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RESEARCH PAPERS

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Copper sulfate inhibits seizure activity induced by pentylenetetrazole in mice

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A B S T R A C T

Background and Objective: Copper is one of the main micronutrients of body which plays a key role as a cofactor in the function of metabolic enzymes. Previous studies have shown that copper sulfate ($CuSo_4$) inhibits long-term potentiation (LTP) in slices of hippocampal CA1 region. Whereas LTP is involved in learning and epilepsy, it seems that copper effects on LTP could be associated with its effects on epilepsy and seizure. Therefore, the aim of this study was to evaluate the effect of $CuSo_4$ on seizure induced by pentylenetetrazole (PTZ).

Materials and Methods: The effect of various doses of CuSO4 (10, 50 and 100 mg/kg, i.p.), saline (as a control group) or sodium valproate (50, 150 and 100 mg/kg, i.p.) on seizure parameters induced by PTZ (100 mg/kg i.p.) was evaluated in NMRI mice. Twenty minutes after injection of saline or $CuSo_4$, PTZ (100 mg/kg) was injected to induce seizures in animals and seizure parameters were recorded.

Results: Comparison of the effect of $CuSo_4$, saline or sodium valproate on seizure parameters such as stage 2 latency, stage 5 latency and stage 5 duration showed that $CuSo_4$ dose-dependently reduced seizure.

Conclusion: This study showed that $CuSo_4$ significantly inhibits seizure parameters compared with the saline and sodium valproate.

Key Words:
Copper sulfate
Seizure
Pentylenetetrazole
Valproic acid

1. Introduction

Copper is one of the essential micronutrients of the body which plays a key role as a cofactor in the function of metabolic enzymes such as cytochrome oxidase C, superoxide dismutase, metallothionein, dopamine-beta-hydroxylase, lysyl oxidase as well as coagulation factor V and VIII. It is also involved in cellular processes like energy production in mitochondria, detoxification of free radicals, melanin structure, synthesis of neurotransmitters, and stability of connective tissue (1). Previous studies have revealed that disruption in copper homeostasis will cause diseases such as Wilson disease and Menkes syndrome (2).

Seizure is one of the neurological complications in Menkes and Wilson's diseases. Previous studies have shown that oral administration of copper inhibits long-term potentiation in the CA1 region of rat hippocampal slices (3,4). Since long-term potentiation is believed to involve in learning and also seizures, it seems that the effect

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of copper on long-term potentiation could be associated with effects of this element on epilepsy and seizures (5-7). The aim of this study was to assess the influence of intraperitoneal injection of $CuSo_4$ on seizures induced by pentylenetetrazole in mice, and comparing it with sodium valproate.

2. Materials and Methods

In this experimental study, male NMRI mice, weighing 25 to 30 g, were housed five per cage with free access to food and water. Mice were kept in a vivarium under controlled laboratory conditions (temperature, 22-26°C) with an artificial 12-h light/dark cycle. All animals were allowed to acclimate for ≥5 days before testing with food and water "ad libitum". All experimental procedures were approved by the Ethics Committee of the Arak University of Medical Sciences. Animals were randomly divided into seven groups with eight mice per group. Animals received saline, copper sulfate (10, 50 and 100 mg/kg, i.p.) and sodium valproate (50, 150 and 300 mg/kg, i.p.). Twenty minutes after injection, all mice received 100 mg/kg of pentylenetetrazole (PTZ) intraperitoneally (8). Mice behaviors were monitored for seizures immediately after PTZ injection for a period of 30 min and seizure responses were assessed. Seizure stages were classified into four phases; stage 1: hypoactivity, stage 2: partial clonus (clonic seizure activity affecting face, head, and/or forelimb or forelimbs), stage 3: generalized clonus (sudden loss of upright posture, whole-body clonus involving all four limbs and tail, rearing, and autonomic signs), and stage 4: tonic-clonic (maximal) seizure (generalized seizure characterized by tonic hindlimb extension) (9). Tonic-clonic maximal seizures were associated with death. Some mice recovered spontaneously. It was not unusual for mice to exhibit multiple episodes of tonic hind limb extension within the 30 min observation period. In this research, latency to stage 2 seizure, stage 3 seizure and from the onset of stage 3 to death were recorded.

2.1. Data analysis

Data of seizure stages are expressed as means± standard error of the mean (S.E.M.). Data were analyzed by one-way analysis of variance (ANOVA) and followed by Tukey's post hoc test. In all experiments, a P-value <0.05 was considered as the significance level between the groups.

3. Results

Figure 1 shows the effect of intraperitoneal administration of different doses of CuSO4 (10, 50, 100 mg/kg) on PTZ-induced seizure. One-way ANOVA ($F_{3, 31}=37.184$, $p<0.0001$) and Post-hoc analysis revealed a significant increase

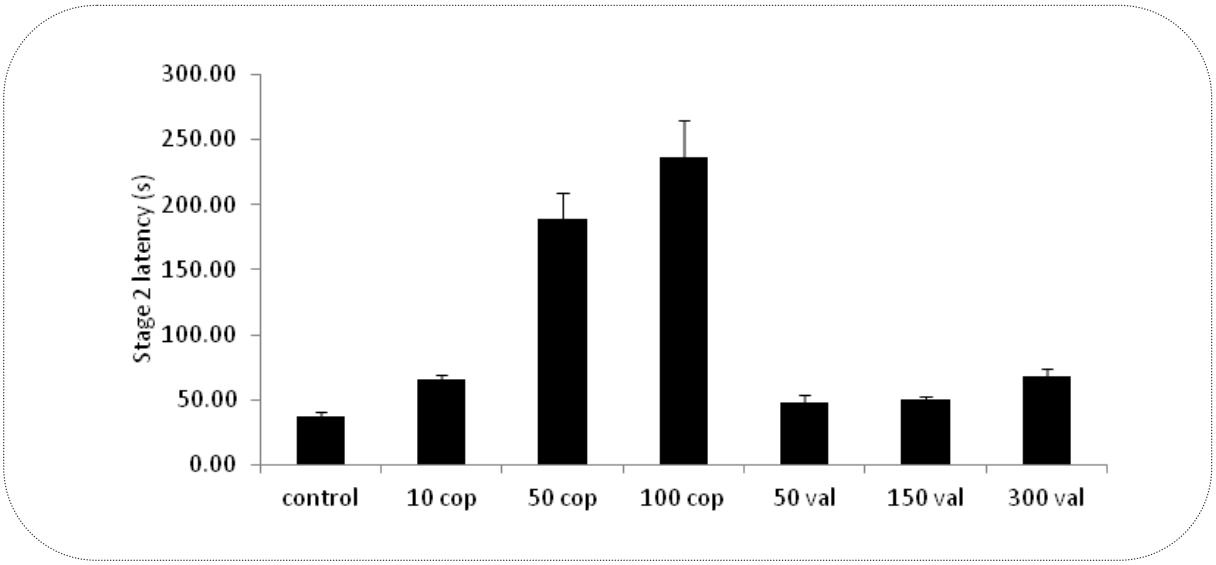

Figure 1. Comparison of the effect of copper sulfate and sodium valproate on stage 2 latency. Statistical analysis (analysis of variance) revealed that both copper sulfate and sodium valprote dose-dependently increase stage 2 latency.

in latency to stage two seizures (S2L) for CuSO4 at doses of 50 and 100 mg compared with saline-treated control animals. Intraperitoneal injections of sodium valproate (50, 150 and 300 mg/kg) also increased S2L in valproate-treated animals as compared to controls (Figure 1). Statistical analysis with ANOVA revealed no significant difference between two groups ($F_{6, 58}=41.667$, $p<0.0001$).

All doses of sodium valproate and copper sulfate significantly decrease the reverse of time that need to reach to stage 3 seizure multiplied to 100 as compared with the control group ($F_{6,58} = 153.736$, $p <0.001$) (Figure 2). The reverse of time between onset of stage 3 seizure to animal death multiplied to 100 showed that this parameter significantly decreases in all groups compared with control group ($F_{6,58} =13.656$, $p<0.001$). It means that time of animal death after the fifth stage of seizures increases in all groups as compared to controls (Figure 3).

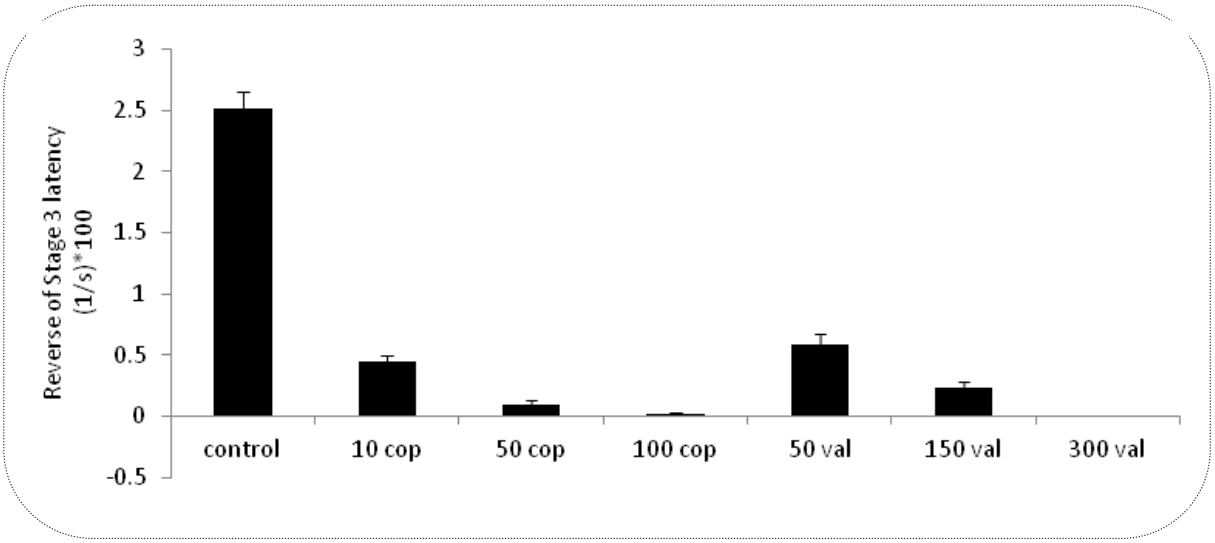

Figure 2. Comparison of effect of copper sulfate and sodium valproate on stage 3 latency. Statistical analysis (analysis of variance) revealed that both copper sulfate and sodium valprote dose-dependently increase stage 3 latency.

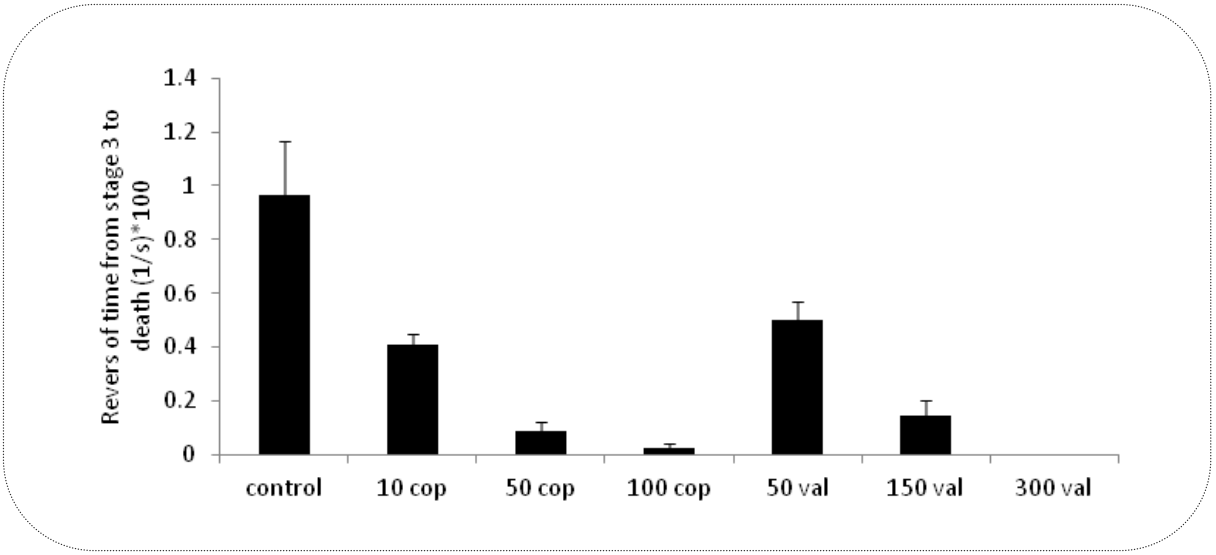

Figure 3. Comparison of the effect of copper sulfate and sodium valproate on revers of time from stage 3 to death. Statistical analysis (analysis of variance) revealed that both copper sulfate and sodium valprote dose-dependently decrease this parameter.

4. Discussion

These results showed that intraperitoneal injection of copper sulfate has an inhibitory effect on seizure parameters in mice. Many studies have been conducted on the relationship between copper and epilepsy, and the identification of toxic effects of copper on the body systems (10). In line with results obtained in this study, it has been reported that seizures and epilepsy are neurological symptoms in patients with Menkes disease. In this x-linked disease, copper transport is impaired and serum levels of copper and ceruloplasmin protein decrease (11). Also, in animal models of Menkes disease, there is increased risk of seizures and neuronal damage that can be removed with copper-containing supplements. Many articles attribute the seizures in this disease to a reduction of serum copper concentration (12-14). Routine injection of copper to mice also eliminates the seizure potentials created by the combination of microwaves and chlorpromazine and convulsive seizures by adding penicillin to sensorimotor cortex of the brain (15). Brunia et al in 1972 reported that plasma copper concentrations increase in patients with different types of epilepsy (16).

On the other hand, Kuzuya and colleagues in 1993 acclaimed that although serum copper increases in epileptic patients who use anticonvulsant drugs, this increase is not significantly different from control group (17). Verroti and his colleagues in 2002 also showed that before and after treatment with anticonvulsant drugs, there is no relationship between plasma copper and epilepsy (18). However, Doretto et al in 2002 reported that serum copper concentrations in mice with audiogenic seizures is more than normal mice (19). The results of our study showed inhibitory effects of copper sulfate injection on seizures induced by PTZ.

Conclusion

The results of this study showed that intraperitoneal injection of copper sulfate has inhibitory effect on seizures induced by intraperitoneal injection of PTZ in NMRI strain mice. These results can help to investigate the chronic effects of copper sulfate on the seizure and anticonvulsant effects of copper and other metals used in enzymes building.

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Synthesis and study of anticonvulsant effect of 1-[1-(4-methoxyphenyl) (cyclohexyl)] 4-piperidinol as a new derivative of phencyclidine in PTZ-induced kindling model in male mice

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A B S T R A C T

Background and Objective: Epilepsy is a common disease in communities. Since there is no cure for it and current treatments are not effective for every patient, new method for medical treatment of epileptic patients is necessary. As NMDA receptors antagonists are the most prominent anti-epileptic drugs, in this study we synthesized and investigated anti-epileptic effect of a new piperidine derivate 1-[1-(4-methoxyphenyl) (cyclohexyl)] 4-piperidinol as a new NMDA receptors antagonist in chemical kindling model.

Materials and Methods: In this study, 48 male mice (NMRI), weighting 25-30 g, were selected and randomly divided into 4 groups (n=12 in each group). 1: PTZ 2: 1-[1-(4-Methoxyphenyl) (Cyclohexyl)] 3: piperidinol and 4: valproic acid (positive control). Chemical kindling was induced by PTZ (35 mg/kg, i.p.) injection 11 times one other days (for 22 days). In challenge dose at day 24, PTZ was applied at 75 mg/kg to the animals. Thirty minutes after PTZ injection, the animals were followed for convulsion scores (0-5). Finally, the mean of convulsion phases, threshold and duration of 2 and 5 phases were considered as data and the statistical analysis was done.

Results: Data analysis showed that administration of the new piperidine derivate Methoxy-PCP has a prominent anticonvulsant effect than PCP, especially in reduction of phase 5 duration.

Conclusion: The results suggest that administration of the new piperidine derivate, 1-[1-(4-Methoxyphenyl) (Cyclohexyl)] 4-piperidinol could yield a prominent anticonvulsant effect in epilepsy. Regarding changes in conformation of the new drug as a non-competitive antagonist, it may potentially block the NMDA receptors than other piperidine derivates.

Key Words:
Convulsion
Piperidine
Chemical kindling
Pentylenetetrazole

1. Introduction

Prevalence of epilepsy is about 0.5-2 % in the world, and it may occur in all of the ages. Epilepsy is due to CNS malfunction in which some regions of brain will be activated spontaneously (1). Epilepsy is an unusual neurologic state which has influences on psycological, emotional and educational parameters. More than 50% of epileptic patients suffer from some kind of cognitive problem

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with abnormal behavior (2). Although control of seizure attacks has benefits, but the drugs may have side effects on the patients' cognition (3, 4).

Therefore, searching for new drugs that inhibit epileptic seizures and also improve the cognitive state of the patients is important. Chemical kindling is a method to study epilepsy. In this method, animals will be stimulated gradually and repeatedly for seizure by chemical drugs (5). Seizure may emerge as shaking movements or other forms of neurologic activities such as sensational, cognitive or emotional dysfunctions (6). Drugs which are used for treatment of epilepsy are not able to cure seizures. Therefore, it seems that trying to find new drugs for treatment of epilepsy is very important (7, 35). For this purpose, it is important to know the mechanisms of seizure attacks (8, 9). There are two important mechanisms: 1) mechanisms which reduce the inhibitory factors: a) dysfunction of inhibitory receptors: GABAa & GABAb, b) dysfunction in activation of gabaergic neurotransmitters (10, 2) mechanism which increases the excitatory factors, i.e. increase activities of NMDA receptors.

Phencyclidine is a derivative of piperidine that works as antagonist of NMDA receptors, and in this way can be used as a treatment of seizure attacks (11, 12). Phencyclidine (1-(1-phenylcyclohexyl) piperidine (PCP) and its analogues are highly potent and widely abused psychotomimetic drugs which influence the central nervous system and display analgesic, stimulant, depressant and hallucinogenic effects because of specific binding sites in the brain (13).

Recently, many analogues of phencyclidine have been synthesized (14-25) and their pharmacological activities have been studied. As part of our efforts to reach selective, non-competitive antagonists at the PCP binding site on NMDA receptor complex, we have prepared 1-[1-(4-Methoxyphenyl) (Cyclohexyl)] 4-piperidinol, as an analogue of PCP with a methoxy group on the aromatic ring (m-position) and a phenyl group with cyclohexane ring (a conjugated cyclic ketone, 1-tetralone) to examine its anticonvulsant effect in PTZ-induced kindling model in mice. The results were also compared to PCP and valproic acid. It was anticipated that incorporation of methoxy group on the aromatic ring of the molecule will produce pronounced effects on electron distribution and dipole moments because of the high electron donating character of this group (14).

2. Materials and Methods

1-Tetralone [1, 2, 3, 4-Tetrahydro-1-naphthalenone], cyclohexanone, piperidine, bromo benzene, magnesium turning, diethyl ether, 3-bromo anizole, and all other chemicals were purchased from Merck chemical Co. (Darmstadt, Germany). Melting points (uncorrected) were determined using a digital electrothermal melting point apparatus (model 9100, Electrothermal Engineering Ltd., Essex, UK). 1H and 13C NMR spectra were recorded on a Bruker 300 MHz (model AMX, Karlsruhe, Germany) spectrometer (internal reference: TMS). IR spectra were recorded on a Thermo Nicolet FT-IR (model Nexus-870, Nicolet Instrument Corp, Madison, Wisconsin, U.S.A.) spectrometer. Mass spectra were recorded on an Agilent Technologies 5973, Mass Selective Detector (MSD) spectrometer (Wilmington, USA). Column chromatographic separations were performed over Acros silica gel (No.7631-86-9 particle size 35-70 micrometer, Geel, Belgium). Adult male mice (Razi Institute, Tehran, Iran), weighing 22 -26 g were used for pharmacological testing.

2.1. Synthesis of compounds (Schemes 1 and 2)

(1-(1-phenylcyclohexyl) piperidine (PCP) I

This compound was prepared according to reported method (15) from 1-piperidinocyclohexanecarbonitrile (IV) and phenyl magnesium bromide. The hydrochloride salt of I was prepared using 2-propanol and HCl and was recrystallized from 2-propanol (15).

1-Piperidinotetralylcarbonitrile V

To a solution of containing 0.582 g (0.0068 mol) of piperidine in 0.253 g of HCl (37%) and 1.36 g of cold water, 1 g (0.0068 mol) of 1, 2, 3, 4-tetrahydro-1-naphtalenone (1-tetralone) was added. Then, 0.465 g of KCN in 1.02 ml of water, 50 ml of ethanol and 0.1 g of tetra-n-buthylammonium bromide (0.0003 mol) were added and stirred in ambient temperature (25˚ C).

The progress of reaction was controlled by TLC (7:3 ethyl acetate/n-Hexane). After one week no additional progress was seen, so the reaction was extracted with chloroform (75 ml, 3 times). Then, organic layer was separated, dried and concentrated. The oily residue was obtained, which was passed through a silica gel column using ethyl acetate-hexane (7:3) as the eluent to afford 1.13 g of V (69% yield).

IR (KBr): 3066, 2941, 2560, 1454, 1436, 1324, 1287, 1225, 764 cm-1.

1H N.M.R. (CDCl3) (p.p.m.): 1.5-2.85 (16H, m), 6.93-7.01 (4H, m).

13C N.M.R. (CDCl3) (p.p.m.): 25.4, 26.2, 26.8, 31, 37.9, 46.7, 52.7, 117.7, 125.5, 128.1, 139.2.

MS: m/z (regulatory intensity): 240 [M]+ (76), 241 [M+ H]+(15).

1-[1-(4-Methylphenyl) (Cyclohexyl)] 4-piperidinol III

A solution containing 4 g (0.016 mol) of nitrile compound (V) in 10 ml of dry THF was added to a refluxing solution of (3-methoxylphenyl) magnesium bromide (Grignard reagent) (prepared from 24.77 g 3-bromoanisole and 3.075 g of Mg in 17 ml of dry ether), refluxed for 5 additional h in 65-67 oC, left overnight at ambient temperature (25 oC) and then poured into ice-NH4Cl. The organic layer was separated and washed with water and the base was neutralized with 10% H2SO4, washed with 20% NaOH, re-extracted with n-Hexane, dried and concentrated. The oily residue was obtained, which was passed through a silica gel column using ethyl acetate-hexane (7:3) as the eluent to afford 2.28 g of III (42% yield).

The hydrochloride salt of III was prepared using 2-propanol and HCl and was recrystallized from 2-propanol.

IR (KBr): 3066, 2941, 1602, 1483, 1454, 1436, 1324, 1287, 1225, 764 cm-1.

1H N.M.R. (CDCl3) (p.p.m.): 1.5-2.85 (16H, m), 3.73 (3H, s), 6.59-7.1 (8H, m).

13C N.M.R. (CDCl3) (p.p.m.): 26.2, 27.5, 31.8, 44.8, 47.4, 56, 63, 111.6, 114, 120.2, 120.7, 125.8, 126.2, 128.8, 130, 139.3, 142.8, 144, 162.5.

MS: m/z (regulatory intensity): 321 [M]+ (100), 322 [M+ H]+(7).

2.2. Experimental procedures

In this experimental research, a total of 60 mice (NMRI), weighing 22-26 g (Razi Institue, Tehran, Iran), were randomly divided into six groups including; 1- control, 2- PTZ, 3- positive control (PTZ and valproate 100 mg/kg; i.p. as an anti-convulsant drug), 4, 5 PCP and its new compound methoxy PCP, respectively. Ten mice were housed in each cage at a temperature of 21±2ºC and 12 h light-dark cycling. The mice had free access to standard food and tap water ad libitum. The experimental protocol was approved by the Ethic Committee of Shahed University.

2.3. Kindling

All animals but control group (group 1) were kindled by a total of 11 period injection of PTZ (35 mg/kg; i.p.). Each administration was carried out every second day for 22 days. The challenge dose of 75 mg/kg of PTZ was injected in kindled mice on day 24 (test day). The challenge dose injection of PTZ produced convulsions (clonic and tonic) and lethality. All kindled mice were tested for PTZ challenge dose (75 mg/kg)-induced seizures and status. However, the exhibited phases of seizure (0-6) were observed and categorized using following scale [18] for 30 minutes after PTZ injection. The scale introduces six phases as follows:

0: no response

1: ear and facial twitching

2: convulsive waves axially through the body

3: myoclonic body jerks

4: generalized clonic convulsions turn over into side position

5: generalized convulsions with tonic extension episode and status epilepticus

6: mortality.

2.4. Statistical analysis

Data were expressed as means ± S.E.M. Statistical analyses was carried out using

repeated measure one way analysis of variance (ANOVA) followed by Tukey post-hoc test and p values less than 0.05 were considered as significant differences.

3. Results

3.1. Chemistry

Phencyclidine (I), and 1-[1-(3-methylphenyl) (tetralyl) piperidine (III) were synthesized by reaction of substituted Grignard reagents and carbonitrile compounds (IV, V). To obtain higher electron distribution and dipole moment properties, a methyl group was substituted on the aromatic ring of the molecule (III). Known procedures were applied for the synthesis of compounds I and IV with the appropriate modifications described previously (26, 27).

Bromobenzene and its m-methoxy (II) derivative were reacted with magnesium to form Grignard reagents, which were then reacted with appropriate piperidinocyclohexanecarbonitrile (IV) and piperidinotetralylcarbonitrile (V). Reaction between the Grignard reagents and the carbonitriles were slow and incomplete. So to overcome this problem, molar ratio of Grignard reagents to carbonitriles were increased (26).

Spectroscopic data (IR, 1H and 13C NMR, Mass) confirmed the structure of compounds III and V. The melting points of known compounds could also confirm their identity. The purity of each compound was checked by TLC using ethyl acetate/n-hexane as the eluent.

3.2. Effect of Methoxy-PCP on the PTZ-induced kindling intensity

Statistical analysis of results (as are shown in figure 1) indicates that there are no significant differences among experimental groups in the seizure intensity till 5th injection. As it is shown in figure 1, PCP injection (5.6 mg/kg) at 9, 11 and specially 12th injection is able to significantly reduce PTZ-induced seizure ($p<0.05$). However, valproate (150 mg/kg) significantly reduced seizure intensity in all periods ($p<0.05$).

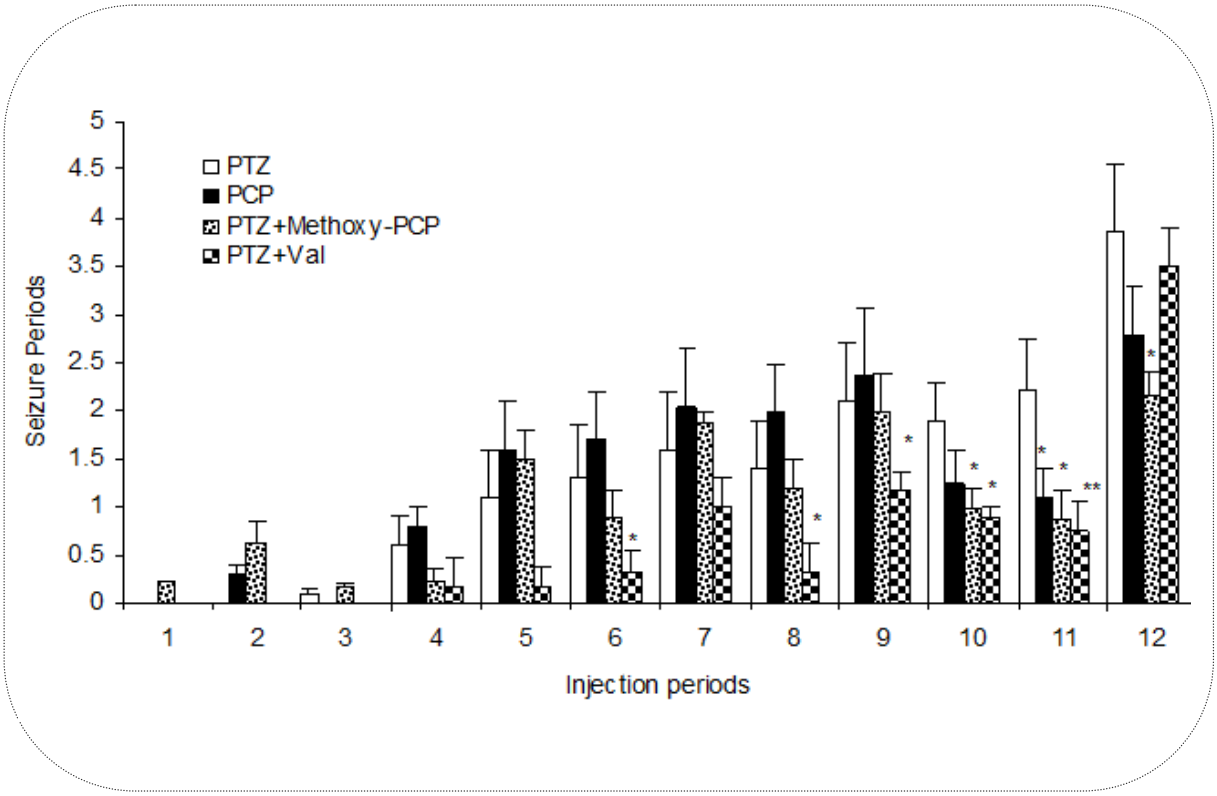

Figure 1. The effect of PCP and Methoxy-PCP pretreatment on the PTZ-induced kindling intensity. *P<0.05 and **P < 0.01 indicate significant differences as compared to PTZ-kindled group

3.3. Effect of Methoxy-PCP on the PTZ-induced kindling factors

As could be seen in Table 1, pretreatment of animals with PCP and methoxy-PCP have significant effect on the duration time of phase 5 ($p<0.05$) and also there was significant difference between valproate150 mg/kg and PCP ($p<0.05$). In addition, Table 2 indicates that only pretreatment of mice with Methoxy-PCP and valproate 150 mg/kg are able to significantly reduce the period that mice remain in phase 5 of seizure ($p<0.05$) and ($p<0.01$).

Table 1. The effect of valproate (150 mg/kg), PCP and methoxy-PCP on the latency of arriving to phase 5 of seizure.

Group test	Phase 5 latency time (s)	Phase 5 duration time (s)	Mortality (%)	Chimney test analysis
				% of mice showing motor impairment
PTZ	3.86 ± 0.70	4.51 ± 0.58	10.20	0
PTZ + VA	3.50 ± 0.60	2.15 ± 0.45 *	0	0
PCP	2.79 ± 0.85	4.15 ± 0.48	12.50	8.33
PTZ + Methoxy-PCP	3.78 ± 0.38	2.66 ± 0.55 *	20	6.33

n=8 in each group.

Table 2. Effect of valproate (150 mg/kg), PCP and Methoxy-PCP on the remaining time in the phases 2 and 5.

Group test	Phase 2 latency time (s)	Phase 2 duration time (s)	Phase 5 latency time (s)	Phase 5 duration time (s)
PTZ	4.41 ± 0.52	27.19 ± 2.19	3.33 ± 0.86	4.11 ± 0.48
PTZ + VA	3.66 ± 1.17	23.50 ± 1.55	2.12 ± 0.60	1.63 ± 0.45 **
PCP	5.81 ± 0.55	19.72 ± 0.74	4.75 ± 0.90	2.58 ± 0.64 *
PTZ + Methoxy-PCP	5.22 ± 1.35	21.98 ± 1.70	4.02 ± 0.48	2.18 ± 0.87 *

n=8 in each group.

4. Discussion

According to the studies on NMDA receptor complex, cationic channels will be opened by the effect of glutamate on its receptor, so Ca2+ and Na+ flow through the channel and the Ca2+ will stimulate the seizure attacks.

Phencyclidine is a non-competitive antagonist of NMDA, so the new piperidine derivative (methoxy-PCP) may work as a non-competitive antagonist of NMDA on the channel and may inhibit epileptic seizures (29).

In one study, it has been demonstrated that the site which PCP will block on the NMDA channel is separate from the known glutamate ligand site, and is different from the channel which will be blocked by Mg2+ ion. Presence of Mg2+ blocks the PCP function and, phencyclidine acts when the channel is not blocked by Mg2+ ion. Furthermore, there is a close interaction between receptors of PCP and NMDA on the channel and sometimes they work together and inhibit the seizure attacks (30). The researches have shown that PCP reduces the activated NMDA channel and in higher concentrations will decrease opening duration of the channel (31). Glycin is a modulator that increases the frequency of opening of activated channels and PCP works oppositely (32). According to the researches, methoxy-PCP acts on NMDA channel and blocks

the flow of Ca2+ through the channel, so decreases the seizures attacks.

According to the earliest studies, the PCP ligand attachment site on NMDA receptors has a high sensitive affinity to the ions with positive charges (33). So, the methoxy-PCP may act as a selective ligand in positive charge environments and has more affinity to the PCP receptor and inhibits the epileptic seizures by this way.

Carter in his study showed that the NMDA receptor has two subunits (NR1, NR2) that will be activated with glutamate and also glycin aminoacid, spermin polyamine and spermidin, will facilitate the glutamate function on the receptors. In fact, they increase NMDA responsibility (34).

In this study, the blocking of NMDA receptors with two types of piperidine derivatives may be by the way of interacting with polyamine sites. The results showed that these two derivatives will antagonize the stimulatory effect of the polyamine and acts on NMDA receptors instead of spermin and spermidin. Therefore, methoxy-PCP might antagonize function of the stimulatory actions of polyamins and in this way blocks the glutamate function and so reduces the seizure attacks and its progression.

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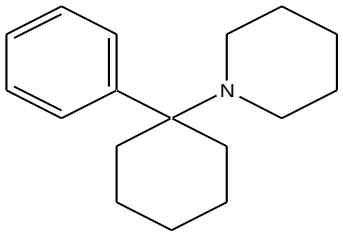

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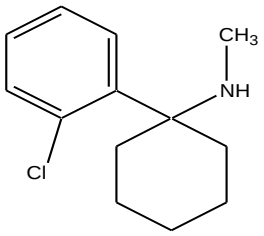

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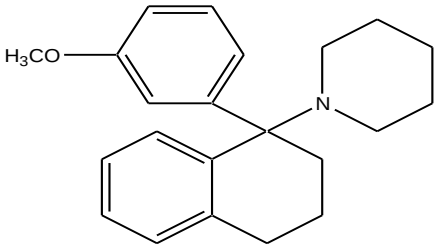

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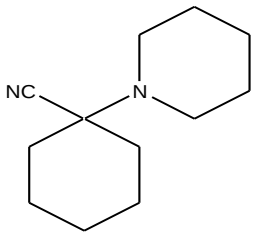

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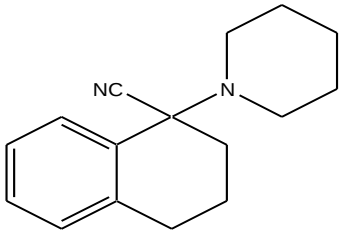

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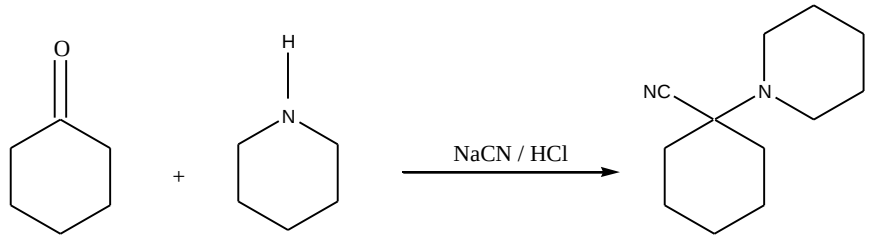
Scheme 1. Structure formulas of PCP (I), Ketamine (II), PCP-OCH_3-tetralyl (III) and Carbonitrile intermediates IV and IV.



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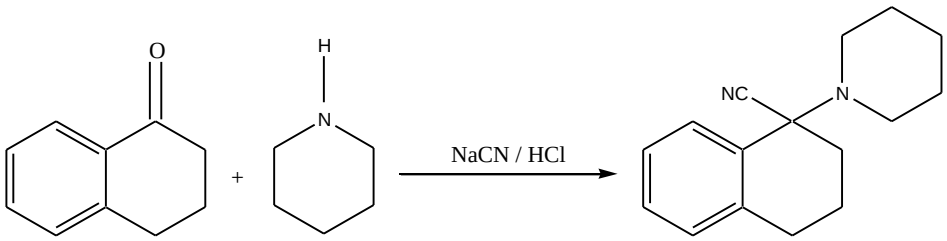
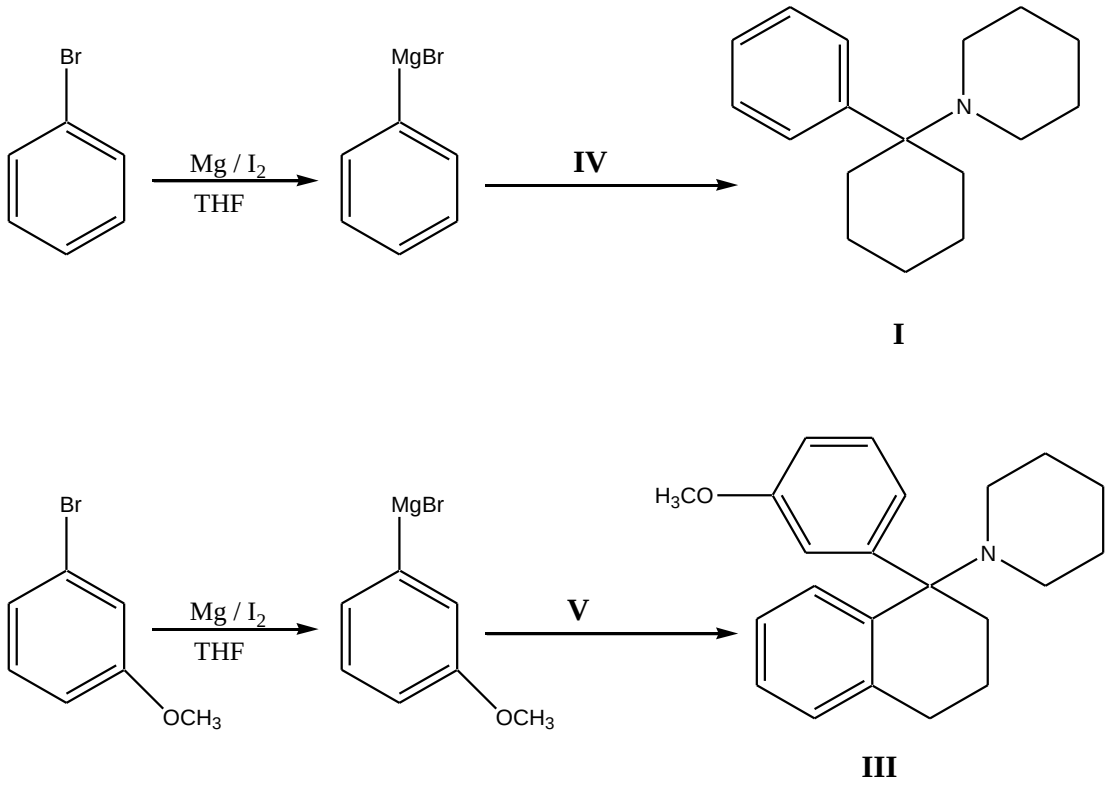


V

Scheme 2. Synthesis of intermediates IV and V.



Scheme 3. Synthesis of compounds I and III.

Methadone and haloperidol combination effect on the acquisition and expression of morphine tolerance and dependence in male mice

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A B S T R A C T

Background and Objective: Today, opioids are used to control and relieve acute and chronic pain. However, the incidence of both tolerance and dependence phenomena for these drugs is a major problem. So, in this study, the combination effect of haloperidol and methadone on the acquisition and expression of morphine dependence and tolerance was examined.

Materials and Methods: Ninety-eight mice were randomly divided into groups of acquisition and expression. Each group was divided into seven subgroups, saline, morphine, methadone, haloperidol, haloperidol + methadone, methadone + haloperidol ratio of 2 to 1, methadone + haloperidol ratio of 1 to 2. All groups were addicted with gradually increasing doses of morphine for 7 consecutive days. All drugs in the acquisition group were injected 30 minutes before morphine injected for 7 days and in the expression group 30 minutes before morphine injected in the eight day (test day). Morphine tolerance was measured by tail immersion test for 30 minutes before and after administration of morphine in test day. To assess dependence, mice were administered with naloxone and withdrawal behaviors were observed for 30 minutes.

Results: Chronic morphine injections induced tolerance and dependence in mice. Percentage of MPE as a tolerance index was significantly increased in acquisition and expression groups in drugs combination methadone1+haloperidol2 than morphine ones. Also, in dependence group, a marked decrease was shown in withdrawal behaviors in the combination therapy groups.

Conclusion: Our results showed that probably methadone and haloperidol combination treatment, especially at a ratio of 1 to 2, could reduce tolerance and dependence more than single drug treatment in animal groups.

1. Introduction

Opioids such as morphine are currently used widely to control and relief acute and chronic pain cases. Morphine exerts its strong analgesic effect due to attachment to μ opioid receptor (1). But, long term prescription of opioids, results in resistance and dependence, and stopping using them, will result in symptoms such as inquietude, anxiety, aggression, and irritability, which are known together as withdrawal syndrome (2). According to such phenomena, high restriction has been made against using these compounds. Despite many researches have been made on this issue, the true mechanism of resistance, dependence, and withdrawal according to some of the studies,

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among mechanisms involved in resistance and dependence on opioids, neurotransmitter systems such as nitric oxide (3), glutamate (4), dopamine (5), and stimulatory amino acid receptors, especially NMDA (6), are significant. The role of NMDA glutamate receptors in opioid-related synaptic shape ability has been proven (6). According to previous studies, calcium entry into the cell increases by activation of these receptors. Obviously, higher intracellular calcium concentration can result in activating some of calcium-dependent second messengers, and many effects such as amplification of calcium calmodulin protein kinase II (CaMKII) activity (7), protein kinase C (PKC) positive feedback regulation (8), nitric oxide synthetase (NOS) activation, and finally, nitric oxide (NO) production (9). NO is a neural moderator derived from L-arginine by NOS enzyme. The NOS enzyme is activated by calcium calmodulin protein kinase II (CaMKII) (10). Many researches suggests NO interference in resistance and dependence to morphine. Evidence suggests cooperation of NO with other neurotransmitter systems such as glutamatergic system and NMDA to perform its role (11). Also, studies suggest a mutual relation between NO production and dopamine release (12,13). Methadone therapy is currently known as the most suitable opioid detoxification. Methadone is a unique industrial opioid which is also used instead of morphine for severe pain treatment (14,15), and is the agonist to opioid receptor, and antagonist to NMDA receptor (16). But unfortunately, some of the patients under treatment with methadone develop mental disorders such as anxiety or depression (17), and some of the patients do not respond to this treatment.

Haloperidol as anti-psychotic butirophenon medication is the antagonist to dopamine and has high tendency to D_2 dopamine receptors. This medication is used to treat various mental diseases such as schizophrenia, mania, and psychosis (18). Researches indicate that this medication is CaMKII inhibitor, and therefore, can be used to decrease opioid resistance and dependence (19). According to this, and regarding new research on effects of compound medications on diseases, this study addresses compound effect of methadone and haloperidol on acquisition and expression of morphine resistance and dependence.

2. Materials and Methods

In this experimental study, 98 NMRI mice (obtained from Razi Institute, Tehran) in the weight range of 20-25 g were used. Animals were kept in clear Plexiglas cages in groups of four, and were moved to animal house of the faculty few days before the experiment, in order to get adapted to the environment. The animal house was in suitable 12 hour day-night period situation and 30-40% humidity, and the temperature was fixed at 21±2°C. Also, all of the animals of every group had unlimited access to sufficient food and water, and each animal was assessed only once. In all phases, all morale principles about animals were respected.

In this study, 98 mice were divided into acquaintance (chronic) and expression (acute) groups. Each group consists of seven sub-categories:

1. saline, 2. morphine, 3. methadone (10 mg/kg) 4. haloperidol, 5. methadone+haloperidol (5 mg/kg and 0.15 mg/kg, respectively), 6. 2 methadone+1 haloperidol (7 mg/kg and 0.1 mg/kg, respectively), 7. 1 methadone+2 haloperidol (3.5 mg/kg and 0.2 mg/kg, respectively).

Morphine was injected to acquaintance group for seven days, twice a day, and once in the eight day, in order to study acquaintance of tolerance and dependence. In this group, in each sub-category, all medications were injected 30 minutes before receiving morphine doses.

Morphine was injected to expression group for seven days, twice a day, and in the eight day, a single dosage of the medication was injected 30 minutes before receiving the last morphine dosage, in order to study expression of tolerance and dependence.

2.1. Tolerance and dependence circumstances

Morphine was injected to all mice with staircase dosages of mg/kg for seven days, twice a day (8 a.m., 4 p.m.), and once in the eight day (8 a.m.), according to the following program, in order to form tolerance and dependence:

First day: 10, second day: 20, third and fourth day: 40, fifth day: 60, sixth day: 80, seventh day: 100, and eighth day: 100.

2.2. Pain tolerance test

In this study, pain threshold of all mice was assessed in two phases (30 minutes after injection of the medication and 30 minutes after injection of morphine) by exposing their tails to hot water. One cm from the end of the tail was exposed to 56±0.5°C water and a stopwatch was immediately started, and stopped when the animal pull the tail out of water as a reflex. A 10 second limit was considered, in order to prevent tissue damages. If the animal did not show any reflex in 10 seconds, the tail was pulled out of water. The procedure was executed with 3 minute gaps, three times in each phase, and the average was calculated. Then, the averages were put into the following formula, maximum possible effect was given in percentages, and the final data was used for statistical analysis.

MPE % = [Delay before morphine injection (sec) - Delay after morphine injection (sec) / Delay before morphine injection (sec) - Stop time (sec)] × 100 %

2.3. Withdrawal syndrome induction and behaviors under study

Two hours after the last morphine injection in the test day, 5 mg/kg of naloxone was injected to each mouse in every group and then each animal was put into a clear box measuring 20×20×30 cm, in order to exhibit withdrawal symptoms, and then behavioral symptoms were observed and noted for 30 minutes. Mice show different behaviors during induction of withdrawal syndrome. In this study, we discussed jumping, standing, licking, and diarrhea. Among these symptoms, jumping is of a high importance, as in many researches, it is the only mentioned symptom.

2.4. Drugs

In this study, morphine sulphate (Tamad, Iran), methadone (Tamad, Iran), haloperidol (Minou, Iran), and naloxone (Tolid darou, Iran) were used. Morphine sulphate and methadone were dissolved in normal saline, and haloperidol was dissolved in methanol. All of the injections were made i.p. at a volume of 0.2 ml.

2.5. Statistical analysis

In this study, SigmaStat software (version 3.5) was used for statistical analysis. All data were reported as average ± variance. Statistical comparison between test groups was made by variance analysis test and then by Tukey post-test, and $p<0.05$ differentiation level was regarded significant. Non-parametric data analysis was done by Kruskal-Wallis test and the related post-test.

3. Results

3.1. The effect of methadone, haloperidol, and their compounds on acquisition of morphine dependence

As shown in figure 1, jumping (A) as an indicator of morphine withdrawal syndrome, in treatment groups of haloperidol, 2 methadone+1 haloperidol, and 1 methdone+2 haloridole, had an outbreak with averages of 30±6.25, 28±8.4, and 12±0.64, respectively, with a significant decrease as compared to morphine group with an average of 79.25±9.89. Frequency of diarrhea (B) in treatment groups of haloperidol, 2 methadone+1 haloperidol, and 1 methdone+2 haloridole, occurred with an average of 1.83±0.74, 2.25±0.9, and 2±0.36, respectively, with a significant decrease as compared to morphine group with an average of 5.16±0.4. Frequency of licking (C) in treatment groups of haloperidol, 1 haloperidol+1 methadone, 2 methadone+1 haloperidol, and 1 methdone+2 haloridule, decreased by 48±15, 49.42±16.74, 55.14±16.43, and 57.75±24, respectively, which was obviously significant, as compared to morphine group 138.28±17.71 ($p<0.05$). Frequency of standing (D) in treatment groups of haloperidol and 2 methadone+1 haloperidol with averages of 20±5.27 and 23±6.94 had an obvious decrease as compared to average of 54±6.86 of morphine group ($p<0.01$).

3.2. The effect of methadone, haloperidol, and their compound on expressing morphine dependence

As shown in figure 2, jumping (A) in treatment groups of methadone and haloperidol with averages of 52.4±11.78 and 45±14.81, and in compound treatment groups of 1 methadone+1 haloperidol and 2 methadone+1 haloperidol with averages of 37±7.87 and 29.75±9.37, respectively, showed a significant decrease as compared to morphine group with an average of 106±16.76. Frequency of diarrhea (B) in treatment groups of

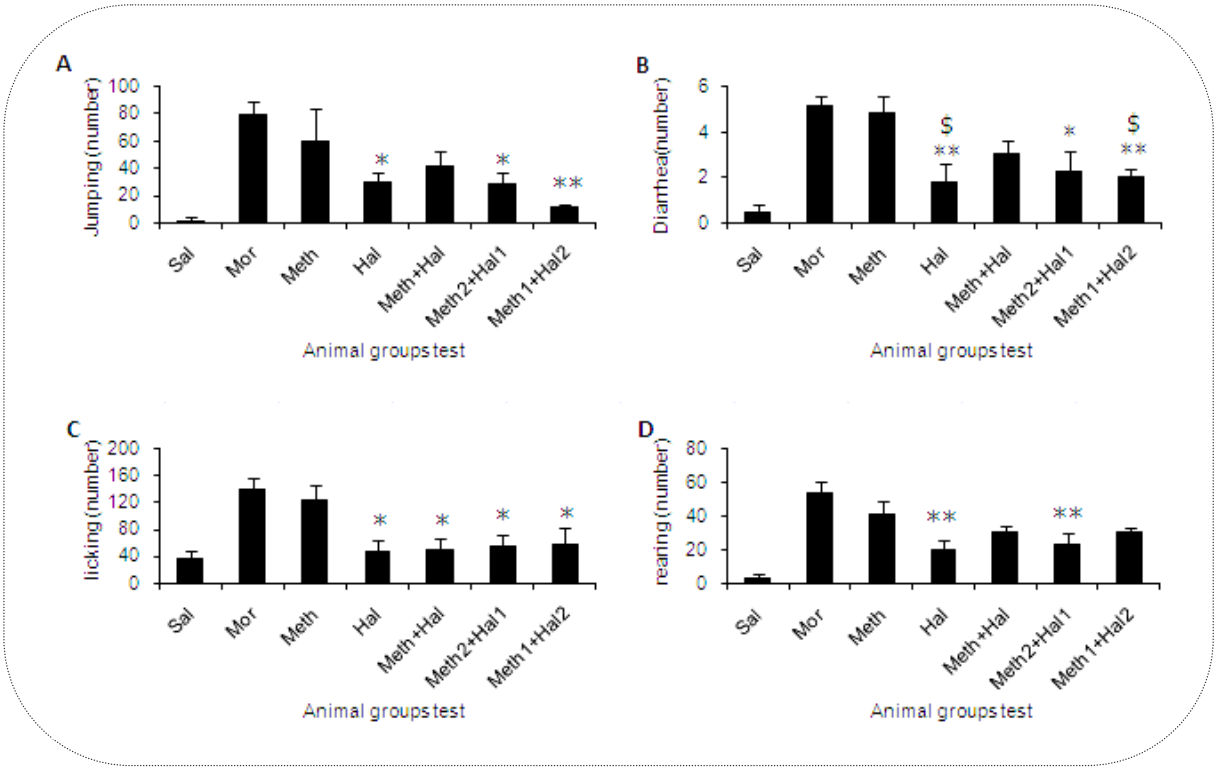

Figure 1. Effect of methadone, haloperidol, and their compound on acquisition of morphine dependence

A: jumping, **B:** diarrhea, **C:** licking, **D:** standing.

Sal: normal saline, Mor: morphine (addicted group), Meth: methadone, Hal: haloperidol.

Columns show Mean ± SEM (n=7).

* and ** show significant difference with morphine group (with P<0.05 and P<0.01, respectively).

$ shows a significant difference from methadone (P<0.05).

haloperidol, 1 methadone+1 haloperidol, and 1 methadone+2 haloperidol, decreased by 2±0.2, 1.6±0.8, and 1.57±0.64, respectively, which was significant as compared to morphine group with an average of 5.16±0.54.

Frequency of licking (C) as another sign of dependence in treatment groups with haloperidol and 1 methadone+2 haloperidol, with averages of 14.66±8/65 and 37±11.78, respectively, showed a significant decrease as compared to morphine group with an average of 91.33±11.95. Number of standings (D) in methadone and 1 methadone+2 haloperidol treatment groups, with averages of 22±4.6 and 23±2.4, respectively, decreased significantly as compared to morphine group with an average of 47.83±5. In addition to these two groups, haloperidol and 2 methadone+1 haloperidol treatment groups with averages of 16.14±2.07 and 18.6±5 also showed a significant decrease as compared to morphine group.

3.3. The effect of methadone, haloperidol, and their compound on acquisition of morphine tolerance

As shown in figure 3, the MPE percentage in 1 methadone+2 haloperidol with an average of 19.49±2.34 shows a significant increase as compared to morphine group with an average of 6.44±1.67 (p<0.001). In other words, it can be said that 1 methadone+2 haloperidol group has increased the analgesic response to morphine, and has caused a decrease in tolerance expression.

3.4. The effect of methadone, haloperidol, and their compound on expression of morphine tolerance

As shown in figure 4, none of the groups could cause a significant change in morphine tolerance

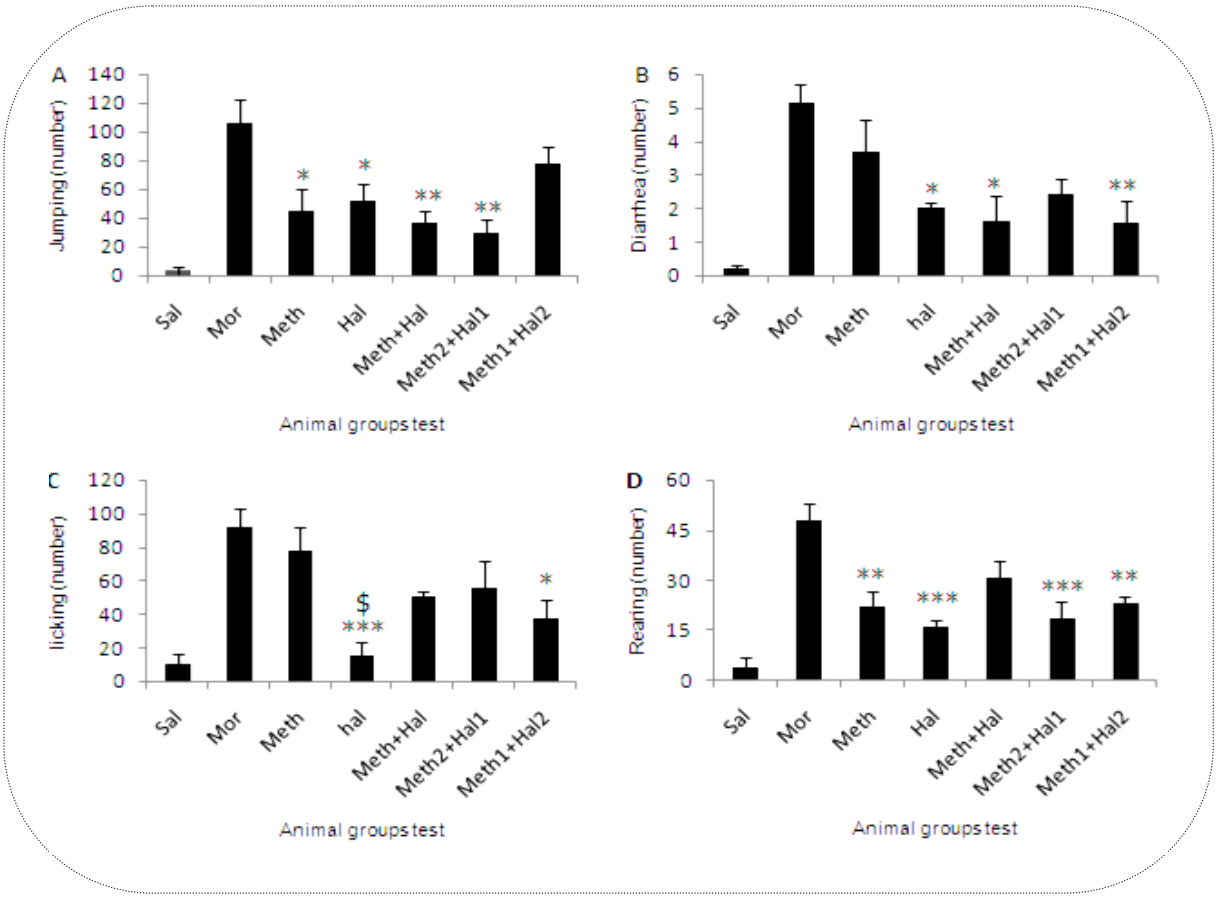

Figure 2. Effect of methadone, haloperidol, and their compound on expressing morphine dependence

A: jumping, **B:** diarrhea, **C:** licking, **D:** standing.

Sal: normal saline, Mor: morphine (addicted group), Meth: methadone, Hal: haloperidol.

Columns show Mean ± SEM (n=7).

* and ** show significant difference with morphine group (with P<0.05 and P<0.01, P<0.001, respectively).

$ shows a significant difference from methadone (P<0.05).

as compared to morphine group, except 1 methadone+2 haloperidol treatment group with an average of 17.92±3.33 could increase the MPE percentage as compared to morphine group with an average of 7.05±1.49, and decrease the tolerance expression significantly (p<0.05).

4. Discussion

The obtained results showed that morphine prescription for 7 days will cause tolerance against its analgesic effects, and stopping using morphine will cause symptoms of withdrawal syndrome, which indicates dependence on morphine. Prescription of pharmaceutical composition of methadone + haloperidol with a ratio of 1 to 2 in acquisition and expression groups can decrease tolerance to analgesic effects of morphine significantly. Therefore, it can be said that this pharmaceutical composition increased analgesic effect of morphine. Results of withdrawal syndrome symptoms showed that methadone in expression group only has been able to cause a significant decrease in jumping and standing on both feet as compared to morphine group, and has reduced none of the other behaviors significantly, but all of the symptoms of withdrawal syndrome reduced significantly in both acquisition and expression groups by haloperidol as compared to morphine.

Many studies have shown that using NMDA receptors antagonists prevent tolerance and dependence on morphine (20,21). Methadone is the antagonist to μ receptor and NMDA receptor, therefore, in addition to analgesic effects, can

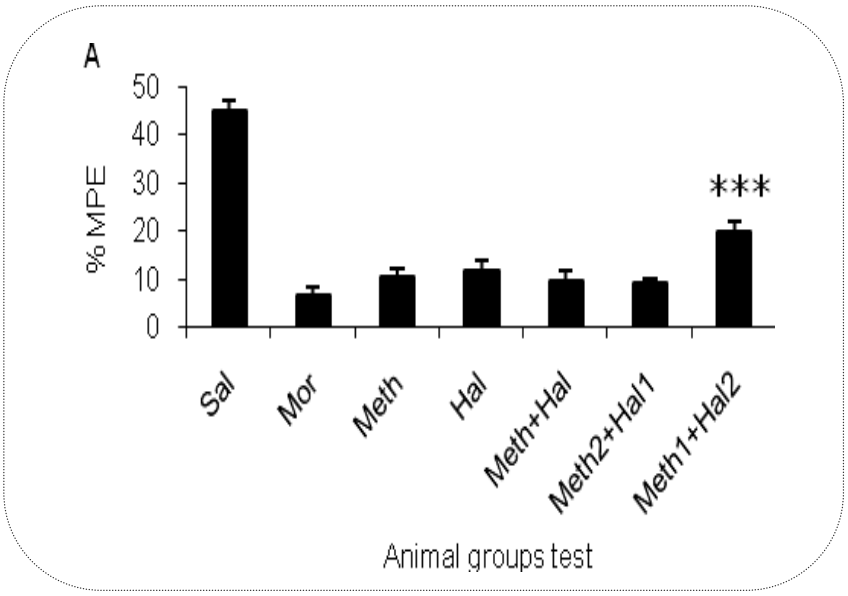

Figure 3. Effect of methadone, haloperidol, and their compound on acquisition of morphine tolerance

Sal: normal saline, Mor: morphine (addicted group), Meth: methadone, Hal: haloperidol.

Columns show Mean ± SEM (n=7).

*** shows significant difference with morphine group (with P<0.001).

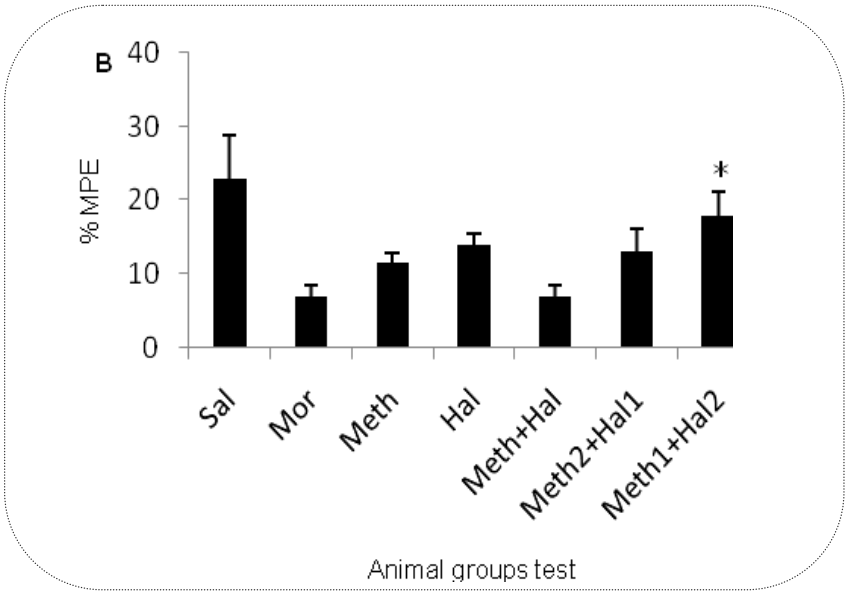

Figure 4. Effect of methadone, haloperidol, and their compound on expression of morphine tolerance

Sal: normal saline, Mor: morphine (addicted group), Meth: methadone, Hal: haloperidol.

Columns show Mean ± SEM (n=7).

* shows significant difference with morphine group (with P<0.05).

prevent tolerance and dependence on morphine (16). A research by Whistler et al showed that prescription of morphine alongside with low dosages of methadone can increase analgesic effect of morphine in treatment of chronic pains. Also, patients in need of long term opioid usage, are able to use a composition of methadone with opioids in order to reduce dependence (1). As shown before, long term treatment using morphine can cause CaMKII activity in the body. Also, it has been proven that spinal and supra-spinal inhibition of CaMKII not only causes prevention, but also inverts tolerance to analgesia and physical dependence on opioids in some rodents (22,23). A study performed by Young et al has shown that haloperidol as an antipsychotic medication can reduce tolerance and dependence on opioids by inhibition of CaMKII activity (19). Also, as mentioned above, haloperidol is mostly known as D_2 dopamine receptors antagonist. Previous studies indicate the ability of dopamine receptor antagonists in movement inhibition (24), conditioned place preference (25), and morphine self-prescription (26) in mice. But what is certain, is that the combination of methadone and haloperidol has been more effective in reducing tolerance and dependence on morphine than the effect of each medication. Perhaps the reason for such phenomenon is that according to experts, CaMKII can phosphorylase NMDA receptor, which can cause an increase in NMDA receptor activity and calcium penetration through the channels. This positive feedback between CaMKII and NMDA receptor can be a CaMKII and NMDA receptor activity booster in tolerance and dependence on opioids (19); which explains reduction of tolerance and dependence due to CaMKII activity inhibition by haloperidol and NMDA receptor inhibition by methadone in this study. However, the results of this study showed that prescription of pharmaceutical combination of methadone and haloperidol can reduce acute or chronic acquisition and expression of tolerance and dependence on morphine. Since the usage of methadone as a common medication for addicted people today, and also according to antipsychotic effects of haloperidol as an effective medication in mental disorders, it is suggested to use a combination of above-mentioned medications as a more effective way of prevention and treatment of tolerance and dependence on opioids such as morphine. Further research is necessary in order to be considered in future studies.

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The effect of simvastatin in prevention of histological changes of substantia nigra and behavioral abnormalities in an experimental model of Parkinson's disease in rat

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A B S T R A C T

Background and Objective: Parkinson's disease (PD) is a rather common neurological disorder in elders that is due to degeneration of dopaminergic neurons within mesencephalic substantia nigra pars compacta. With regard to protective and antioxidant effect of simvastatin, this study was conducted to evaluate its neuroprotective effect in an experimental model of PD.
Materials and Methods: In this experimental study, male rats (n =40) were divided into 5 groups, i.e. sham-operated, simvastatin20-treated sham-operated, lesioned and simvastatin10 and simvastatin20-treated lesioned groups. The hemi-PD early model was induced by unilateral intrastriatal injection of 5 microliter of saline-ascorbate (left side) containing 12.5 microgram of 6-hydroxydopamine (6-OHDA).Treated sham and lesioned groups received simvastatin i.p. at doses of 10 and 20 mg/kg once a day before surgery for two times at an interval of 24 h. Two weeks after surgery, the animals were tested for rotational behavior by apomorphine for an hour and the number of dopaminergic neurons in the substantia nigra pars compacta (SNC) was counted.
Results: Two weeks after surgery, apomorphine caused a significant contralateral turning ($P<0.0001$) in 6-OHDA-lesioned group and a reduction in the number of neurons on the left side of the SNC in the lesioned group was observed in comparison with sham group ($P<0.01$). In addition, simvastatin pretreatment at both doses significantly decreased the rotational behavior in lesioned rats ($p<0.05$ and $p<0.01$, respectively) and also at a higher dose significantly attenuated the reduction in the number of SNC neurons ($p<0.05$). On the other hand, pretreatment of sham group with simvastatin had no significant effect on the number of apomophine-induced rotations and neurons of SNC.
Conclusion: Intraperitoneal administration of simvastatin exhibits neuroprotective effect against 6-OHDA toxicity in an experimental model of PD, as was shown by a lower rotational behavior and attenuation of neuronal loss.

Key Words:
Simvastatin
Parkinson's disease
6-hydroxydopamine
Rotational behavior
Apomorphine
Substantia nigra

1. Introduction

Parkinson's disease (PD) is regarded as a neurological and debilitating disease, which involves the neurodegeneration of dopaminergic neurons within the substantia nigra pars compacta, with the subsequent loss of their terminals within the striatum. The ensuing loss of the neurotransmitter dopamine leads to the debilitating motor disturbances in PD (1). Oxidative stress and increased lipid peroxidation, low glutathione level, damage to DNA and iron accumulation are reported as the main pathogenic causes of dopaminergic neurons degeneration in PD (2). Oxidative stress not only damages the

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dopaminergic neurons, but it also endangers mitochondrial oxidative phosphorylation, leading to decreased energy output by these organelles and eventually to subsequent death of these cells (3). Despite great achievements in innovation of chemicals to treat PD, none yet address the underlying problem associated with it, i.e. the progressive and gradual degeneration of dopaminergic neurons (4).

Statins are known as inhibitors of the enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase that could inhibit cellular synthesis of cholesterol and isoprenoids. Statins have been known for their potency in reducing serum cholesterol level and prevention of cardiovascular disorders, while growing evidence has shown the efficacy of statins in treating neurodegenerative diseases and in conditions of brain injury. The neuroprotective and beneficial effects of statins is related to their properties such as endothelial protection, anti-oxidant, and anti-inflammatory effects. In this respect, statins could suppress inflammatory processes following brain insult and inhibit cytokines release in neurological disorders (5-7). Simvastatin is a statin with neuroprotective and antioxidant activity in in vitro and in vivo environments (8, 9). Therefore, this study was carried out to investigate the possible neuroprotective potential of simvastatin administration in an early model of Parkinson's disease in rat.

2. Materials and Methods

Adult male Wistar rats (220-280 g; n = 40) (Pasteur's Institute, Tehran, Iran) were housed three to four per cage in a temperature-controlled colony room under light/dark cycle with food and water available ad libitum. Procedures involving animals and their care were conducted in conformity with NIH guidelines for the care and use of laboratory animals. The animals were held in the colony room for at least one week before being tested. Only rats not showing any biased rotational behavior (net rotations less than 30/hour) following intraperitoneal injection of apomorphine hydrochloride (2 mg/kg) were selected for the present study. The animals were randomly divided into five groups: sham-operated group, simvastatin-treated sham-operated group (at a dose of 20 mg/kg), lesion group, and two simvastatin-treated lesion groups (at doses of 10 and 20 mg/kg). Unilateral intrastriatal 6-OHDA injection (left side) was performed through a 5 μl Hamilton syringe on anesthetized rats (ketamine 100 mg/kg and xylazine 5 mg/kg, i.p.) using stereotaxic apparatus (Stoelting, USA) at the coordinates: L –3 mm, AP +9.2 mm, V + 4.5 mm from the center of the interaural line, according to the atlas of Paxinos and Watson. At the end of injection, the needle was left in place for an additional 5 min and then withdrawn at a rate of 1 mm/min. The lesion group received a single injection of 5 μl of 0.9% cold saline containing 2.5 μg/μl of 6-hydroxydopamine-HCL (6-OHDA, Sigma) and 0.2% ascorbic acid (W/V). The sham group received an identical volume of ascorbate-saline solution. Simvastatin was administered at doses of 10 and 20 mg/kg for two days presurgery. Simvastatin was dissolved in propylene glycol.

2.1. Behavioral testing

The animals were tested for rotational behavior by apomorphine hydrochloride (2 mg/kg, i.p.) one week before (baseline) and two weeks after the surgery. The rotations were measured according to a method as described previously. Briefly, the animals were allowed to habituate for 10 min and then 1 min after the drug injection, full rotations were counted in a cylindrical container (a diameter of 33 cm and a height of 35 cm) at 10-min intervals for 60 min in a quiet isolated room. Net number of rotations was defined as the positive scores minus the negative scores.

2.2. Histological study

Five animals in each group were used for histological assessment. Following behavioral experiment, the rats were deeply anesthetized with a high dose of ketamine (150 mg/kg) and perfused through the ascending aorta with 50 ml of 0.9% saline followed by 150 ml of fixative solution containing 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4) followed by 100 ml of 0.1 M PB containing 10% sucrose. Following perfusion, the brains were removed from the skull, the blocks of forebrain and brainstem were prepared, and after final steps of preparation (30% sucrose for 2 days), sections were cut at a thickness of 30 μm on a freezing microtome (Leica) and collected in PB (0.1 M). Every second section was Nissl-stained with

0.1% cresyl violet (Sigma).

2.3. Neuronal counting

For each animal, mesencephalic sections (Interaural 2.9-4.2 mm) were examined by a method as described previously. Briefly, Nissl-stained neurons of the SNC were counted (Light microscopy; X200) using a superimposed grid to facilitate the procedure. At least two sections representative of each of four Paxinos-Watson planes (4.2, 3.8, 3.2, 2.97; Interaural) were examined by scanning the entire extent on each side. Counting was done blind to the treatments received.

2.4. Statistical analysis

All data were expressed as mean ± S.E.M. For rotational behavior, one-way ANOVA followed by Tukey post hoc test was used. For each group, the values of Nissl-stained cells for the injected and non-injected sides were compared using two-tailed student's t-test for paired samples and the inter-group differences were analyzed using one-way ANOVA followed by Tukey's post-hoc test. In all analyses, the null hypothesis was rejected at a level of 0.05.

3. Results

The beneficial effect of simvastatin was evaluated on apomorphine-induced rotations for a period of 1 hour (Table 1). There were no significant differences among the sham groups. Statistical analysis of the total net number of rotations made over a 60-min period (Figure 1) in lesioned groups showed that apomorphine caused a very significant contralateral turning in the rats of 6-OHDA group ($p<0.001$) and induced lower rotations in 6-OHDA+simvastatin10 ($p<0.05$) and 6-OHDA+simvastatin20 ($p<0.01$) in comparison with 6-OHDA group.

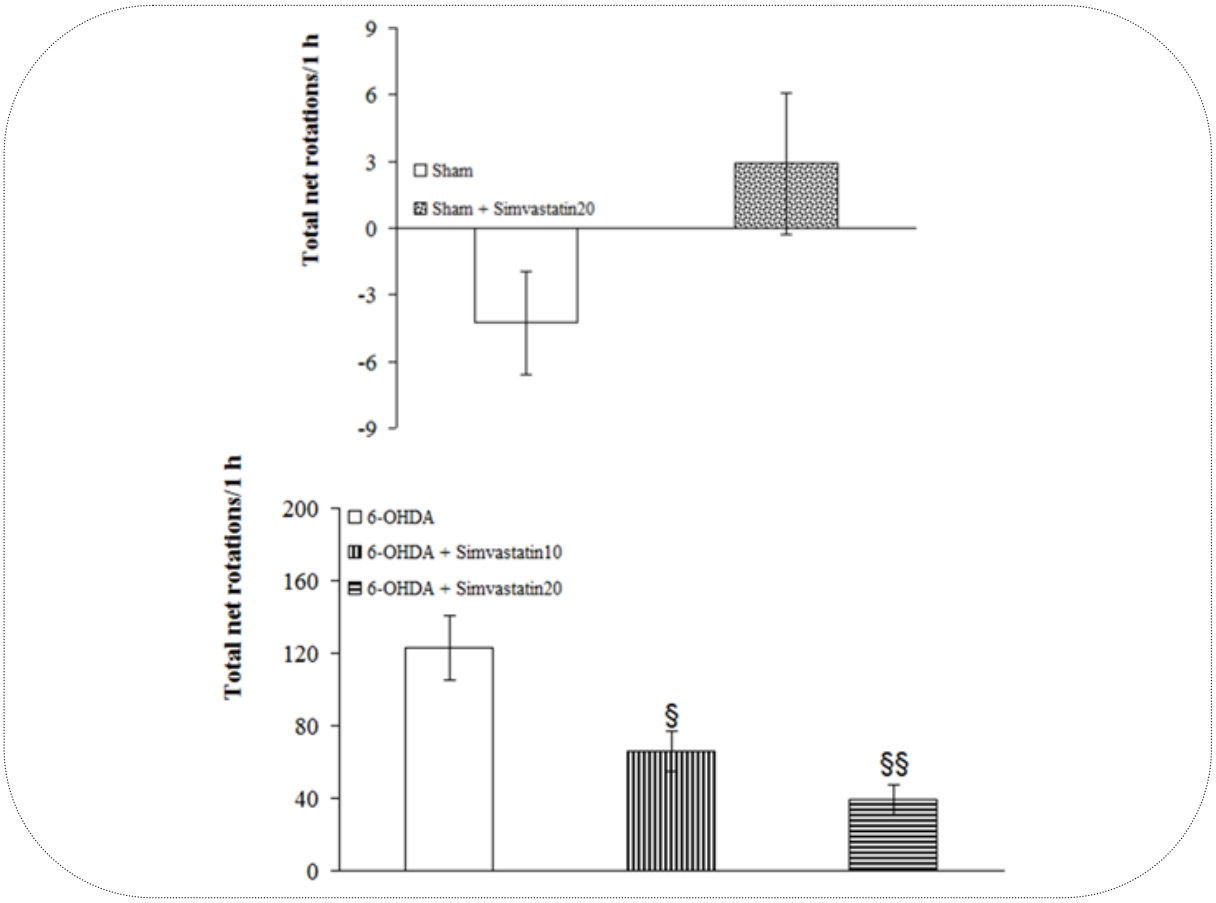

Figure 1. Total net number of rotations (mean ± S.E.M.) induced by apomorphine (2 mg/Kg, i.p.) after 1 week over a period of 60 min in sham (upper panel) and 6-OHDA-lesioned (lower panel) groups. Note that the positive values indicate contralateral rotations. 6-OHDA stands for the neurotoxin 6-hydroxydopamine. \$ $p<0.05$, \$\$ $p<0.01$ (versus 6-OHDA)

The results of histochemical studies (Figure 2) showed that there is no significant difference for the number of Nissl-stained neurons between the two sham groups, a significant reduction was observed for 6-OHDA group (P<0.01) and 6-OHDA+simvastatin10 group (p<0.05), and no such difference was obtained for 6-OHDA+simvastatin20 group. Meanwhile, in the latter group, the number of neurons was significantly higher than 6-OHDA group (p<0.05).

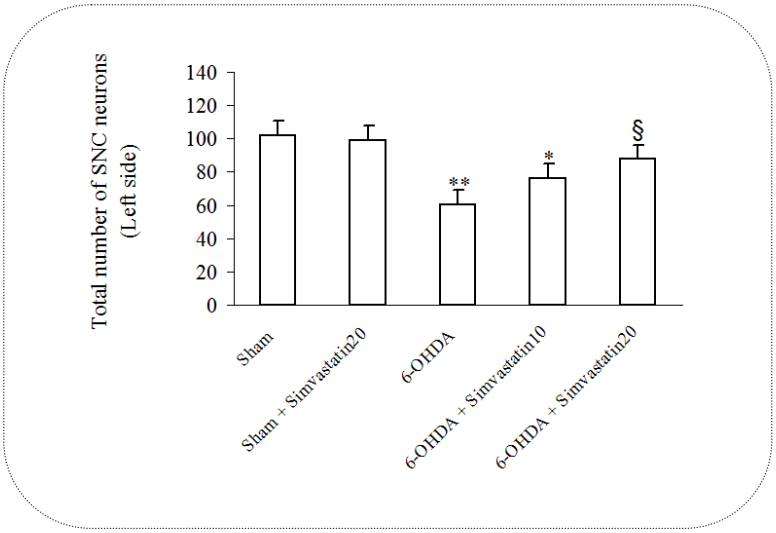

Figure 2. Total number of Nissl-stained neurons on the left side of substantia nigra pars compacta (SNC) in different groups after 1 week post-surgery. 6-OHDA stands for the neurotoxin 6-hydroxydopamine.
* p<0.05, ** p<0.01 (in comparison with Sham)
$ p<0.05 (in comparison with 6-OHDA)

4. Discussion

According to literature, unilateral damage of the nigrostriatal dopaminergic system through intrastriatal injection of neurotoxins like 6-OHDA is associated with a reduction in the striatal dopamine level and an upregulation of dopaminergic postsynaptic receptors at the same side. These changes produce a prominent functional and motor asymmetry that can be evaluated by direct acting (apomorphine) and indirect-acting (amphetamine) dopaminergic agonists (2). These rotations, especially those induced by apomorphine are considered as reliable indicators of nigrostriatal dopamine depletion (10). In the present study, a significant attenuation of the apomorphine-induced rotational behavior was observed in simvastatin-pretreated 6-OHDA-lesioned group after 1 week. The observed attenuation of rotational behavior in simvastatin-pretreated lesioned group in the present study could be attributed to possible protective effect of this agent against nigral neurodegeneration and maintenance of striatal dopamine at a level that is not accompanied with a marked turning behavior. On the other hand, nigrostriatal neurons within SNC were mainly preserved against neurodegenerative effects induced by the neurotoxin 6-OHDA. In this respect, it has been reported that reactive oxygen radicals are involved in the toxicity of 6-OHDA-induced nigrostriatal lesions that is used as an experimental model of unilateral Parkinsonism (2).

Oxidative stress is considered an important pathogenic factor that could affect the survival of dopaminergic neurons in PD. Neuronal cells mostly depend on energy produced by mitochondria and are simultaneously faced with high levels of reactive oxygen species (ROS) as well as increased levels of free iron, which can promote OH formation (11). Overload of the free radical formation may lead to cell death. In addition, auto-oxidation of dopamine or levodopa overdosing may produce dopamine quinine (12).

Formation of species such as semiquinones and other free radicals could especially damage nucleic acids, proteins, and membrane lipid components (13). Therefore, the therapeutic approach is aimed at attenuation of oxidative stress. In addition, free radical scavengers may also be helpful in prolonging survival time of dopaminergic neurons (14,15). In this respect, simvastatin may have attenuated neuronal damage and loss through counteracting oxidative stress in this study, as has been reported before (6-8).

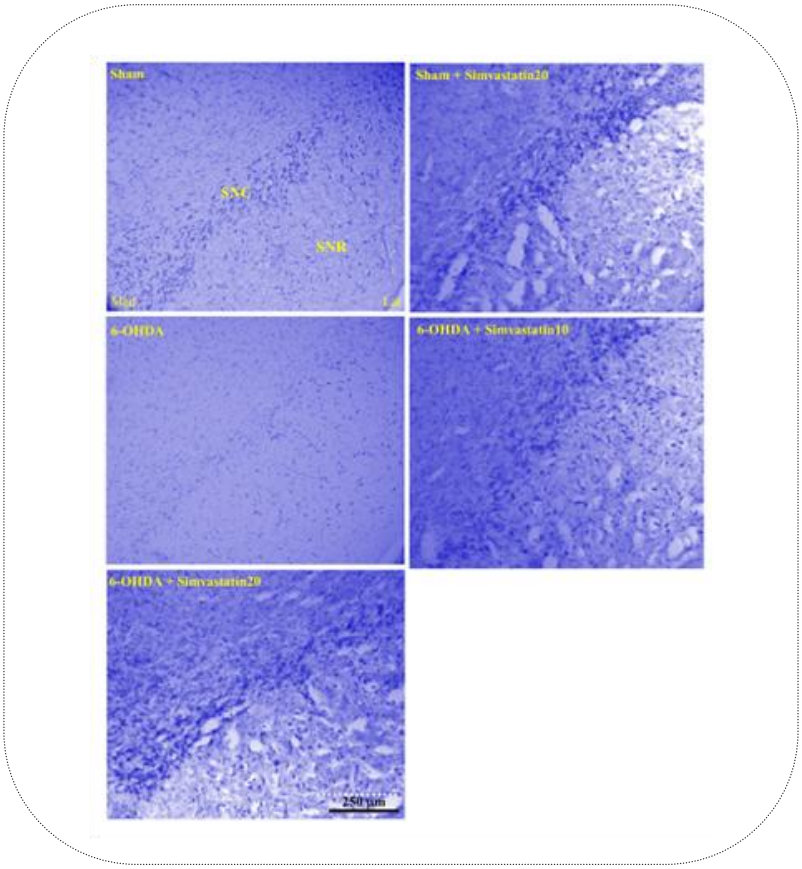

Figure 3. Photomicrograph of coronal sections through the midbrain showing Nissl-stained neurons in experimental groups. A severe reduction in the number of neurons in SNC was observed in the 6-OHDA group, but no such marked decrease was noted in the simvastatin20-treated 6-OHDA group in comparison with 6-OHDA. Scale bar = 250 µm (SNC and SNR = Substantia nigra pars compacta and pars reticulate, respectively)

To conclude, this study showed that intraperitoneal administration of simvastatin exhibits neuroprotective effect against 6-OHDA toxicity in an experimental model of PD, as was shown by a lower rotational behavior and attenuation of neuronal loss and this may be put forward as a novel adjuvant treatment for early PD in clinical settings.

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Pathological characteristics of uterus in rats with polycystic ovary

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Background and Objective: Uterus of rat with polycystic ovary (PCO) may show pathological features. We provided pathological evidence for the rat uterus with NO-induced PCO.

Materials and Methods: Wistar rats (weighing 200-250 g) were kept diestrous to receive L-arginine (50 mg/kg) intraperitoneally (i.p.) for 9 days/once a day. Control group solely received saline (1 ml/kg, 9 days/once per day). At the end of the treatment period, all animals were surgically studied. The rats' ovaries and uteri were examined biometrically and collected in 10% formalin. The pathological data were collectively determined.

Results: The treated ovaries of rats showed polycystic characteristics when compared with the control. The uteri of treated rats also showed pathological changes as compared to those that belonged to the controls.

Conclusion: The pathological aspect of rat uterus may be linked with the cystic characteristic of ovary in PCO model. This study provides pathological evidence for uterus of rat with PCO.

1. Introduction

One of the endocrine disorders of women is known as polycystic ovary (PCO) which is characterized by polycystic feature of ovary along with the hyperandrogenism and ovulatory dysfunction (1,2). Also, one who suffers from PCO may show a higher exhibition of pro-inflammatory agents such as nitric oxide (NO) (3).

We have already shown that the chronic use of L-arginine, a precursor of NO, in rats with diestrous phase may induce the PCO alongside with lipid metabolism malfunction (3), the characteristics that mark the PCO syndrome (PCOS).

Since, by reviewing of literature, the uteri of those suffering from PCO have not been much studied, we sought to evidence pathologically the PCO model's uterus. In this work, the female Wistar rats were treated chronically with Larginine to provide the PCO model. Also, the experimental animals' uteri were examined to provide valuable data representing the involvement of NO in pathophysiology of the disorder.

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2. Materials and Methods

2.1. Animals

Wistar rats (body weight 200-250 g) were purchased from Pasteur Institute of Iran and were retained under standard conditions (21 ± 3°C and 12-h light/dark cycle) with food and water ad libitum. All experiments were approved by local Ethical committee.

2.2. Drugs

L-arginine (Merck Co., Germany) was injected i.p. for a 9-day period/ once per day. The vehicle (saline at 1 ml/kg, i.p.) was used in control group.

2.3. Female Cycle Test

Since female rats with 4-5 day sexual cycle are always in diestrous (4,5) unless in case of having mating with the male rat, so, the rats were kept virgin in the present study to avoid the change in female sexual cycle.

2.4. Drug administrations

Animals were randomly divided into the L-arginine (50 mg/kg), and saline control (1 ml/kg) groups (n=6). They were injected the agent or saline intraperitoneally (i.p.) once a day during the 9 days period.

2.5. Surgery procedure

The treatment groups were anesthetized by an overdose of diethyl ether. Then, a midline incision in the lower abdomen area was performed. The ovaries and uteri were biometrically examined and dissected out. They were collected in 10% formalin for histological examination.

2.6. Histological investigation

The collected tissues were processed and sectioned at a thickness of 3-4 µm. They were stained by Hematoxylin and Eosin (H&E) method (6). The thin sections were then dehydrated, cleared, and eventually mounted with entellane (Merck Co., Germany) and coverslipped. The prepared slides were evaluated with light microscope (Olympus, Japan) at 4-40X.

2.7. Image analysis

The photomicrographs were assessed in areas of 100-µm^2 with an aid of Image Tool program (UTHSCSA, version 2.03), the free image processing and analysis program for Microsoft Windows.

2.8. Statistical analysis

All data were first assessed by Kolmogorov-Smirnov (K-S) to show the equality to analysis by variance (ANOVA). The ANOVA was then performed using SPSS software (version 13.0; SPSS, Inc., Chicago, IL), followed by post-hoc test. Statistical significance was considered at $p< 0.05$. All data are expressed as Means ± SEM. The photos were examined in an area of 100-µm^2 using the Image Tool program.

3. Results

3.1. Histology

The ovaries from the L-arginine-treated group (50 mg/kg, chronically) showed cystic formations (Fig. 1B) as compared to control samples (Fig. 1A), the aspects certifying the polycystic ovary (PCO) example.

The uteri samples of those received chronically L-arginine (50 mg/kg) (Fig. 1C) presented pathological evidence to those belonged to the controls (Fig. 1D). Due to activation of the L-arginine-related metabolic pathway, the uterus wall revealed the aspects of swelling, the proliferation and angiogenesis, suggesting an inflammatory process involvement.

3.2. Biometrical value (diameters of uteri)

The uteri diameters were calculated in all groups. They showed changes in L-arginine-treated group as compared to those obtained from saline group. The uteri of rats treated with L-arginine showed a significant increase when compared with the control group ($p<0.05$) (Fig.2).

4. Discussion

This research study showed that chronic treatment of rats with a nitric oxide (NO) agent, L-arginine, induces the polycystic ovary (PCO) formation as well as the uteri inflammation.

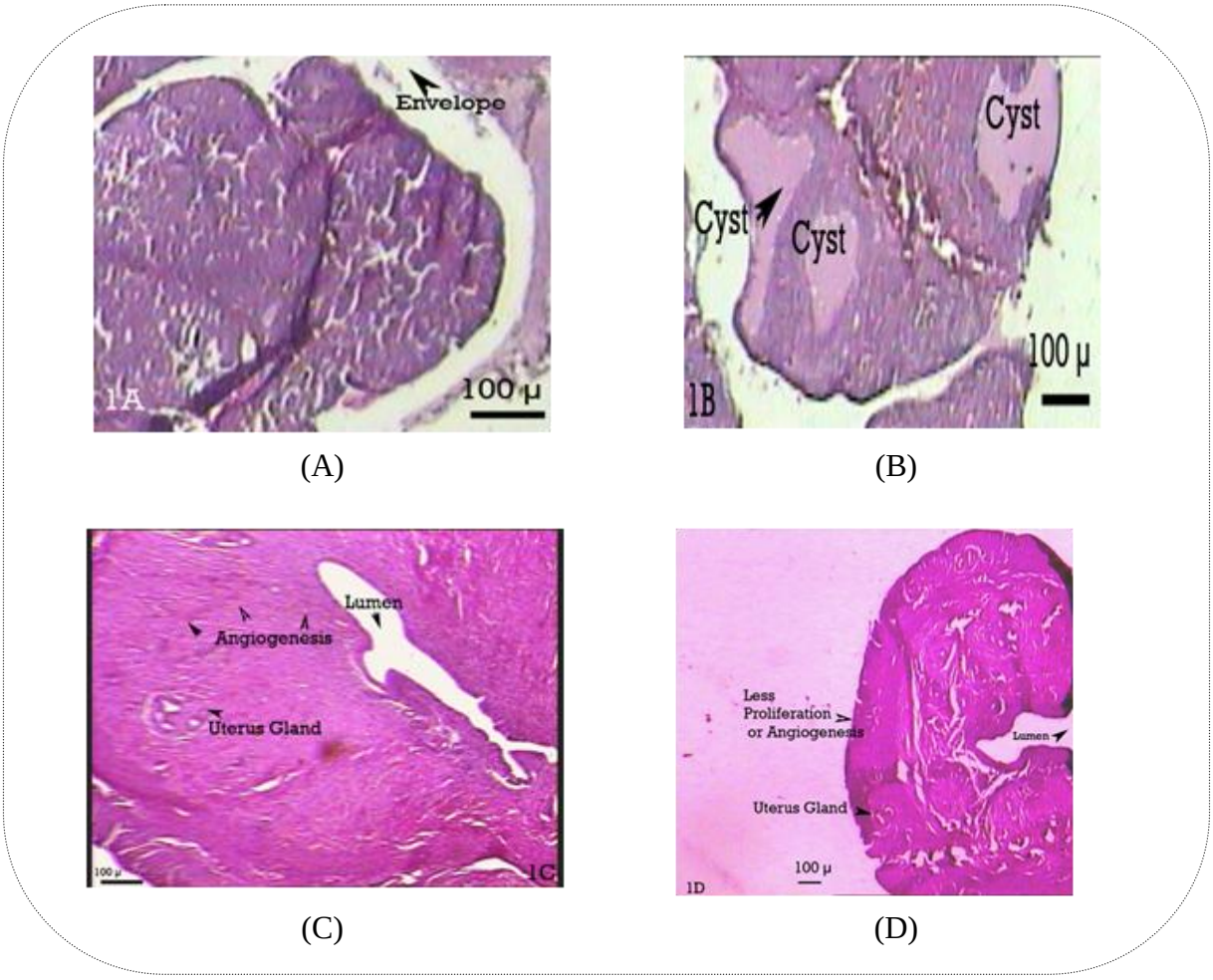

Figure 1. Photomicrographs of ovaries and uteri from control (A, C), and L-arginine-treated (B, D) rats.

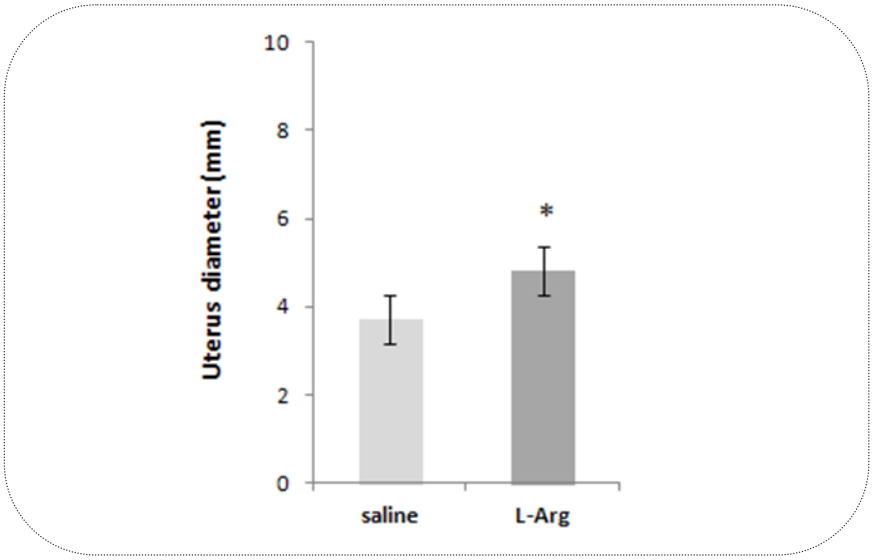

Figure 2. The diameter of uteri in rats. X axis denotes the control and experimental groups (n=6). Control was injected saline (1 ml/kg, i.p., 9 days/once per day). The experimental rats received L-arginine (50 mg/kg, i.p. for 9 days). Values are means ± S.E.M.* $p < 0.05$ vs. control (based on *Post-hoc* test).

The pro-inflammatory NO participates in endocrine physiological and pathophysiological events (7). The enzyme nitric oxide synthase (NOS) produces the NO by the oxidation of terminal guanidino nitrogen of arginine (7). We have already demonstrated the NOS activation with NADPH-diaphorase (8). The molecule NO is well introduced as a local inflammatory generator (9). The present work also provides support for a functional role of the NO in the ovarian and uterine events. This plan further supports our previous study (8), denoting that hyperactivity of enzyme NOS due to chronic usage of L-arginine induces polycystic formation in treated rats' ovaries. In agreement with this idea, it has been indicated that the presence of large cysts due to treatment by NO producer, L-arginine, accords with common characteristics of PCOS (10).

In addition of significant changes in feature of ovary, the uterus of the L-arginine-treated rats also showed differences as compared to the saline control group. These finding may suggest that the NO as a pro-inflammatory element may induce the inflammatory changes in uteri as well as ovaries. Although the exact effect of the L-arginine in this study remained elusive, the metabolic pathway may involve the NO which is known as a short-lived cytotoxic mediator (11). By viewing of the uterus diameter that increased in L-arginine-treated rats, it appears that inflammatory processes play crucial role in reproduction at all levels from the follicles and ovarian function to the accessory sex organs (i.e. uterus). We aimed to involve the NO by chronic use of L-arginine in PCO and pathology of rats' uteri. We have now evidenced that production of pro-inflammatory NO may induce significant change in the ovary and uterus parameters. It should be notified that role of NO in activation of NOergic neurons of the pelvic plexus has been previously shown (12). The NO has also been involved in the control of uterine smooth muscle via NOergic terminals (13). Based on our results, however, the exact mechanisms to involve the inflammatory processes in ovary and uterus events rest elusive.

In conclusion, this study indicates the pathological evidence in reproductive system of Wistar rats with PCO. Because of activation of the L-arginine-related metabolic pathway, the uterus wall illustrated the aspects of swelling, the proliferation and angiogenesis, suggesting an inflammatory process involvement.

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Conflict of interest

The authors state that they do not have any conflict of interest.

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Antidiabetic effect of *Teucrium polium* aqueous extract in multiple low-dose streptozotocin-induced model of type 1 diabetes in rat

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Background and Objective: *Teucrium polium* (TP) has shown hypoglycemic effect in type 1 diabetes induced by single high dose of the cytotoxic agent streptozotocin (STZ) in rats. This study was conducted to evaluate whether its aqueous extract could have such an effect in multiple low-dose STZ-induced model of type 1 diabetes in rats.

Materials and Methods: Male Wistar rats were divided into control, TP-treated control, diabetic, TP-treated diabetic groups. For induction of autoimmune model of type-1 diabetes, streptozotcin (STZ) was administered at a dose of 20 mg/kg/day for 5 days (multiple low-dose; MLD). Aqueous extract of TP was administered at a dose of 100 mg/kg for 3 weeks, started on 4th day post-STZ injection. Serum glucose level was determined before the study and at 2nd and 4th weeks after the study.

Results: TP extract-treated rats had a significantly higher weight versus diabetic rats at 4th week ($p<0.01$). In addition, serum glucose was significantly lower in TP-treated diabetic rats at 2nd and 4th weeks as compared to untreated diabetics ($p<0.005$). Meanwhile, treatment of control rats with TP extract did not significantly change serum glucose level.

Conclusion: Subchronic TP aqueous extract treatment of rats with autoimmune model of diabetes could attenuate abnormal changes in serm glucose and this may be of potential benefit in patients with type 1 diabetes.

1. Introduction

Diabetes mellitus is known as a heterogeneous complex of metabolic disorders characterized by the common phenotype hyperglycemia due to disturbances in insulin secretion, action or both (1). The development of chronic hyperglycemia in diabetes leads to severe damage in bodily tissues, organ dysfunctions and finally the irreversible failure of some critical organs of the body, especially the eyes, kidneys, nerves, and cardiovascular system (2). In addition to hyperglycemia, diabetes is itself followed by dyslipidemia and hyperlipidemia in affected patients with ensuing development of cardiovascular disorders, which are the major causes of morbidity and mortality (3). Deranged functioning of antioxidant system in diabetes leads to

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enhanced lipid peroxidation, inactivation of proteins, and protein glycation (4).

Type 1 diabetes is regarded as an autoimmune disease characterized by the infiltration of T-cells and macrophages in and around the islets of Langerhans (that is referred to as insulitis) with simultaneous and selective demolition of insulin-producing beta cells. These mononuclear cells may cause this event either directly and/or through the production and secretion of pro-inflammatory cytokines (5). This model is usually induced in rodents like rat via administering STZ at a low dose for five consecutive days (6).

Several approaches are presently used to lower the hyperglycemia in diabetes mellitus including insulin therapy which suppresses glucose production and increases glucose utilization, treatment by agents like sulfonylureas, which stimulates insulin secretion from pancreatic islet cells, agents like metformin with ability to reduce hepatic gluconeogenesis; inhibitors of α-glucosidase, which interfere with glucose absorption. Unfortunately, all of these therapies have limited efficacy and various side effects and thus searching for new classes of compounds is essential to overcome these problems (7). Recent interests have focused on the use of medicinal plants with antidiabetic and antioxidant potential in lowering the ensuing complications in diabetic patients (8). Plant-based pharmaceuticals have been employed in the management of various mankind diseases (8). They are as essential part of human diet and are present in plant extracts that have been used for centuries in oriental medicine. Antioxidant properties, ROS scavenging and cell function modulation of medicinal plants and their effective substances could mainly account for their pharmacological activity (8).

Teucrium polium L. is one of the species of the genus Teucrium from Lamiaceae family. T. polium is a perennial shrub, 20-50 cm in height, distributed widely in the dry and stony places of the hills and deserts of Mediterranean countries, South Western Asia, Europe and North Africa. T. polium (locally called as Kalpooreh) is abundantly and widely found in Iran (9). Phytochemical investigations have shown that T. polium contains various beneficial compounds including terpenoids and flavonoids. T. polium has been used in Iranian traditional medicine to treat many diseases such as abdominal pain, indigestion, common cold and urogenital diseases. The aqueous extract of the aerial parts of T. polium has been used by many type 2 diabetic patients, especially in the Southern Iran as an antidiabetic drug. In the Mediterranean countries, T. polium has been routinely used for various types of pathological conditions, such as gastrointestinal disorders, inflammations, diabetes and rheumatism (9-11). The aim of this study was to assess the hypoglycemic effect of subchronic administration of Teucrium polium aqueous extract in multiple low-dose streptozotocin-induced model of type 1 diabetes in rats.

2. Materials and Methods

2.1. Animals

Male albino Wistar rats (Pasteur's institute, Tehran, Iran) weighing 190-240 g were housed in an air-conditioned colony room at 21 ± 2 °C and supplied with standard pellet diet and tap water ad libitum. Procedures involving animals and their care were conducted in conformity with NIH guidelines for the care and use of laboratory animals.

2.2. Preparation of *Teucrium polium* aqueous extract

Fresh leaves of *Teucrium polium*, known by the local name Kalpooreh in Persian language, were collected from Alborz province in 2012. The leaves were botanically identified by the taxonomist of the Department of Botany, Shahid Beheshti University. A voucher specimen of the plant was deposited at the University's Botany Departmental herbarium. Leaves were air-dried at room temperature under shade. One hundred g of the air-dried leaves of the plant was milled into fine powder in a commercial blender. The powdered leaves were macerated and boiled in 1000 ml of distilled water for 10 min, extracted, and filtered three times. The combined aqueous extract was concentrated to waxy extract under reduced pressure in a rotary evaporator. The resulting crude aqueous extract was waxy in nature with a yield of 23% (w/w). The extract stock was kept in a 20 °C freezer until being used. Aliquot portions of the crude extract were weighed and dissolved in normal saline for use on each day of our experiment.

2.3. Experimental protocol

Male Wistar rats (n=32) were divided into equal-sized control, TP-treated control, diabetic, and TP-treated diabetic groups. Autoimmune model of type 1 diabetes mellitus was induced in rats by multiple low dose intraperitoneal injections of STZ (20 mg/kg body weight), freshly dissolved in normal saline, daily for five consecutive days. Age-matched normal animals that received an injection of an equivalent volume of normal saline comprised a non-diabetic control group. Diabetes was confirmed by the presence of hyperglycemia, polyphagia, polydipsia, polyuria and weight loss. Four days after the first STZ injection, TP aqueous extract was administered at a dose of 100 mg/kg for three weeks. Body weight and serum glucose level were recorded during the experimental period before the study (baseline) and at weeks 2 and 4.

2.4. Data and statistical analysis

All values were given as means ± SEM. Statistical analysis was carried out using repeated measure and one-way ANOVA followed by Tukey post hoc test. A statistical p value less than 0.05 considered significant.

3. Results

Body weight and serum glucose measurements (Figures 1 and 2) indicated that before diabetes induction, there were no significant differences among experimental groups. At 4th week, the weight of the vehicle-treated diabetic rats was found to be significantly decreased as compared to control rats ($p<0.05$) and TP-treated diabetic rats showed no decrease in body weight as compared to vehicle-treated diabetics and its weight was significantly higher versus diabetic rats in the same week ($p<0.01$).

Untreated diabetic rats had also an elevated serum glucose level over those of control rats ($p<0.001$-0.0005) and treatment of diabetic rats with TP extract caused a significant decrease in the serum glucose level at 2nd and 4th weeks ($p<0.005$) relative to vehicle-treated diabetics. In addition, TP extract treatment of control rats did not produce any significant change regarding serum glucose level.

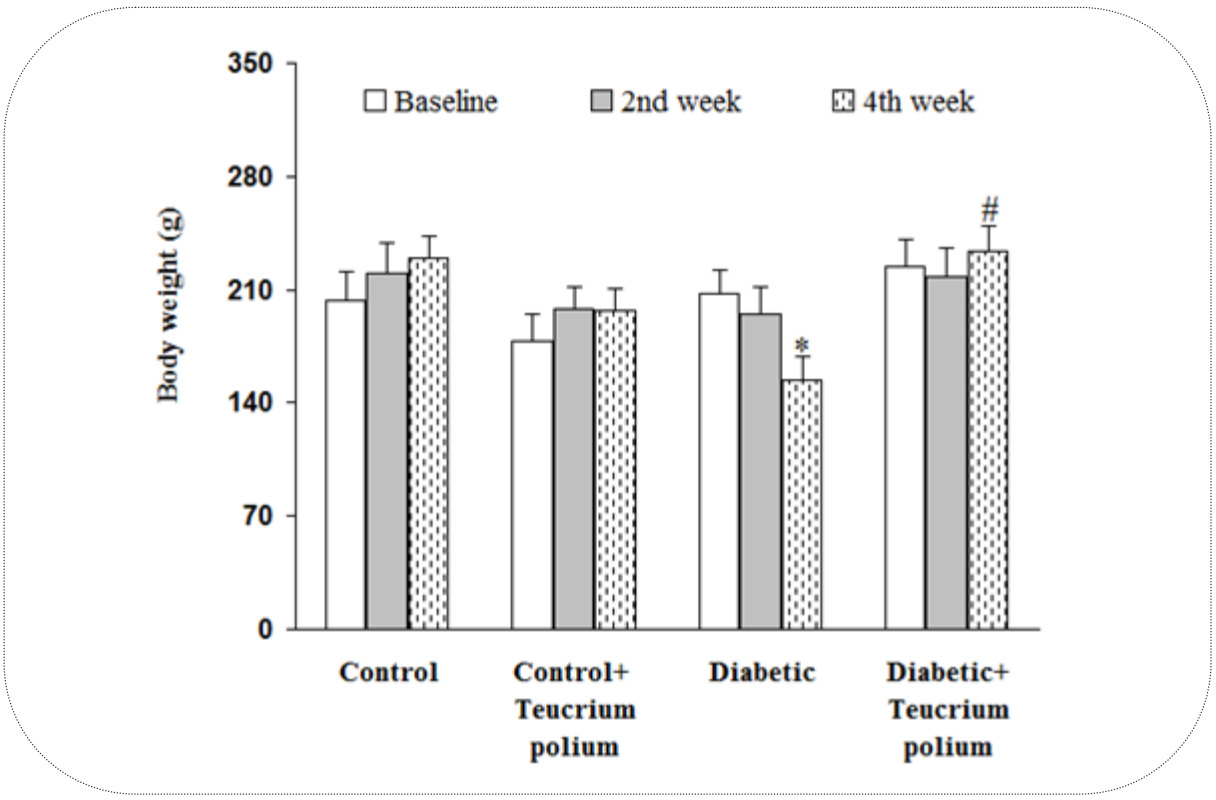

Figure 1. Body weight in different weeks (means ± S.E.M) * $p<0.05$ (as compared to baseline in the same group); # $p<0.01$ (as compared to diabetic in the same week)

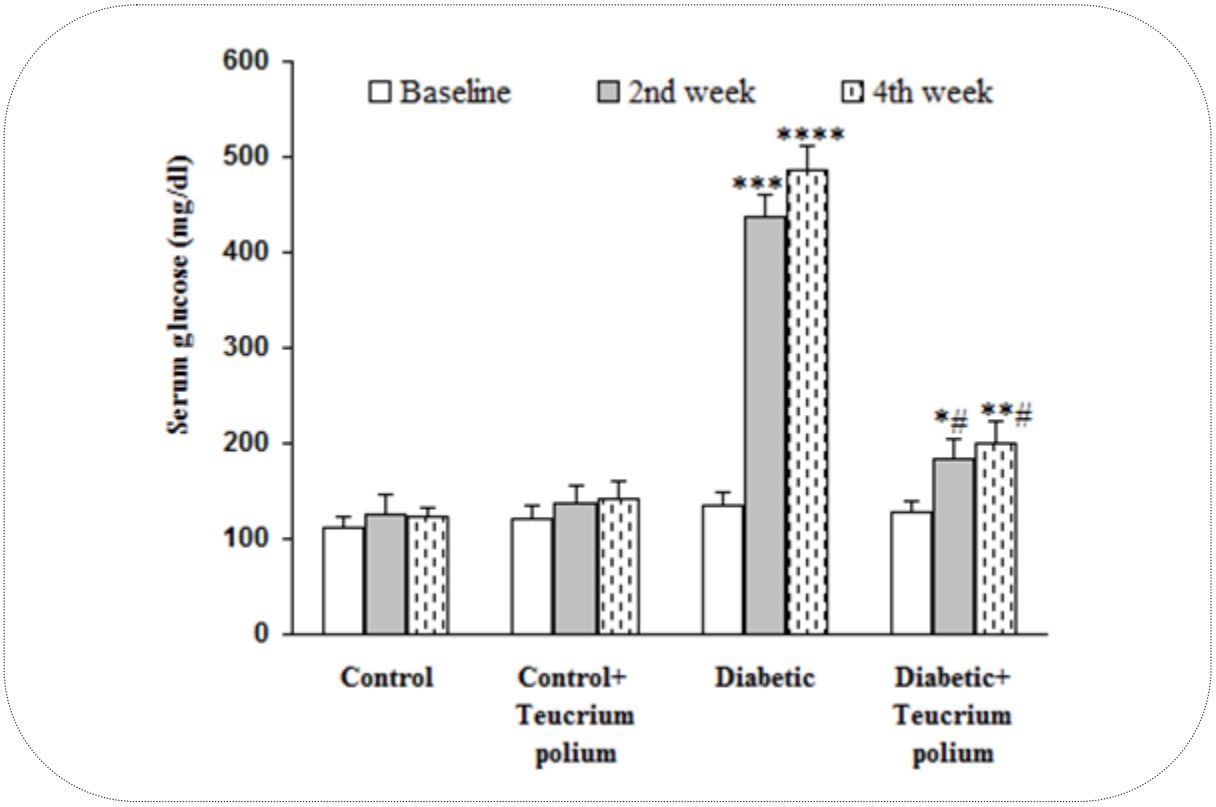

Figure 2. Serum glucose level in different weeks (means ± S.E.M) * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0005 (as compared to baseline in the same group); # p<0.005 (as compared to diabetic in the same week)

4. Discussion

In this study, TP extract-treated rats had a significantly higher weight versus diabetic rats at 4th week, serum glucose level was significantly lower in TP-treated diabetic rats at 2nd and 4th weeks as compared to untreated diabetics, and treatment of control rats with TP extract did not significantly change serum glucose level.

Although glucose-lowering effect of Teucrium polium aqueous extract was not significantly observed for control group in this study, but subchronic Teucrium polium treatment showed a marked hypoglycemic and antihyperglycemic effect in diabetic rats, indicating hypoglycemic mechanism of this medicinal plant to be different and specific in diabetic condition. The results of the previous studies have shown that TP administration to single dose STZ diabetic rats could protect and in part restore secretory function of beta cells in pancreatic tissue, in this way exerting its antihyperglycemic and antidiabetic effect (12). In addition, some flavonoids of the plant could have anti-diabetic and hypoglycemic potential (13). Such compounds have been suggested to inhibit hepatic gluconeogenesis through a ROS-dependent pathway (11). In addition, these flavonoids could exert an insulinomimetic effect and produce the cellular effects of insulin such as reducing gene expression of rate-limiting gluconeogenic enzymes (14). Furthermore, these flavonoids like the hormone insulin could increase tyrosine phosphorylation of the insulin receptor and insulin receptor substrate-1 and it reduces phosphoenolpyruvate carboxykinase gene expression in a phosphoinositide 3-kinase-dependent manner (14).

Since oxidative stress due to an increased production of ROS plays an important role in pathophysiology of diabetes, TP extract has the ability to attenuate oxidative stress and lipid peroxidation (11), and in this way maye have affected carbohydrate metabolism in this study.

In conclusion, subchronic TP aqueous extract treatment of rats with autoimmune model of diabetes could attenuate abnormal changes in serm glucose and this may be of potential benefit in patients with type 1 diabetes. More studies are

required to evaluate whether such therapy can be administered as an auxiliary beneficial therapeutic regimen in diabetic population.

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The effect of oral consumption of olive leaves on serum glucose level and lipid profile of diabetic rats

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Background and Objective: Alleviation of serum glucose level and lipid profile in diabetic patients using herbal medications is of great importance. In the present study, the effect of oral consumption of olive leaves on serum glucose level and lipid profile was investigated.

Materials and Methods: Male Wistar rats were divided into four groups including control, control under treatment, diabetic and diabetic under treatment. A single dose of streptozocin (60 mg/kg) was used to induce diabetes in rats. The two groups under treatment were fed with olive leaves powder mixed with the standard food at a ratio of 6.25% for 6 weeks. Serum glucose level and lipids profile were measured before, and at 3rd and 6th weeks after the treatment.

Results: In diabetic rats under the treatment with olive leaves, serum glucose level was significantly lower at 6th week as compared to the diabetic rats without treatment ($p<0.05$). Moreover, there was a significant decrease regarding triglyceride ($p<0.05$) and total cholesterol ($p<0.01$) in diabetic group under treatment with olive leaves as compared to diabetic rats without treatment. Also, treatment with olive leaves led to significant improvement of HDL ($p<0.05$) and LDL ($p<0.01$) as compared to untreated rats.

Conclusion: Oral consumption of olive leaves in experimental model of diabetes had hypoglycemic effect and exerts some beneficial changes in lipid profile.

1. Introduction

Diabetes mellitus (DM) is considered as one of the most important clinical risk factors involved in some disorders like nephropathy, retinopathy, neuropathy, and cardiovascular diseases, which its prevalence is predicted to be increased daily (1). DM prevalence in Iran is about 5-6%, and at the present, about 4 million Iranians are living with DM or prone to become diabetic (2). Although the most common treatment is insulin and drugs with hypoglycemic effects, but their side effects such as increasing body fat storages, body wasting at the injection site, and hypoglycemic shock are worth to mention; however, these drugs do not have many impact on long term debilitating morbidities. Regarding our daily increasing knowledge about the disease, it is really needed to seek for new medications with fewer side effects (3). Herbal medication usage is of great importance in traditional medicine and such plants have been used for the treatment of many diseases for a long time, whereas there are not still enough scientific evidences about the

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effects of most of them (4). From the plants used for diabetes, olive (*Olea europia*) is of great importance.

Olive is a shrub from oleace species with permanent green leaves, which grows wild with about 5m or more height. Used parts of the tree include the fruit and leaves. This plant has been mentioned in traditional medicine as having the following effects; antihypertensive, anti-atherosclerotic, laxative, giving strength, effective on urinary tracts infection, headache treatment, and antioxidant (5). Also, there are some reports available about the effects of olive leaves in the treatment of malaria (6).

In this study, rats were treated with olive leaves to investigate its anti-diabetic effects. For this purpose, serum glucose level and lipid profile were measured during the study.

2. Materials and Methods

2.1. Collection, identification and preparation of the plant

Olea europia leaves were collected and identified by Karaj Agriculture Faculty taxonomists. Plant leaves were dried at 25°C under shade, then powdered with mechanical grinder. Olive leaves powder was mixed with rats food at a ratio of 6.25%, and the mixture was used as the food for rats under treatment (7).

2.2. Animals

This investigation was an experimental study, in which 30 adult male Wistar rats (weight range from 200 to 250 g) were used (Razi Institute). Rats were kept at animal room, and water and food were provided ad libitum.

2.3. Methods

In this study, only male Wistar rats with a serum glucose level lower than 250 mg/dl at normal conditions without fasting were used (8). Rats were randomly divided into 4 groups including control, control under treatment, diabetic and diabetic under treatment. Control and diabetic groups used only standard food, while control group under treatment and diabetic group under treatment used standard food in combination with the olive leaves powder. Treatment lasted for six weeks. Streptozocin at a dose of 60 mg/kg (single dose) dissolved in normal saline was used intraperitoneally to induce diabetes in rats (8). Diabetes signs including weight loss, polydipsia, and polyuria appeared after 5-7 days. More assurance was obtained after detecting glucosuria and serum glucose level of more than 250 mg/dl.

Blood samples were taken 3 times from rats, the first and second times were performed using capillary tubes from retroorbital capillary vessels, and the third time was performed from the heart. Samples were kept in microtubes at -70°C to measure glucose, cholesterol, triglyceride, HDL and LDL using available commercial kits (ZistChem Co., Tehran).

2.4. Statistical analysis

Data were expressed as positive and negative mean standard deviation. ANOVA test with repeated measures was used to compare each parameter in each group before and after the treatment. Also, one-way ANOVA and Tukey's post-test were used to compare groups with each other. In addition, $p<0.05$ was considered statistically significant.

3. Results

3.1. Serum glucose level

Serum glucose level significantly increased in diabetic rats as compared to control rats ($p<0.0001$). Also, serum glucose level showed a 38% statistically significant decrease in diabetic group under treatment with olive leaves as compared to diabetics without treatment ($p<0.005$).

3.2. Serum lipids

Serum triglyceride level was higher in diabetic group ($p<0.005$). After the 6th week, triglyceride level showed a 77% statistically significant decrease in diabetic group under treatment as compared to untreated rats.

Moreover, diabetes caused significant elevation of cholesterol and olive leaves consumption led to a significant decrease of cholesterol level in diabetic group under treatment as compared to those without treatment ($p<0.005$). HDL cholesterol level showed a 48% increase at 6th

week in diabetic group under treatment which was also significant versus diabetics (p<0.01). Despite a significant increase of LDL cholesterol in diabetic group, its level significantly decreased in diabetic group under treatment as compared to those diabetics without treatment (p< 0.01).

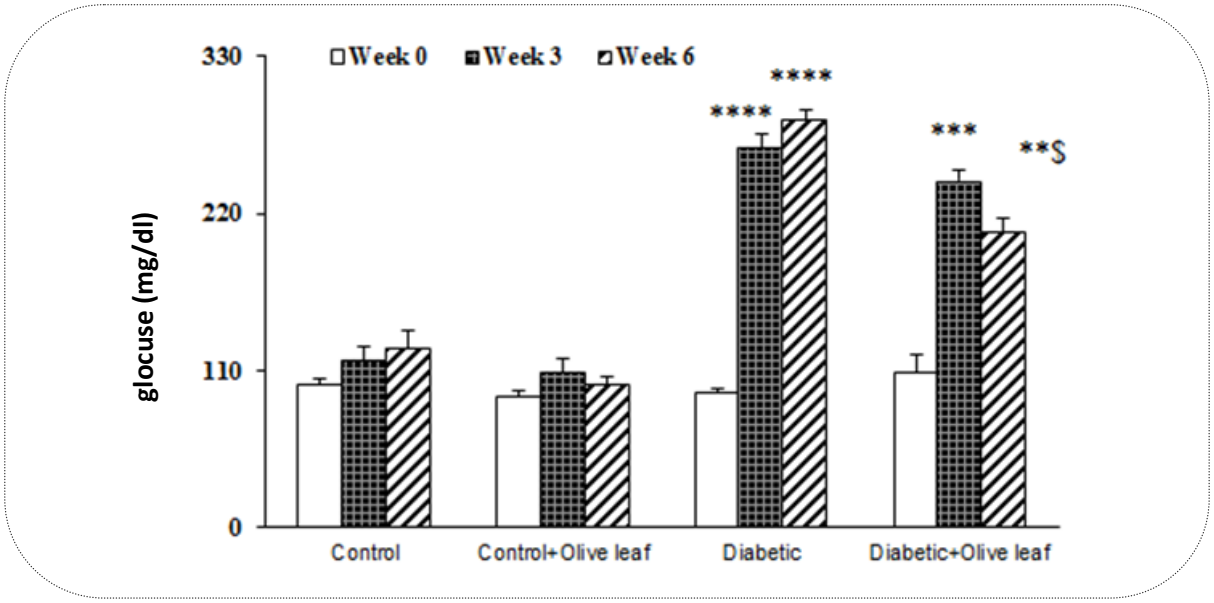

Figure 1. The effect of long-term oral consumption of olive leaves on serum glucose level of control and diabetic rats. ** p<0.005, *** p<0.001, ****p<0.0001 (as compared to week 0 in the same group), $ p<0.01 (as compared to diabetic group in the same week)

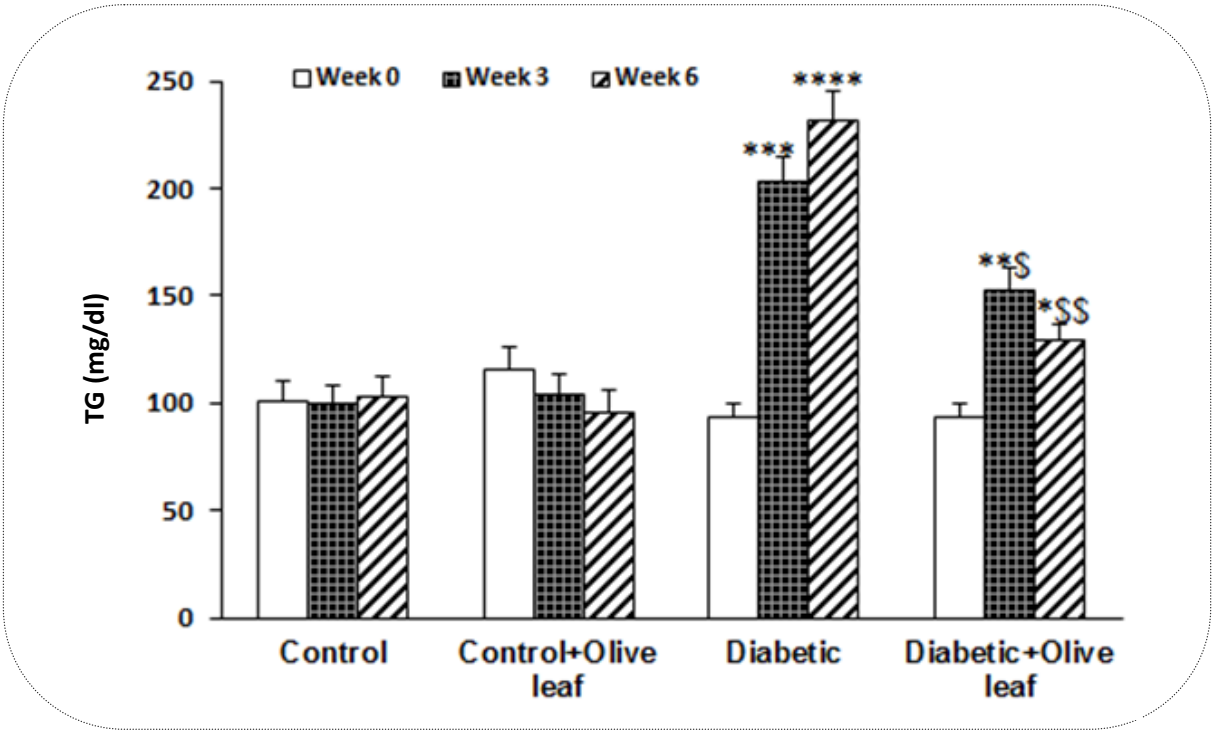

Figure 2. The effect of long-term oral consumption of olive leaves on serum triglyceride in control and diabetic rats. * P<0.05, ** p<0.01, *** p<0.005, **** p<0.001 (as compared to week 0 in the same group), $ p<0.05, $$ p<0.005 (as compared to diabetic group in the same week)

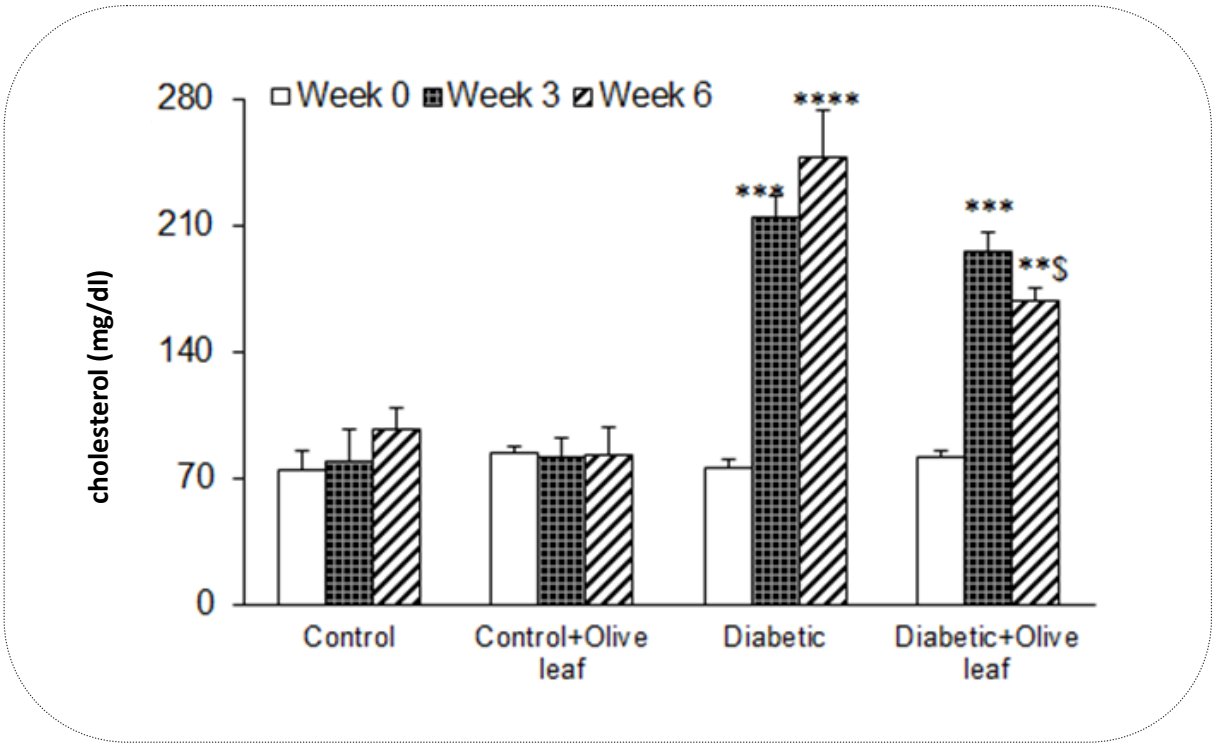

Figure 3. The effect of long-term oral consumption of olive leaves on serum cholesterol in control and diabetic rats. ** p<0.005, *** p<0.001, **** p<0.0005 (as compared to week 0 in the same group), $ p<0.01 (as compared to diabetic group in the same week)

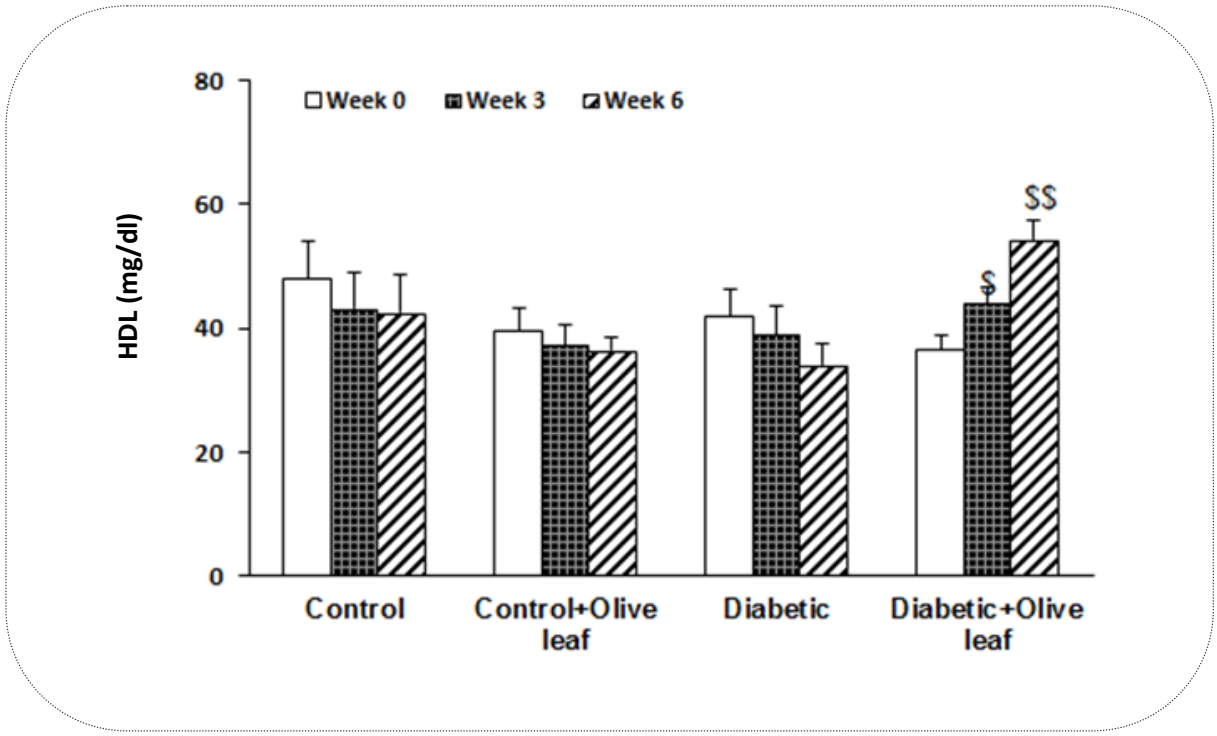

Figure 4. The effect of long-term oral consumption of olive leaves on serum HDL cholesterol level in control and diabetic rats. $ p<0.05, $$ p<0.005 (as compared to diabetic group in the same week)

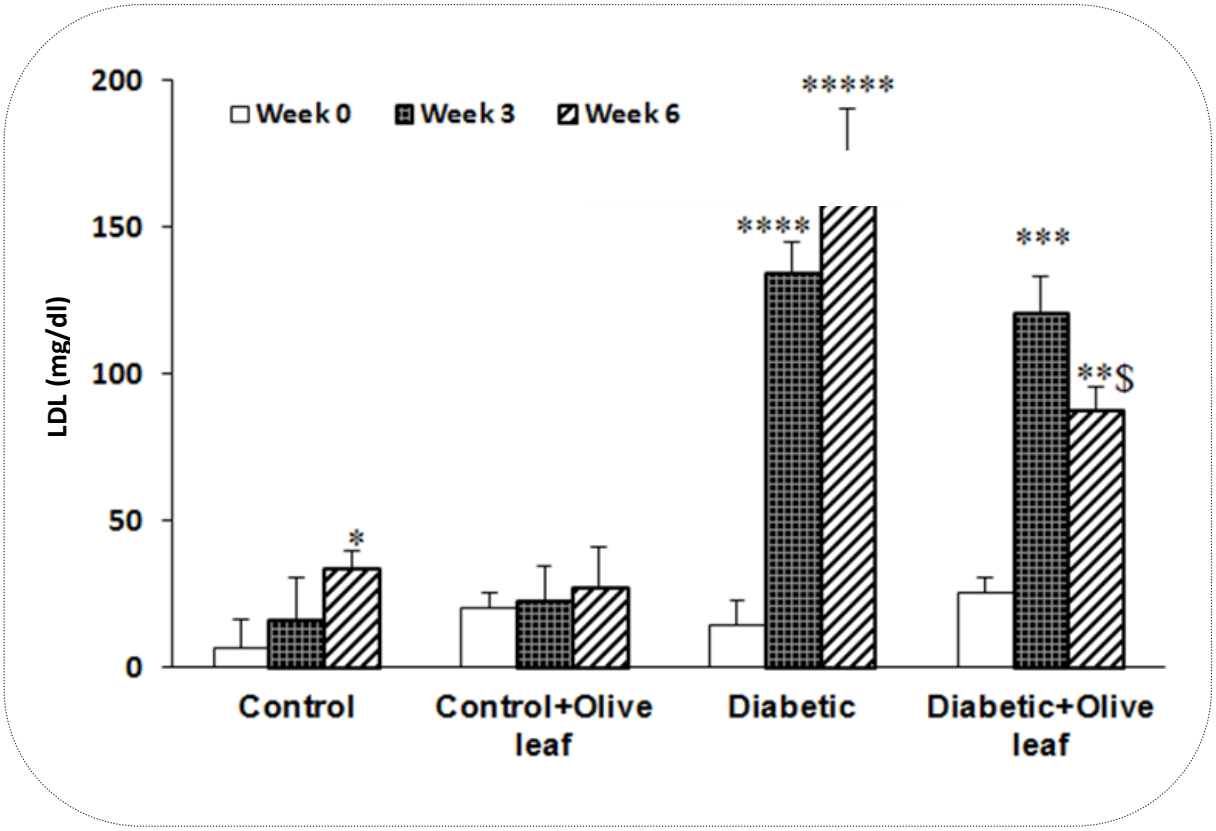

Figure 5. The effect of long-term oral consumption of olive leaves on the LDL cholesterol level in diabetic and control rats. * p<0.05, ** p<0.005*** p<0.001, **** p<0.0005, ***** p<0.0001(as compared to week 0 in the same group), $ p<0.01 (as compared to diabetic group in the same week)

4. Discussion

The results of the present study indicated that oral consumption of olive leaves powder for 6 weeks in diabetic rats has hypoglycemic effect and causes beneficial changes of cholesterol, HDL and LDL levels.

Streptozocin-induced diabetes leads to some changes at metabolic enzymes level due to absence or very low levels of insulin, therefore causes hyperglycemia. Diabetes causes some inappropriate changes of plasma lipids and lipoproteins, in which some body tissues especially liver have a significant role with absorption of serum free fatty acids, then oxidation and metabolic changes of these fatty acids to other undesired molecules, cholesterol and phospholipids synthesis, and secretion of some proteins into the plasma (9). Furthermore, an increase in triglyceride and cholesterol levels in this study is in line with the existing evidence (10). Moreover, increased levels of serum glucose in diabetic rats can increase triglyceride, LDL, VLDL and indirectly decreases HDL (10).

The hypoglycemic effect of olive leaves powder is presumably due to an increase in glucose consumption by the peripheral tissues (11). Olive leaves powder causes glucose consumption maintenance, probably due to continuing the response to insulin and inhibition of intestinal absorption of glucose (12). Eidi et al results showed that olive leaves alcoholic extract leads to a decrease in serum glucose level and an increase in serum insulin level in diabetic rats, but no effect was seen on healthy animals (13).

Moreover, komeyli et al study indicated the hypoglycemic effect of aqueous extract of olive leaves in diabetic rats, blood cholesterol and triglyceride also decreased and HDL cholesterol increased, which is in accordance with our results (14). Although Jamae et al evaluated the effects of some other extracts of olive leaves on alloxan-induced diabetic rats, they concluded that serum glucose and cholesterol levels significantly

decreased and this effect is due to antioxidant properties of olive leaves (15). In an investigation conducted by Alazavi et al on healthy and alloxan-induced diabetic rabbits for 16 weeks, hypoglycemic and antioxidant properties of olive leaves were approved and long-term administration of olive leaves led to a decrease in lipid peroxidation products like MDA (16).

In conclusion, long-term oral consumption of olive leaves by streptozocin-induced diabetic rats has hypoglycemic effect, decreases serum triglyceride, total cholesterol and LDL cholesterol and increases HDL level.

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Evaluation of the effect of oral administration of Hab-o Shefa on morphine withdrawal syndrome in rats: a behavioral study

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A B S T R A C T

Background and Objective: Traditional Iranian Medicine (TIM) has a long history in the field of diagnosis and treatment of various diseases, particularly addiction. Different therapeutic methods have been recommended in this respect. One of these methods is the replacement of natural narcotics instead of opium. Hab-o Shefa is a natural product of TIM which has been used as an alternative for opium in the treatment of addiction since centuries ago. In this study, the effect of Hab-o Shefa was investigated on behavioral quantities of morphine withdrawal syndrome.

Materials and Methods: A total of 30 rats were divided into three groups of ten cases each. The control group received solely morphine at a dosage of 10 mg/kg daily for 8 days by the intraperitoneal route. In the second group, in addition to morphin with the same dosage, methadone at the dosage of 25 mg/kg was daily administered by gavage. Hab-o Shefa at a dosage of 2000 mg/kg through gavage was administered in addition to 10 mg/kg of morphine daily for the third group. Finally and 4 to 24 hours after the last injection of morphine, naloxone was injected i.p. at a dosage of 2.5 mg/kg and the desired withdrawal parameters were evaluated.

Results: Considering uncountable parameters, a significant difference was seen when comparing methadone and Hab-o Shefa with placebo in regarding diarrhea symptoms ($p<0.05$). Regarding jumping between Hab-o Shefa and placebo and also between methadone and placebo, the difference was also statistically significant ($p<0.05$).

Conclusion: In summary, Hab-o Shefa better controlled the withdrawal symptoms in comparison with placebo and it also better improved the symptoms of diarrhea and salivation as compared to methadone.

1. Introduction

Opiate abuse and dependence is one of the world's major health problems. (7,11). Different therapeutic methods have been presented for the treatment of opiate addiction. Although these drugs have been successful in controlling some of withdrawal symptoms, they have not been able yet to control the symptoms completely, some of them are not available everywhere or are abused in other ways. (5,10). Therefore, herbal medicines can be an excellent option for solving this problem given the high

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social acceptance due to the low complications, high effectiveness, their availability and low cost (13). According to the World Health Organization recommendations regarding the use of traditional medicine in health systems (12,14), addiction treatment has been reviewed from the perspective of TIM scientists so that they can play their role in solving this problem. Various therapies for addiction treatment have been introduced in TIM (8). One of these methods is the replacement of opium with natural narcotic drugs (16,2). Several natural narcotic drugs as isolated or in a combination have been introduced in TIM text books (8,16,2,4,15,17,2,3). Hab-o Shefa is a natural combination of drugs which most of TIM scientists have a consensus on the positive effects of this combination in addiction treatment and it has been used for several centuries as a viable alternative for opium in the treatment of opium addiction. This compound contains Datura stramonium, Rheum palmatum, Zingiber officinale and Acacia Arabica (8,16,1,4,15,16). In this study, the efficacy of this drug in controlling morphine withdrawal symptoms was evaluated in rats.

2. Materials and Methods

Habo- Shefa is a natural combination of Datura stramonium, Heum palmatum, Zingiber officinalea and Acacia arabica which was prepared in the pharmaceutical Laboratory of Traditional Medicine of the Shahed University of Tehran (Iran).

Methadone and morphine were purchased in the form of powder from the Daroupakhsh Company.

2.1. Animals

In this study, 30 male rats of the NMRI strain (Razi Institute of Iran), weighing 250-330 g, were used. These rats were placed in cages quaternary and the temperature was set between 22-25 °C. Ambient light as a 12- hour light 12-hour dark cycle was controlled by a timer. The required amount of water and food was available to each rat. In all the performed experiments the research ethics on animals were fully observed.

2.2. Dependency induction

For creating dependency in animals, morphine at a daily dosage of 10 mg/kg for 8 days was injected i.p. (18).

2.3. Behavioral evaluation

For the study of behavioral dependency after the 8-day morphine injection and 4 to 24 hours after the final injection of morphine to one of the rats in each group, 2.5 mg/kg of naloxone was injected i.p. and the behavioral symptoms were assessed during the following forty minutes. Subsequently, the other rats in each group were also studied one by one. Behavioral indices of withdrawal syndrome including diarrhea, ptosis and teeth chattering are indicative of morphine dependency of such animals. In general, behaviors that occur during the withdrawal syndrome in terms of being countable or non-countable are divided into two categories by the standard agreement; graded signs and chocked signs. Graded signs included jumping, abdominal contraction, wet dog shakes and chocked signs included diarrhea, teeth chattering, salivation, ptosis and genital grooming/ejaculation.

2.4. Experimental groups

In this study, 30 rats were divided into three groups of 10 cases each; the first group named as the control group only received morphine 10 mg/kg daily for 8 days injected i.p.; the methadone group received the same dose of morphine injection plus 25 mg/kg of methadone daily for 8 days by gavage. In the Hab-o Shefa group besides administering the same dose of morphine injection, Hab-o Shefa was prescribed at a dosage of 2000 mg/kg daily for 8 days through gavage.

2.5. Statistical analysis

For data analysis, the graded signs data were recorded as the number of behaviors occurring in a certain time duration in each rat. After confirming that behaviors of the withdrawal syndrome have a quantitative entity, the number of such behaviors was compared in different groups with ANOVA and Tukey post-hoc test.

3. Results

Figure 1 shows the number of diarrhea, ptosis and salivation in the three groups. Comparing these numbers between the methadone and Hab-o Shefa groups did not reveal a statistically significant difference ($p>0.05$), but the difference

was significant when comparing these two groups with the placebo group ($p<0.05$). Regarding genital grooming/ejaculation and teeth chattering, no statistically significant difference was observed between the three groups ($p>0.05$).

Figure 2 shows the number of jumps in the three groups. Comparing the number of jumps between Hab-o Shefa and methadone with placebo showed a statistically significant difference ($p<0.05$). No statistically significant difference was observed when comparing the number of abdominal contractions and wet dog shakes between the three groups ($p>0.05$).

4. Discussion

In the previous study by Mohsen Khalili and Mohsen Naseri et al (9), the effectiveness of datura seeds extracts in controlling morphine withdrawal symptoms in rats was studied. In the latter study, the efficacy of datura seeds extract was significantly superior to placebo, whereas no significant difference was seen in comparison with methadone. Hab-o Shefa acted better than datura extract in controlling symptoms such as diarrhea, salivation and ptosis as compared to methadone. Considering that Hab-o Shefa is a natural compound and datura is its main

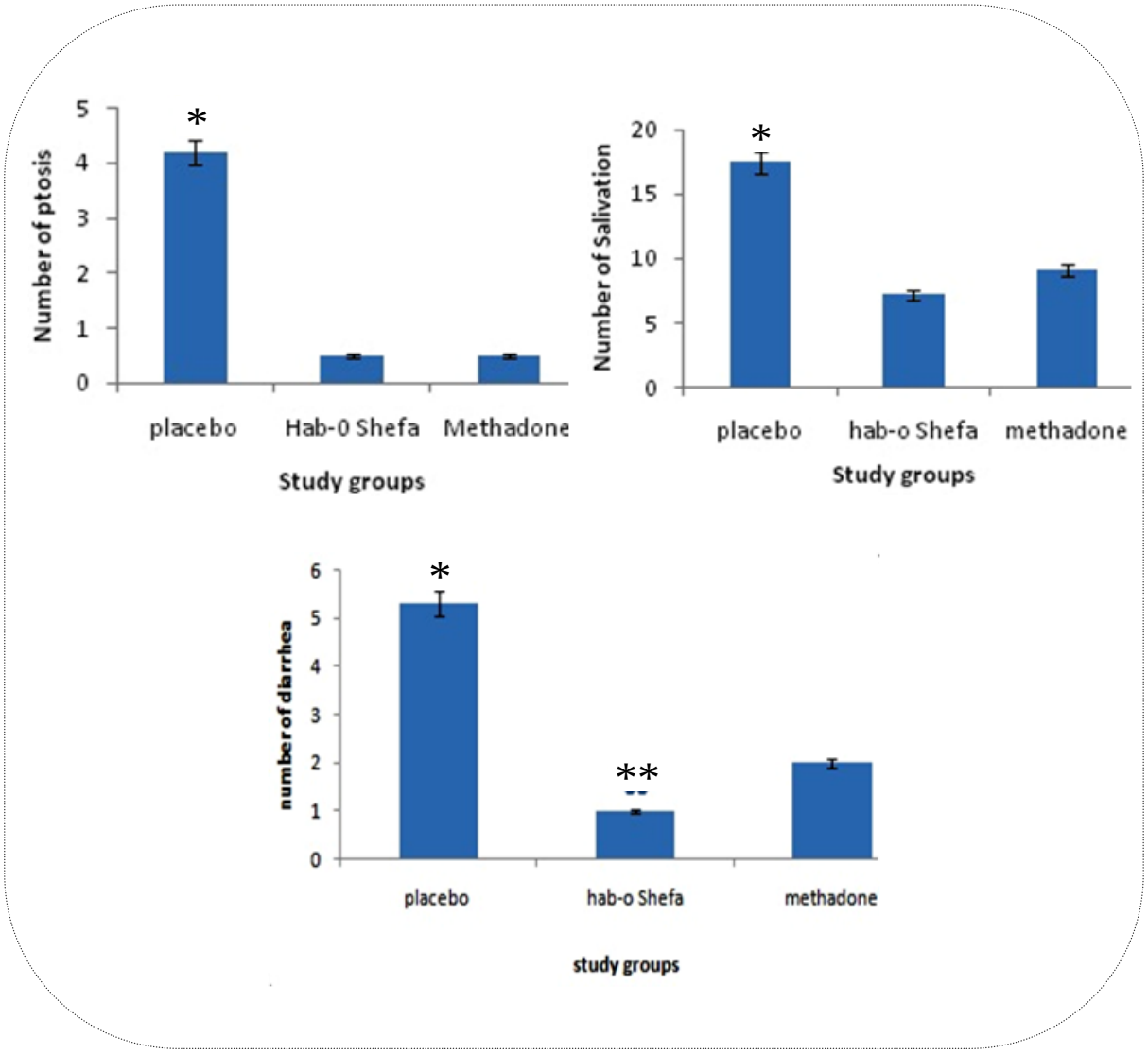

Figure 1. Comparison of innumerable withdrawal symptoms in rats. As can be seen, the parameters of diarrhea, ptosis and salivation for Hab-o Shefa and methadone groups as compared together was not significant ($p>0.05$) and between these two and placebo was significant ($p<0.05$) * $p<0.05$ ** .

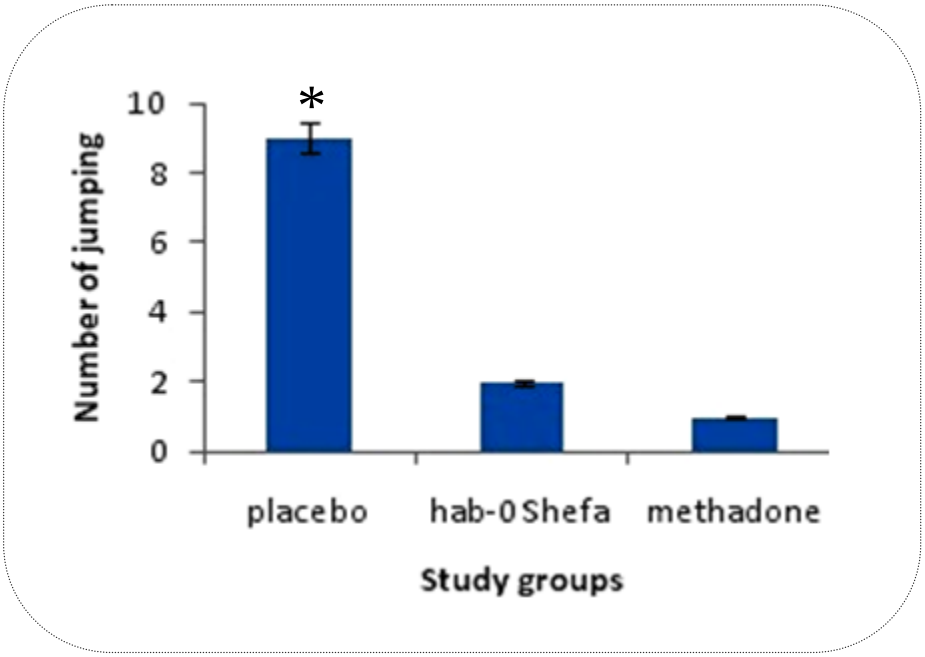

Figure 2. Comparison of countable jumps in the three groups. As can be seen, jumping between the Hab-o Shefa and methadone was not statistically significant ($p>0.05$) and was statistically significant between these two groups and the placebo * $p<0.05$

component and the other herbs in this combination act as modifiers of stramonium effects, it can be concluded that natural drug combinations have a better efficacy than natural isolated drugs and also reduce the risk of adverse drug reactions. In addition, in relation to mechanism of action of natural combination drugs, TIM scientists believe that these compounds have their unique properties in general and certain effects cannot be attributed to a specific substance contained in the combination; for example, regarding Hab-o Shefa, the effectiveness of its pharmaceutical composition cannot be attributed to the alkaloids contained in datura plant or to antiserotoninergic substances contained in ginger or the flavonoids contained in Rheum palmatum or the tannin contained in Acacia arabica.

Conclusion

Due to the better efficacy of Hab-o Shefa in comparison to datura extract in controlling morphine withdrawal symptoms, the use of TIM combination drugs could result in lower drug side effects and higher efficacy. In complementary future studies, clinical trials will be performed on the natural combination of Hab-o Shefa in controlling withdrawal symptoms of opioids.

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