Original Article

Microinjection of a Dopamine-D1 Receptor Agonist into the Ventral Tegmental Area Reverses the Blocked Expression of Morphine Conditioned Place Preference by N-Methyl-D-Aspartate Receptor Antagonist

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Abstract

Background: The release of dopamine (DA) in the posterior ventral tegmental area (pVTA) plays an important role in cue-related learning, reward, and relapse. On the other hand, studies have shown that the use of N-methyl-D-aspartate receptor (NMDAR) antagonist (AP5) inhibits the expression of morphine (5 mg/kg, s. c) conditioned place preference (CPP). In this study, we have tried to show the interaction effect of the DA stimulatory agents through D1-like receptor (D1R) agonist (SKF38393) and D2-like receptor (D2R) antagonist (eticlopride; through disinhibition) with NMDAR antagonist into the pVTA on the expression of morphine CPP. **Materials and Methods:** The SKF38393 and eticlopride, individually and simultaneously (in ineffective doses), were injected into the pVTA with the AP5 in rats, and animals were then placed in a CPP apparatus. **Results:** Concomitant administration of D1R agonist (4 μg/rat) with NMDAR antagonist (1 μg/rat) induced the expression of morphine CPP, but the administration of D2R antagonist with NMDAR antagonist was unaffected on the expression of morphine CPP. Furthermore, concomitant administration of ineffective doses of D1R agonist and D2R antagonist with NMDAR antagonist had no effect on the expression of morphine CPP. **Conclusions:** The results showed using higher doses of D1R agonist with NMDAR antagonist could reverse the blocked expression of morphine CPP by NMDAR antagonists, while, the use of D2R antagonist with NMDAR antagonist could not. Therefore, presynaptic receptors such as D1R probably through releasing other stimulatory neurotransmitters can play a vital role in the expression of morphine CPP and cue-related learning.

Keywords: *Receptors, N-Methyl-D-Aspartate, morphine, microinjections, Receptors, Dopamine D1, Dopamine agonists, Dopamine D2 Receptor antagonists, ventral tegmental area*

Introduction

Many studies have shown natural stimuli and drug abuse-related rewarding effects are mediated through the mesocorticolimbic dopamine (DA) system.[1-3] One of the most important areas in reward pathway comes from dopaminergic (DAergic) neurons of ventral tegmental area (VTA), especially in the posterior region of VTA (pVTA) that reciprocally has a connection to the various area such as medial prefrontal cortex (mPFC) and nucleus accumbens (NAc) involved in motivational behavior, memory and cue-related learning.[4]

Consumption of abuse drugs lead to the increased activity of glutamatergic inputs into the VTA that increases firing rate in DAergic neurons and subsequent DA release in the NAc and mPFC, axonally,[5] and also in the VTA, somatodendrically.[6] DA increased in the VTA can affect D1R located in the terminal of glutamate axon inputs in the VTA that facilitates glutamate release and subsequent excitatory effects on DAergic neurons, especially in the pVTA. This DA enhanced by sustaining the activity of DAergic neurons has a role in cue-related learning.[7] In many of various studies have shown the roles of excitatory inputs such as serotonergic,[8] orexinergic,[9] and cholinergic inputs in the VTA[10,11] in the activation of DAergic neurons and the induction of cue-related learning. Of course, there is little mention in the literature about the release of these agents, presynaptically, by D1R in VTA. It has been made clear that blockade

How to cite this article: Ahmadian SM, Ghahremani P, Alaei H. Microinjection of a dopamine-D1 receptor agonist into the ventral tegmental area reverses the blocked expression of morphine conditioned place preference by N-methyl-D-aspartate receptor antagonist. Adv Biomed Res 2020;9:54.

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Received: 14 January 2020
Revised: 17 February 2020
Accepted: 22 April 2020
Published: 30 October 2020

Access this article online
Website: www.advbiores.net
DOI: 10.4103/abr.abr_11_20
Quick Response Code:

of D1R and N-methyl-D-aspartate receptor (NMDAR) by D1[7] and glutamate receptors antagonist,[12] inhibit the expression of drug abuse-induced conditioned place preference (CPP). Therefore, the presence of NMDA and D1Rs are necessary for reward-related learning. On the other hand, increased DA concentration also can through D2 autoreceptor, inhibit the VTA DAergic neurons firing; therefore, the elimination of inhibitory effects of D2R, using D2R antagonist, may lead to facilitating the DAergic neurons firing.[13] This dual-function of DA is necessary for the performance of the reward system. It is suggested that the infusion of D1R agonist and removing DA inhibitory effect by inhibiting D2R, can have a stimulatory effect on the VTA DAergic neurons activity. Hence, in this study, we have investigated the presynaptic role of D1R (in releasing different neurotransmitters) and postsynaptic D2R on the DAergic neurons in the pVTA, to determine whether the excitation of D1Rs (using D1R agonist) and also the inhibition of D2Rs (using D2R antagonist) in the pVTA can reverse the blocked expression of morphine CPP by an NMDA receptor antagonist or not. Hence, this way can be applied to study the details of the reward circuit of the brain in cue-related learning.

Materials and Methods

Subjects

Adult male Wistar rats (Royan; Isfahan Iran), weighing 230–300 g (n = 6-9) at the time of surgery were used. They had free access to food and water, were housed four in a cage, and kept at (22 ± 2ºC) under a 12/12 h light-dark cycle (light beginning at 7:00 a. m). Experimental groups consisted of eight animals, and each animal was tested once. The Ethics Committee of Animal Use of the Isfahan University of Medical Sciences approved this study, and all tests were performed in accordance with the instructions for Animal Care and also the use of Laboratory Animals (National Institutes of Health Publication No. 85-23), revised in 2010.

Drugs

Morphine sulfate (Temad, Tehran, Iran) was dissolved in saline, and injected subcutaneously (5 mg/kg; SC, pH = 7.4), S-(−)-Eticlopride hydrochloride a D2 receptor antagonist,®-(+)-SKF-38393 hydrochloride a D1 receptor agonist and 2-amino-5-phosphonopentanoic acid an NMDA receptor (AP5) antagonist (Sigma-Aldrich, Germany) were dissolved in saline and were injected in the pVTA.

Surgery and drug microinjection

Rats were anesthetized intraperitoneally with a ketamine/xylazine mixture (100 and 10 mg/kg, respectively) and placed in a stereotaxic frame (Stoelting, USA) with the flat-skull position.

Two stainless steel, 23-gauge guide cannula, were bilaterally placed 1 mm above the VTA (AP = −5.6 mm; ML= ±2.1 mm; DV = −8.4 mm),[14] and anchored to the skull with dental cement. Bilateral stainless steel stylets (30 gauges) were implanted into the guide cannula, to be kept free of debris. Each rat was placed separately in the cage, and the opportunity given to recover for 7 days.

To drug microinjections, stylets brought out, and 30G injector needles were inserted 1 mm beneath the tip of the guide cannula, into the VTA. Subsequently, different doses of the SKF38393, eticlopride, AP5, or saline were administered by the microinjection apparatus (KD Scientific, USA) bilaterally in a total volume of 0.6 µl/rat (0.3 µl in each side), over a 60s period.

Apparatus

The best method for measuring drug reward is the apparatus of CPP. The CPP apparatus was included from three chambers (A, B, and C). Two large chambers (A and B) with equivalent size. The walls and floor of the A chamber are black with a grid floor, while they were white and checkered with a smooth floor in the B chamber, respectively. The C chamber was tiny, and it was jointed to other chambers by a guillotine door. The time animal spent in each chamber and its locomotor activity was recorded by using a video track software (ANY-maze**,** Stoelting Co., USA**)**. The CPP was accomplish using a biased method, in which the animal was devoted to the nonpreferred chamber, following the administration of effective dose of morphine (5 mg/kg). The behavioral procedure of CPP was done in nine successive days with four different phases: Habituation, pre-conditioning, conditioning, and postconditioning.[15]

Habituation

On the 1st and 2nd days, each rat was placed in the start chamber C, and after 1 min, the door was open. The animal was then allowed to explore the entire apparatus for 15 min. The experiments were started 2 days after habituation.

Preconditioning

On the 3rd day, each rat was inserted into the C chamber, while the guillotine door was open, and the rat was permitted to move freely for 15 min. Software any maze was used for recording the time spent and locomotor activity of the animals.

Conditioning

It was included a 5-day plan that contained ten sessions (5 for saline and 5 for morphine), and each session took time of 45 min. The guillotine gate was closed, and also daily infusion was accomplished in two stages, with a 6-h interval. In the morning of the 4th and 8th days, after injectioning morphine, rats were confined to nonpreferred chamber and in the evening, after injection of saline, to the preferred chamber. On the 4th day, rats received morphine in the morning and saline in the evening.

Postconditioning

On the 9th day, similar to the 3rd day, after injection of drugs (SKF38393, Eticlopride, and AP5), each rat was inserted into the C chamber for 15 min, while the guillotine gate was open. The conditioning score was computed as the time spent in the morphine-paired chamber minus the time spent at the same chamber on the 3rd day.

Experimental design

Dose-response curve for morphine

In this study, a fixed ratio schedule of reinforcement was arranged. We examined the effects of four doses of morphine (0.5, 2.5, 5and 7.5 mg/kg, s. c), on the CPP in this experiment. Rats were given saline (1 ml/kg, s. c), in the vehicle group in both chambers (A and B). A dose of morphine (5 mg/kg, s. c), were used as an effective dose.

Intra-VTA microinjection of SKF38393, eticlopride, and AP5

To evaluate the effects of SKF38393 (a D1R agonist like), eticlopride (a D2R antagonist like) and AP5 (an NMDAR antagonist) on the expression (on the test day, postconditioning) of morphine-induced CPP, different doses of eticlopride (1, 2 and 4 μg/rat), SKF38393 (1, 2 and 4 μg/rat) or the combinations of their ineffective doses (1 μg/rat) with AP5 (1 μg/rat), were bilaterally injected into the pVTA, 5 min before subcutaneous injection of morphine.

In addition, there were two more groups, which received eticlopride (4 μg/rat) and SKF38393 (4 μg/rat), without morphine administration. In the saline paired-chamber and the control-morphine groups, saline was microinfused into the VTA without drugs.

Histology

At the end of the experiments, the rats were deeply anesthetized and perfused transcardially with a 10% formalin solution. Then, the brain was dissected and fixed in 10% formalin for at least 5 days. To verify the position of the cannula in the pVTA, transverse sections through the brain were cut using a freezing microtome with a thickness of 50 μm, and examined under a microscope [Figure 1].[16]

Statistic

Analysis of data was evaluated using one-way ANOVA, following a significant *P* value, *post-hoc* analyses (Tukey test), and unpaired *t*-test for comparing specific groups using sigma plot software. All data were expressed as mean ± standard error of the mean, $P < 0.05$ ($P < 0.05$) considered statistically significant.

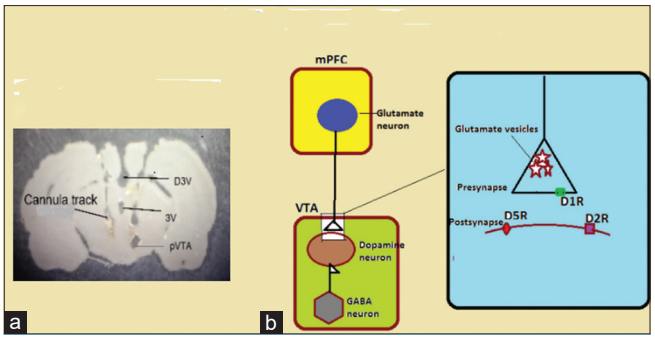

Figure 1: Coronal photomicrograph of bilateral microinjection site in the ventral tegmental area (a). Locations of dopamine receptors in the ventral tegmental area and its association to the medial prefrontal cortex (b). 3V: 3rd ventricle, D3V: Dorsal 3rd ventricle, posterior ventral tegmental area: Posterior ventral tegmental area, mPFC: Medial prefrontal cortex and D1, D2, D5 Rs: Dopamine D1, D2, and D5 receptors

Results

The effect of different doses of morphine on the expression of morphine- conditioned place preference

The results showed that morphine at a dose of 5 mg/kg had a significant difference in the conditioning scores compared to the saline group, but other doses did not have a significant impact [Figure 2a].

The effects of N-methyl-D-aspartate receptor antagonist into the posterior ventral tegmental area on the expression of morphine-induced conditioned place preference

Statistical analysis showed a significant difference for the conditioning scores among groups in the expression of CPP [F (4.31) =3.624, $P < 0.05$; Figure 2b]. The results showed that administration of AP5 (1 and 2 μg/rat) into the pVTA with morphine (5 mg/kg. s. c), decreased the conditioning scores [+$P < 0.05$, Figure 2b] compared to morphine group.

The effects of co-infusions of D1 receptor agonist with N-methyl-D-aspartate receptor antagonist into the posterior ventral tegmental area on the expression of morphine-induced

Statistical analysis showed a significant difference for the conditional scores among groups in the expression of CPP [F (6.44) =3.133, $P < 0.05$; Figure 3]. The results showed that administration of SKF38393 (4 μg/rat) with AP5 (1 μg/rat) into the pVTA in the receiving groups of the effective dose of morphine (5 mg/kg), increased the conditioning scores in comparison to the saline group [*$P < 0.05$, Figure 3].

The effects of simultaneous administration of N-methyl-D-aspartate receptor antagonist and D2 receptor antagonist into the posterior ventral tegmental area on the expression of morphine-induced conditioned place preference

Statistical analysis did not show any significant difference for the conditioning scores among groups on the expression

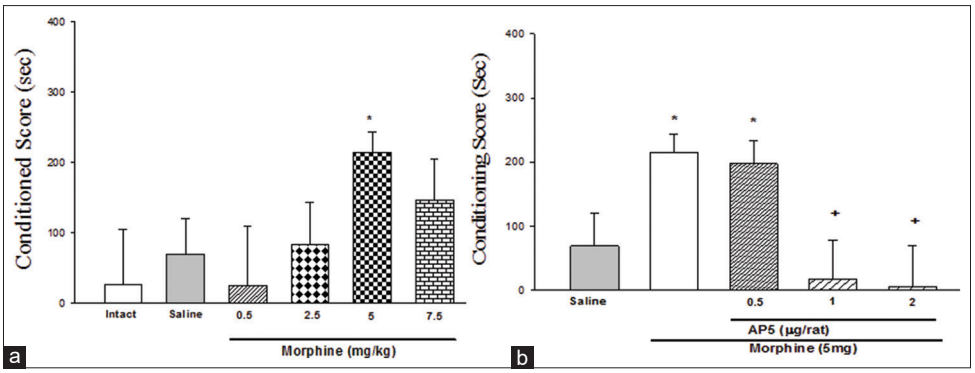

Figure 2: Morphine dose-response curve in the conditioned place preference pattern. The preference of score was calculated as the difference between time spent in the drug-paired compartment on the 9th and 3st days (a). The effect of bilateral administration of AP5, individually within the VTA on time spent. Time spent was recorded either alone or concurrently with an effective dose of morphine (5 mg/kg s.c.) (b). Data are expressed as mean ± standard error of the mean. *$P < 0.05$ different from the saline-control group and $^{+}P < 0.05$ different from the morphine-control group ($n = 6–8$)

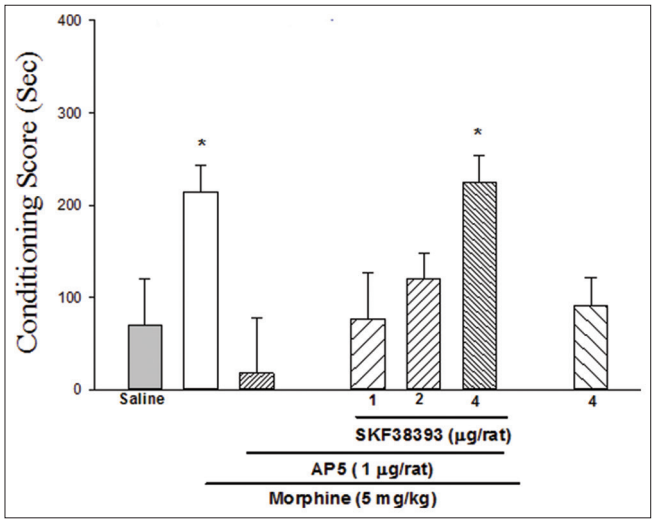

Figure 3: The effect of coadministration of SKF38393 and AP5, within the posterior ventral tegmental area on time spent. Time spent was recorded either alone or concurrently with effective morphine (5 mg/kg s.c.). Data are expressed as mean ± standard error of the mean. *$P < 0.05$, different from the saline-control group ($n = 6–8$)

of CPP. The results showed that co-administration of eticlopride (4 µg/rat) with AP5 (1 µg/rat) into the pVTA in the receiving groups of the effective dose of morphine (5 mg/kg) and without morphine, did not change the conditioning scores [Figure 4].

The effects of concurrent injection of ineffective doses of D1 receptor agonist, D2 receptor antagonist with N-methyl-D-aspartate receptor antagonist into the posterior ventral tegmental area on the expression of morphine-induced conditioned place preference

Statistical analysis did not show any significant difference for the conditioning scores among groups on the expression of CPP. The results showed that co-administration of SKF38393 (1 µg/rat), eticlopride (1 µg/rat) with AP5 (1 µg/rat) into the pVTA in the animals receiving an effective dose of morphine and without morphine, did not change the conditioning scores [Figure 5].

Discussion

The aim of this current study was to identify whether the excitation of D1Rs and also the inhibition of D2Rs in the pVTA can reverse the blocked expression of morphine CPP by an NMDA receptor antagonist or not.

The effective dose of morphine in this study determined to be 5 mg/rat (ip).[15] We found that the administration of D1R agonist through increasing conditioning scores compared to saline-control group caused cue-related learning, while the infusion of D2R antagonist had no effect [Figures 4 and 5, respectively].

Different inputs that come to VTA from different areas of the brain are as follows: Laterodorsal tegmental nucleus and pedunculopontine tegmental nucleus cholinergic inputs,[17,18] neuropeptides such as neurotensin and orexin/hypocretin from lateral hypothalamus,[19,20] mPFC glutamatergic input[21] and dorsal raphe serotonergic input.[22,23] The consumption of morphine increases glutamatergic inputs, especially from mPFC to the VTA.[24] Increased stimulatory inputs into the VTA can be due to the consumption of abuse drugs or natural stimuli (food, water, and sexuality).[25-27] Released glutamate leads to the excitation of DA neurons and consequently increased DA release in NAc, mPFC, axonally,[5,28] and in the VTA, somatodendrically.[6] Increased DA release has a major role in the modulation of the reward system.[29,30] Many studies indicated that blocking NMDAR in the VTA inhibited the expression of drug abuse CPP.[12,31] Our study shown that blockade of NMDAR inhibited the induction of CPP in the morphine group [Figure 3]. Furthermore, DA-D1 receptors located in glutamatergic axon terminals and other entries in the VTA have a regulatory role in the local release of glutamate and other agents and can be caused the stability of the DAergic neurons firing.[7,32] In a study by Galaj *et al*. was observed that the administration of D1R antagonist into the VTA inhibited the expression of cocaine CPP.[7] Therefore, the existence of D1R is necessary for the expression of drug abuse CPP in this region. Hence, the

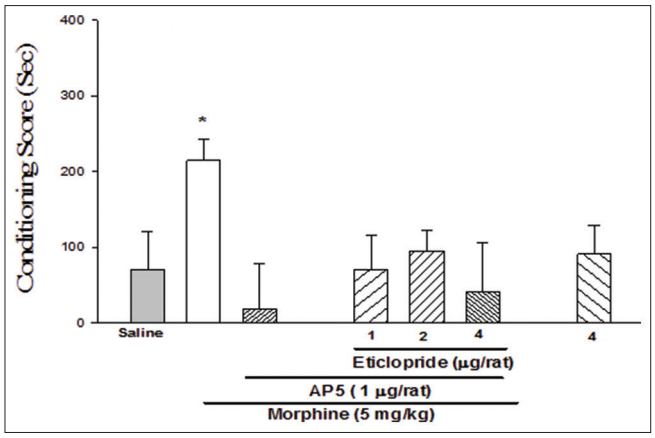

Figure 4: The effect of coadministration of eticlopride and AP5, within the posterior ventral tegmental area on time spent. Time spent was recorded either alone or concurrently with an effective dose of morphine (5 mg/kg s.c.). Data are expressed as mean ± standard error of the mean **P* < 0.05, different from the saline-control group (*n* = 6–8)

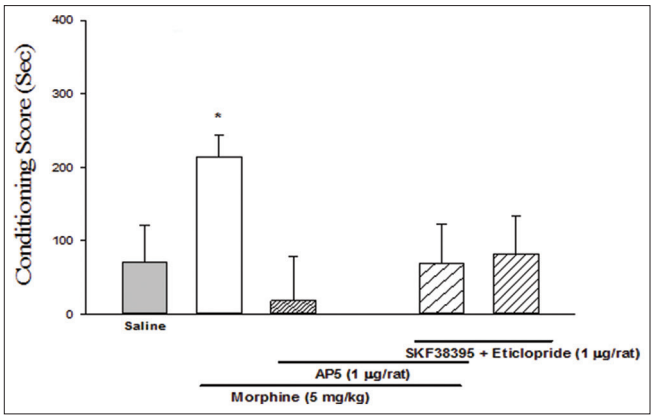

Figure 5: The effect of co-administration of ineffective doses of eticlopride and SKF38393 with AP5, within the VTA on time spent. Time spent was recorded either alone or concurrently with effective dose of morphine (5 mg/kg s.c.). Data are expressed as mean ± standard error of the mean **P* < 0.05, different from the saline-control group (*n* = 6–8)

presence of glutamate (NMDAR) and D-1 receptors are necessary for the expression of CPP, but it was little known about the role of the release of other neurotransmitters by the D1R, presynaptically [Figure 1b].

Our study showed that simultaneous use of D1R agonist with NMDAR antagonist in the morphine-receiving group induced the expression of morphine CPP (increased conditioning scores) compare to the saline group [Figure 3]. Many studies have been reported that the induction of CPP is consistent with the increased firing of DAergic neurons in the VTA.[4,33-35] Hence, it is probably that infusion of D1R agonist through pathways other than glutamate and with the release of various factors, presynaptically, such as the inputs of cholinergic,[17,18] orexinergic,[19,20] endocannabinoid,[36,37] serotoninergic[22,23] and some hormones[38-40] can produce an alteration of firing in the VTA DAergic neurons and induction of CPP. However, little is known about the release of other agents by D1R. Another possibility is that D1R agonists via D5R located on cell bodies of DA neurons be caused stimulatory effects on these cells. Of course, no studies have indicated a direct role for this receptor in the expression of abuse drugs CPP in VTA.

Furthermore, the D1Rs were found on the terminals of axonal inputs of GABAergic neurons and interneurons located in the VTA, and hence, it can potentiate the release of this neurotransmitter. Hence, increased GABA neurotransmission can have an inhibitory effect on DA neurons. Despite this effect, GABA released can, by inhibiting GABAergic interneurons, impact indirectly on the activation of DA neurons in the VTA. Therefore, the possibility arises that increased DA in the pVTA increases DAergic neurons activity.[41,42] As was previously mentioned, consumption of abuse drugs is caused by an increased DA concentration in the VTA, and that can via D2R have an inhibitory effect on DAergic neurons, postsynaptically.[13] Hence, in this study, in addition to using D1R agonist, we used D2R antagonist until we see eliminating the inhibitory effect of DA how affect through D2R on the expression of morphine CPP. Our findings showed that the co-administration of D2R antagonist with NMDAR antagonist in the morphine-receiving group had no effect on the induction of CPP [Figure 4]. Therefore, the possibility arises that in the absence of glutamate effect, after eliminating the inhibitory effect of DA through D2R located on DAergic neurons in the VTA, it cannot reverse the blocked expression of morphine CPP by an NMDAR antagonist. It is suggested that after administering morphine, DA concentration in the VTA is not high enough to exert its stimulatory effects (in the presence of inhibition of D2R by D2R antagonist) via activating D1R and D5R.

Also, we observed concurrent microinjection of ineffective doses of D1R agonist and D2R antagonists with NMDAR antagonist into the VTA, could not affect morphine-induced CPP [Figure 5]. Hence, the simultaneous use of DA drugs did not show synergistic effects.

Conclusions

It is possible to excite some of the presynaptic receptors that can alter the excitability of postsynaptic neurons by releasing different neurotransmitters. Hence, this study proposes the possibility that the D1R agonist, presynaptically, and in the presence of morphine modulates the activity of pVTA DAergic neurons and induces the expression of morphine CPP through the release of factors other than glutamate in the pVTA in regulating cue-related learning.

Acknowledgments

A special thanks go to Drs; H. Alaei, P. Reisi, for their many helpful assistance during this study.

Financial support and sponsorship

This research was funded by a grant (396127) from the Isfahan University of Medical Sciences, Isfahan, Iran.

Conflicts of interest

There are no conflicts of interest.

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