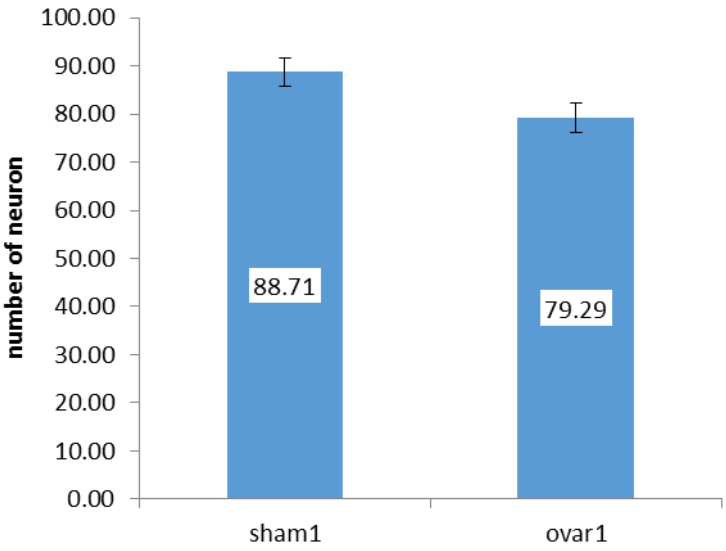

Diagram 3-9: average number of neurons in groups of sham 1 and ovarectomy 1

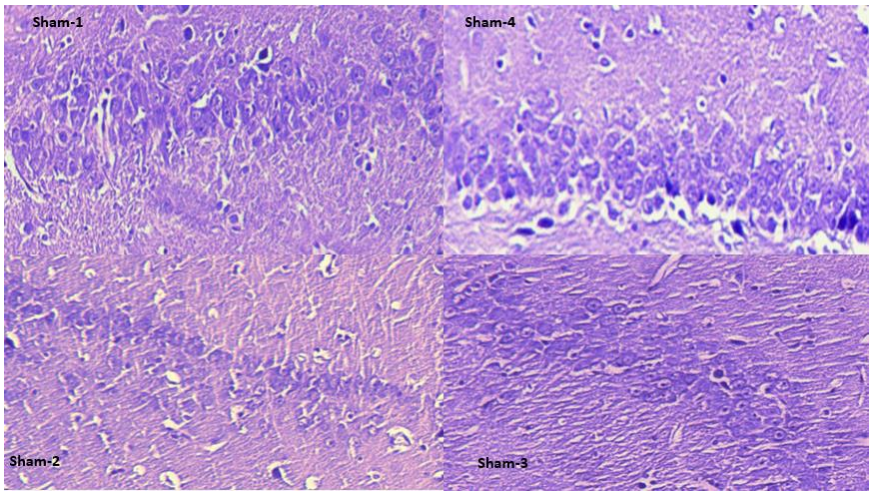

Photo 1: Histological cuts from CA1 region of hippocampus with the cresyl violet coloration for showing nerve cells (group of sham)

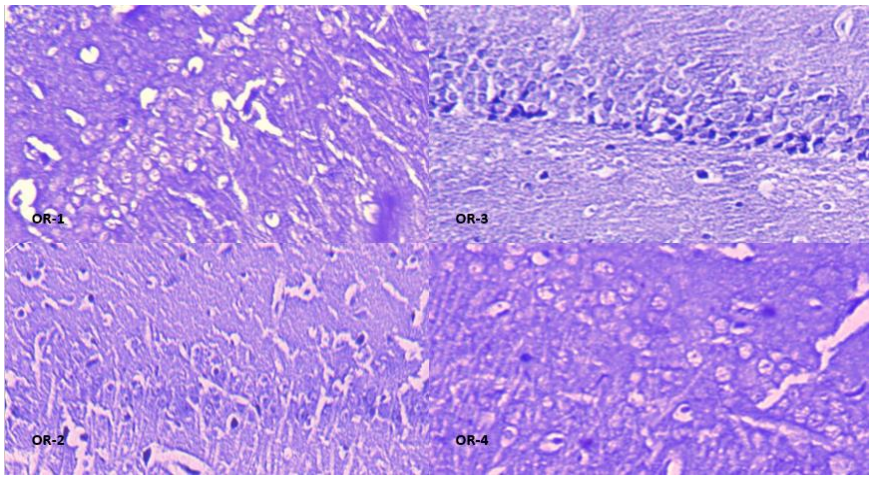

Photo 1: Histological cuts from CA1 region of hippocampus with the cresyl violet coloration for showing nerve cells (group of ovarectomy)

Discussion

The results of the study showed that the administration of Royal Jelly had no impact on the amount of nitric oxide; there was not observed any significant difference in the groups under study. Malekinezhad et al investigated in 2014 the effect of Royal Jelly on the amount of nitric oxide in the tissue of the heart. In this study, Royal Jelly has caused to reduce the amount of nitric oxide and this result indicates the protection of this substance from heart tissue against the destructive effects of nitric oxide in inflammatory and autoimmune diseases [39]. But in our study the royal jelly has not had any impact on the amount and inhibition of nitric oxide and hence on reduction of the effects of nitric oxide in increasing inflammation in the epilepsy. In a study Moradi et al examined the effect of Royal Jelly on the amount of nitric oxide and total antioxidants in improving various parameters in sperm production. In this study, despite our result of our study, the Royal Jelly caused to reduce the amount of nitric oxide. In this study, it was also reported that Royal Jelly has antioxidant activity and reduces the amount of inflammation in the lower dose [40]. Delkhoshe-Kasmaie et al obtained also the same results in studying the effect of Royal Jelly on the inhibition of the injuries caused by the inflammation and nitric oxide on testicular tissue and confirmed the antioxidant activity of Royal Jelly [41]. Perhaps the difference with other studies was because of the amount of the concentration of royal jelly. Because in the study of Malekinezhad there has been not observed any impact in the low dose of Royal Jelly on the amount of nitric oxide [39]. In a study Gasic et al have known the reason for the reduction or increase of influence of Royal Jelly a change in method of extracting this substance. In this study two types of extract of Royal Jelly have been used: the water extract and the dichloromethane one. The result was that the water extract has had a greater impact on reducing inflammation [42].

In another part of this study we investigated the effect of Royal Jelly on the total antioxidant of groups under study. In most studies the royal jelly has been introduced as an antioxidant substance [41, 43]. By inhibiting the formation of active oxygen species and inhibition of cytochrome C release from mitochondria this substance reduces inflammation; it has an antioxidant activity [44]. In the study of Zamiani et al also the royal jelly with antioxidant effects has influenced the rat's learning [45]. Jamnik et al and Nekeety et al and also in two separate study introduced the Royal Jelly as an effective antioxidant Agent [46, 47]. These results are consistent with the results of our study; this shows that with a certain prescribed dose of Royal Jelly the total amount of antioxidants in two sham and ovarectomy groups 3 and 4 has enhanced, compared to Sham and ovarectomy groups 2 that were exposed to seizure only by PTZ. So Royal Jelly has increased the total antioxidants in both groups under study. Other aspect under study in this research has been to investigate the effect of Royal Jelly on the number of neurons in the groups under study. The study of Hattori et al has been oriented to the effect of Royal Jelly on neurogenesis in cerebral tissue and hippocampus of specimens poisoned by Trimethyltin. The result represented the increase in the number of neurons and neurogenesis in the groups under study and an increase in brain activity in mice in eighth day [48]. In another study that has been done by Hattori et al the main property of the constituents of Royal Jelly was the property of its being neurotropic [49]. In another study by the same author, it became clear that Royal Jelly causes the production and duplication of the astrocytes via activation of STAT3 signaling pathway [50]. This author in other research has known the combination of 10-hydroxy-trans-2-decenoic acid which is a fatty acid in Royal Jelly as a cause to generate and multiply the neuron [51]. These results are consistent with the results obtained from our study. In our study the use of Royal Jelly in the Sham and ovarectomy groups has created a tangible and significant change in these groups; this substance causes an increase in the number of neurons in groups 3 and 4 of sham and ovarectomy that have received a Royal Jelly compared to group 2 that has not received it.

Conclusion

In summary in this study there was observed a positive impact of royal jelly on increasing the number of neurons and the amount of total antioxidants in rats with epilepsy induced by PTZ. The groups 3 and 4 of the Sham and ovarectomy having received Royal Jelly showed an increased number of neurons and total amount of antioxidants compared to group 2 of Sham and ovarectomy that did not receive royal jelly and had suffered a seizure by PTZ. But the administration of Royal Jelly has had no effect on the amount of nitric oxide in the groups under study and this composition did not create any significant change in the groups. Therefore, we can conclude that Royal Jelly has antioxidant activity and stimulates the production of neurons in the brain tissue in patients with cerebral tissue damage caused by seizures and epilepsy.

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