Research Paper

Acute Venom Toxicity Determinations for Five Iranian Vipers and a Scorpion

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Background: Poisoning due to the bites and stings of venomous snakes and scorpions is a neglected public health problem, particularly in rural areas. Poor health facilities and inadequate knowledge of health care personnel are the major factors that result in envenomated human victims not receiving adequate care and medical attention. There is a great need for up-to-date and effective healthcare knowledge and awareness of the potency and lethality of venomous creatures in Iran. Assessment of the potency, acute toxicity, and lethal effects of venomous creatures come from a variety of specific tests, such as the 50% median lethal dose (LD50) and ample animal experimentations.

Methods: In the present study, using modified Reed-Muench method, the LD0, LD50, and LD100 values of the venoms from five Iranian vipers and one scorpion were determined. The studied venomous creatures were: Macrovipera lebetina, Vipera albicornuta, Vipera raddei, Caucasicus intemedius agkistrodon, Montivipera latifii, and one scorpion Hemiscorpius lepturus. The venoms were injected in Albino mice (n=204) intraperitoneally, and their toxicities determined.

Results: The results revealed that the LD50 values of the above-mentioned creatures were 3.87, 2.05, 1.63, 1.45, 0.84, and 6.33 mg/kg, respectively. Among the vipers, M. latifii had the most potent venom while M. lebetina’s venom had the lowest toxicity.

Conclusion: Theoretically, the determined LD50 values provide for objective comparisons of the toxicity among of the venoms. However, comparison becomes complicated due to variations in the venoms’ LD50. Further, based on the venoms’ toxicity levels, H. lepturus’ venom caused the lowest toxicity in the Albino mice.

Keywords: Albino mice, LD50 test, Iranian vipers, Scorpion venom, Toxicity test, Venoms

Introduction

The determination of venom’s lethal dose is an important step in protecting human victims against getting poisoned by snake and scorpion venoms. This also permits the assessment of individual venoms for toxicity, selecting an appropriate antivenom, evaluating the effective capacity of specific anti-venoms, and finally determining the venoms’ lethal dosages [1, 2]. The most common test of acute toxicity is LD50 test, which is the reference standard to the dosage or amount of any chemical compound or drug that has proven to be lethal to 50% of the test animals in a particular study. This test was devised by Trevan in 1927, for the determination of the poisonous nature of certain medications, e.g., diphtheria antitoxin, digitalis and insulin. However, over the years this standard has been
steadily extended for the estimation of the relative safety of many other compounds and drugs [3].

There is a wide variety of wild animals in Iran, including venomous species, the characteristics of which, including the venoms’ toxicity, have not been well understood. Therefore, investigating the venoms’ toxicity can directly impact the public health and advance the body of scientific knowledge. The LD50 test is performed under controlled laboratory conditions via well-designed, standard experiments, as the initial step for the estimation of many drugs’ toxicities. This test is also required as part of the development of new therapeutic agents in order to assess how safe it is and to uncover the potential toxic effects and the risk of bodily harms to human consumers [4-6]. The LD50 test can identify the species’ tolerance under study and their susceptibility to specific toxins. Further, LD50 test can define the strength of toxins and hazardous chemicals in various species as a basis for their actual potency. This test use has been built into the law in many nations and is widely employed since it provides useful data to satisfy the legal requirements before marketing drugs and hazardous chemicals.

Venomous creatures especially snakes and scorpions are found in most areas of the world and are serious threats to the public health, and responsible for a large number of human deaths annually worldwide [2, 7]. It has been estimated that the yearly incidents of snakebites are between 4-5 million around the world, causing approximately 400,000 amputations and 20,000-125,000 deaths [8, 9]. Iran has a variety of reptilian species including 83 species of snakes, of which, 27 species are venomous and 11 others are semi-venomous [10]. In 2014, Dehghani et al. demonstrated that the mean incidence of snakebites over ten years per 100,000 populations was 7.42 [11]. The most medically important species which are responsible for the fatal snakebite incidents in Iran belong to the Viperidae family of snakes [10, 12, 13]. Snake venoms contain a mixture of pharmacologically active molecules, including organic and mineral components, small peptides, and proteins [14, 15].

Figure 1. Iranian vipers

Montivipera latifii (A), Macrovipera lebetina (B), Caucasicus intemedius agkistrodon (C), Vipera albicornuta (D), Vipera raddei (E), and scorpion Hemiscorpius lepturus (F). Original vipers’ images are courtesy of Amir A. Mirsepah, Director of “The Exhibition of Animal Sciences Development” Tehran, Iran. The scorpion image is courtesy of Abbas Zare, from Razi Vaccine and Serum Research Institute, Karaj, Iran.
The venoms from Viperidae snakes are typically rich in hydrolytic enzymes, and contain considerable amounts of zinc-dependent metalloproteinase, phospholipase A2, and serine proteinases. However, the relative proportions of these enzymes vary among the venoms from various species [16, 17]. The venoms also contain various proteins that interfere with the haemostatic system and impair the blood coagulation cascade and tissue repair. Consequently, envenomation by these snakes results in persistent bleeding and hemorrhage in critical organs, such as the heart, lungs, kidneys, and brain [18-21], causing critical health conditions. However, precise and reliable epidemiological data are not currently available in Iran due to the absence of a national registry, especially in rural areas.

This study aimed to experimentally evaluate the lethal potency of a series of venoms in a reliable animal model, and to determine the LD0, LD50, and LD100 dosages of the venom of five viper snakes and one scorpion that are endemic to Iran in Albino mice. The results of such a study can improve the available knowledge for physicians and healthcare personnel on the venoms from the snakes and scorpion, and provides a significant help toward adopting effective treatment methods for the human victims. The information also helps toxicology researchers to get the right venoms’ concentration in their studies and need to kill fewer animals. The findings of this study can set guidelines for selecting appropriate doses used in prolonged studies, thus reducing the cost of conducting research on venoms, and offering justifiable basis various research design and protocols. Before presenting the remaining sections of this article, we wish to provide a review of relevant literature primarily to familiarize the readers with the endemic venomous creatures in Iran.

**Literature review**

*Montivipera latifii* (Figure 1A) is endemic to the Alborz Mountain range in Iran and is a member of the M. raddei group. This species is categorized on the red list of International Union for Conservation of Nature’s (IUCN) as endangered species due to its limited distribution and population size [10, 22].

*Macrovipera lebetina* (Figure 1B) is one of the most abundant and venomous snakes from the Iranian plateau in central Asia to areas in the Middle East. Its venom contains several enzymes, proteins and peptides, such as metalloproteases, serine proteases and phosphodiesterase, phospholipase A2s, L-amino acid oxidase, disintegrins and C-type lectins, with numerous toxicological functions, causing local and systemic harms, including the local tissue damage and hemorrhage, abnormalities in the blood coagulation system, necrosis, cytotoxicity, edema and acute kidney injury [10, 23-25].

*Caucasicus intermedium Agkistrodon* (Figure 1C) is fairly abundant in the Central, Gilan, and Mazandaran provinces [10]. The halys complex of the pitviper genus Agkistrodon consists of three distinct polytypic species, halys, intermedius, and blomhoffii, that are differentiated by characters involving the presence or absence of paired apical pits, a number of scale rows at midbody, and the configuration of the dorsal markings [26].

*Vipera albigomuta* (Figure 1D) known as Zanjani viper is a venomous viper species endemic to Iran, found mostly in the Central, Gilan, and East Azerbaijan provinces. It is also prevalent mostly in Zanjan Valley and the surrounding mountains in northwestern Iran [10].

*Montivipera raddei* (Figure 1E) is a venomous viper species distributed in the Hamadan, Kurdistan, and West Azerbaijan provinces and also found in Armenia, Turkey, and Azerbaijan. It is one of the five known taxa of the raddei-complex [10, 27]. Its venom with hemorrhagic activity contains more than 100 proteins with enzymatic activities, such as serine proteinases, zinc-metalloproteinasmes, L-amino acid oxidase, and group II phospholipase A (PLA2). It also has other proteins without enzymatic activities, such as disintegrins, C-type lectins, natriuretic peptides, myotoxins, cysteine rich secretary proteins (CRISP) toxins, nerve and vascular endothelium growth factors, cystein and Kunitz-type proteinase inhibitors [28-30].

*Hemiscorpius lepturus* (Figure 1F) is distributed throughout six countries in the Middle East, including Iran, Iraq, Pakistan, Saudi Arabia, Oman, Yemen, and United Arab Emirates [31]. Its venom is mainly composed of hemotoxins and cytotoxins [32]. The most abundant components of the venom of this scorpion are such enzymes as phospholipases, metalloproteases, hyaluronidases, and proteases [33]. Two main peptides isolated from H. lepturus venoms are hemicalcin and hemotoxin which block calcium and potassium channels, respectively [34, 35].

**Materials and Methods**

**Animals:** A total of 204 albino mice, weighing 25-40 g and 8-10 weeks old, were purchased from the animal house of Mashhad University of Medical Sciences and used for this study. The experimental protocols were ini-
tiated after receiving the approval of the Animal Ethics Committee of the Faculty of Veterinary Medicine at Ferdowsi University of Mashhad (Registered Code: IR.UM.REC.1400.173). The mice were housed in the animal center of the Faculty of Veterinary Medicine at an animal facility in standard rodent cages covered with wood shavings, at standard environmental conditions of temperature 24±2°C; relative humidity 55±10%; and 12:12 hours of alternating light and dark cycles. The mice were fed standard rodent pellet diet and water ad libitum.

Venoms: The lyophilized crude venoms of Macrovipera lebetina, Vipera albicornuta, Vipera raddei, Caucasicus intermedius agkistrodon, Montivipera latifii and Hemiscorpius lepturus were generously provided by “The Exhibition of Animal Sciences Development” in Tehran, Iran. The venoms were stored at 4°C and freshly prepared by dissolving each of them in sterile saline solution. After weighing each mouse and calculating the required dose, the venom was injected intraperitoneally (IP) into the animals at a final volume of 500 μL.

Determination of LD50: The modified Reed-Muench method (Miller & Tainter) was used to determine the LD50 value for each venom in Albino mice. The method was a cumulative analysis of values achieved from the result of the study as follows [36]. For the calculation of LD50, we needed to know the dosage of least tolerance that caused 100% mortality and most tolerance that cause 0% mortality experimentally. We selected several doses between the minimum and maximum tolerance levels, and recorded the mice mortality for each of the doses. The mice were divided into groups of four each, and were treated with only one of the selected doses, ranging from LD0 to LD100. The lowest venom dose did not kill the mice (LD0) while the LD0 dose was multiplied by a factor of 1.25 to obtain the next dose. Each new dose was injected into four mice until we reached a concentration that could kill all the mice in the group, which constituted the LD100 dose (Table 1).

The cumulative mortality and survivors in each group were recorded over 24 hours and the number of deaths were placed in the following formula to determine the LD50 for each venom: M=x100±d/n (Σr-n/2). Where, M=logLD50; X100=log least dose required to causing 100% mortality; N=Number of mice used in each group (n=4); Σr=Total dead mice in the experiment=(e.g. 0+1+1+2+4); d=log (Coefficient of intervals between doses), and 1.25=0.097, if M=x, then Anti log of x=LD50.

Results

In this study, we experimentally determined the LD0, LD50, and LD100 values for the venom toxicity of each of the five viper and one scorpion as listed below. Further details are also presented in Table 2:

- Montivipera latifii: The venom’s LD0, LD50, and LD100 values were 0.46, 0.84 and 2.43 mg/kg of the mice, respectively.
- Caucasicus intermedius Agkistrodon: The venom’s LD0, LD50, and LD100 values were 0.80, 1.45 and 3 mg/kg of the mice, respectively.
- Vipera raddei: The venom’s LD0, LD50, and LD100 values were 1, 1.63, and 4 mg/kg of the mice, respectively.
- Vipera albicornuta: The venom’s LD0, LD50, and LD100 values were 0.65, 2.05 and 5.93 mg/kg of the mice, respectively.
- Macrovipera lebetina: The venom’s LD0, LD50, and LD100 values were 2, 3.87 and 6.40 mg/kg of the mice, respectively.
- Scorpion, Hemiscorpius lepturus: The venom’s LD0, LD50, and LD100 values were 2.44, 6.33 and 11.71 mg/kg of the mice, respectively.

The analysis of experimental LD50 values for these venoms indicated that M. Latifii was the most toxic compared to other vipers whereas the least toxic venom came from Vipera lebetina. The M. Latifii venom was 4.6 folds more toxic than that of Viper lebetina, over twice more toxic than V. albicornuta (2.4 folds), and approximately twice as toxic as that of V. raddei and C. intermedius Agkistrodon (1.9 and 1.7), respectively (Figure 2).

Discussion

In this study, the mean lethal doses (LD0, LD50 & LD100) values for the venoms from five Iranian viper’s and one scorpion were determined. These species, which are endemic to Iran include Macrovipera lebetina, Vipera albicornuta, Vipera raddei, Caucasicus intermedius agkistrodon, Montivipera latifii, and Hemiscorpius lepturus (scorpion). The dose determinations were established in albino mice via IP route of injection. The results of this study revealed that the LD50 of the selected venoms were 0.84, 1.45, 1.63, 2.05, 3.87, and 6.33, respectively. Among these, M. Latifii had the most toxic venom while the Vi-
Pera lebetina had the least potent venom. Based on the results, we were able to compare the venoms at high precision. Our findings are especially useful to medical staff in areas with high rates of snakebite or scorpion sting, and may also help researchers to accurately adjust the necessary concentrations of these venoms in their experiments.

The estimations of the venoms’ toxicity enable researchers to examine the venoms’ potency accurately. Also, the estimations can be valuable criteria for physicians to be aware of the lethality of the venoms and provide more effective treatment to the poisoned human victims. The geographical distribution of the creatures across Iran indicates that serious and lethal attacks are related primarily to the vipers’ stings, justifying the significance of the current study.

Table 1. Determination of the LD0, LD50 and LD100 of venoms.

<table>
<thead>
<tr>
<th>Number of tested mice/group</th>
<th>V. Albicornuta</th>
<th>V. Raddei</th>
<th>V. Lebetina</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dose (mg/kg)</td>
<td>Number of dead mice</td>
<td>Dose (mg/kg)</td>
</tr>
<tr>
<td>4</td>
<td>0.65</td>
<td>0</td>
<td>1.28</td>
</tr>
<tr>
<td>4</td>
<td>1.25</td>
<td>1</td>
<td>1.6</td>
</tr>
<tr>
<td>4</td>
<td>1.95</td>
<td>3</td>
<td>2.048</td>
</tr>
<tr>
<td>4</td>
<td>2.43</td>
<td>3</td>
<td>2.56</td>
</tr>
<tr>
<td>4</td>
<td>3.03</td>
<td>3</td>
<td>3.2</td>
</tr>
<tr>
<td>4</td>
<td>3.8</td>
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<td>4</td>
</tr>
<tr>
<td>4</td>
<td>4.75</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>5.93</td>
<td>4</td>
<td>-</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Number of tested mice/group</th>
<th>M. latifii</th>
<th>C. i. agkistrodon</th>
<th>H. lepturus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dose (mg/kg)</td>
<td>Number of dead mice</td>
<td>Dose (mg/kg)</td>
</tr>
<tr>
<td>4</td>
<td>0.46</td>
<td>0</td>
<td>0.8</td>
</tr>
<tr>
<td>4</td>
<td>0.8</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
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<td>1.25</td>
</tr>
<tr>
<td>4</td>
<td>1.25</td>
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<td>1.6</td>
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<td>1.56</td>
<td>2</td>
<td>2</td>
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<td>4</td>
<td>1.56</td>
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<td>2.5</td>
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<td>4</td>
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<td>2</td>
<td>3</td>
</tr>
<tr>
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<td>1.95</td>
<td>2</td>
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</tr>
<tr>
<td>4</td>
<td>1.95</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>2.43</td>
<td>4</td>
<td>-</td>
</tr>
</tbody>
</table>
value for estimating acute and lethal poisoning in humans [39]. Although the best species for measuring the toxicity of substances in humans are humans themselves [40, 41]; however, this is impossible to achieve ethically. Recently in 2021, Dearden and Hewitt have shown good correlation between the median LD50 values of 36 organic chemicals tested on mouse and rat with the human lethal dosage for the same chemicals. These researchers concluded that conventional median LD50 values derived in rodents could be effectively used to precisely predict the toxicity in humans [42]. Therefore, conducting toxicity studies are clinically important since they provide significant health-related information, such as, dose-response curve, safety assessment of new chemicals, venoms, antivenoms, drugs or food additives, and useful data for epidemiological studies [30, 43]. Although, LD50 tests have their limitations and disadvantages, such as using large number of animals, causing considerable pain in them, and the confounding factors, e.g., species differences in gender, age, diet, genetic strain, health, degree of starvation, and method of dosing [44, 45], they are still being used.

In 1984, a study on mice [46] has reported the LD50 of Macrovipera lebetina, Montivipera latifii and Caucasicus intermedius agkistrodon to be 6.4, 5.5 and 13.7 μg per mouse, respectively. That study concluded that Monti-

Table 2. Lethal toxicity of the studied venoms

<table>
<thead>
<tr>
<th>Venomous animals</th>
<th>LD0 (mg/kg)</th>
<th>LD0 (μg/g)</th>
<th>LD0 (μg/mouse (33g))</th>
<th>LD50 (mg/kg)</th>
<th>LD50 (μg/g)</th>
<th>LD50 (μg/mouse (33g))</th>
<th>LD100 (mg/kg)</th>
<th>LD100 (μg/g)</th>
<th>LD100 (μg/mouse (33g))</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. latifii</td>
<td>0.46</td>
<td>0.46</td>
<td>15.18</td>
<td>0.84</td>
<td>0.84</td>
<td>27.72</td>
<td>2.43</td>
<td>2.43</td>
<td>80.19</td>
</tr>
<tr>
<td>C. i. Agkistrodon</td>
<td>0.80</td>
<td>0.8</td>
<td>26.4</td>
<td>1.45</td>
<td>1.45</td>
<td>47.85</td>
<td>3</td>
<td>3</td>
<td>99</td>
</tr>
<tr>
<td>V. raddei</td>
<td>1</td>
<td>1</td>
<td>33</td>
<td>1.63</td>
<td>1.63</td>
<td>53.79</td>
<td>4</td>
<td>4</td>
<td>132</td>
</tr>
<tr>
<td>V. albicornuta</td>
<td>0.65</td>
<td>0.65</td>
<td>21.45</td>
<td>2.05</td>
<td>2.05</td>
<td>67.65</td>
<td>5.93</td>
<td>5.93</td>
<td>195.69</td>
</tr>
<tr>
<td>V. lebetina</td>
<td>2</td>
<td>2</td>
<td>66</td>
<td>3.87</td>
<td>3.87</td>
<td>127.71</td>
<td>6.40</td>
<td>6.4</td>
<td>211.2</td>
</tr>
<tr>
<td>H. lepturus</td>
<td>2.44</td>
<td>2.44</td>
<td>80.52</td>
<td>6.33</td>
<td>6.33</td>
<td>208.89</td>
<td>11.71</td>
<td>11.71</td>
<td>386.43</td>
</tr>
</tbody>
</table>

The lethal toxicities were determined by IP injections in mice. The comparison of the lethal doses, i.e., LD0, LD50 and LD100, are presented as mg/kg, μg/g and μg/g of mouse body weight (average, 33 g) of the venoms from the five Iranian vipers and one scorpion.

Figure 2. Comparison of the lethal doses of LD0, LD50 and LD100 presented as mg/kg (b.w. of mice) of Iranian vipers, Montivipera latifii, Caucasian intermedius agkistrodon, Viper raddei, Viper albicornuta, Macroviiper lebetina, and Iranian scorpion Hemiscorpius lepturus venoms.
Vipera latifii is the most dangerous snake in Iran [46]. Despite the methodological differences, the findings of that study are in agreement with our results.

Oukkache et al. in 2012 reported the toxicity of Moroccan snakes Macrovipera mauretanica, Cerastes cerastes, and Bitis arietans snake venoms in Swiss mice with the respective LD50 values of 5.97, 5.75, and 52.54 μg per mouse, respectively [47]. This study showed that the LD50 of vipers lebetina venom was 3.87 mg/kg (3870 μg/kg) while, Nalbantsoy, et al. have reported that the LD50 of the same venom in Albino mice was 7580 and 1205 μg/kg, respectively [24, 48]. The exact reason for the differences are not clear but as indicated before, several factors such as sex, age, diet, genetic strain, and health of the animals might have been involved [2, 49]. Further, Oukkache in 2014 has shown that in the case of viper venoms, the LD50 values are strongly dependent on the injection route; i.e., intravenous route causes a 3-fold higher toxicity in the same animals that the IP route [48].

In 2017, Madani, et al. reported the lethal dose (LD50) of Pseudocerastes persicus fieldi or Iranian horned viper venom in Albino mice was 21.9 μg per mouse and concluded that the viper was one of the most poisonous and dangerous ones [50]. It should be noted that, in the recent years several researchers have been tried to introduce an alternative testing method, i.e., cell culture system derived from human tissues or cancer cells, which have their own limitations, since there are differences among laboratory cell culture conditions and testing toxins on live animals [51].

Limitations of the Study: Lack of access to adequate venoms (especially scorpion venom) and budget, limited our experiment to measure the toxicity of these venoms only on mice. We had just one scorpion venom which makes the comparison of this creature’s venom limited and difficult.

Recommendations for Future Studies: We recommend that the median lethal dose of these venoms be measured in other animals such as rats, rabbits, etc., and also measured through various methods of venom administration. In addition, we recommend that other methods of measuring toxicity, such as cell culture, be compared with the median lethal dose.

Conclusions

This is the first report of the LD0, LD50 and LD100 of these vipers and scorpion (apart from M. Lebetina). The result of this study showed that the LD50 value of Macrovipera latifii, Vipera albicormuta, Caucasus intermedius Agkistrodon, Vipera raddei and Vipera lebetina were 0.84, 1.45, 1.63, 2.05 and 3.87 mg/kg, respectively. In theory, these calculated values allow for a standard comparison of these venoms’ toxicity; however, in practice, this may be complicated because of the LD50 variation. Moreover, the achieved doses that are lethal to one species, e.g., mice, will not be equally lethal to another species, or most importantly, to humans. In spite of this fact, we still believe that our reported LD0, LD50, and LD100 values help researchers and physicians in their practice to provide more accurate antivenoms services and patient care.

Based on the reported results, the LD50 of Vipera latifii venom is lowest compared with those in other viper species. However, the potency and therefore the toxicity of this venom is more than those found in others. In contrast, Macrovipera lebetina, possesses the highest LD50, indicative of the weakest toxicity. In comparison between the vipers and tested scorpion, the venom of H. lepturus possesses the weakest LD50, thus posing the weakest toxicity. It’s LD0 (2.44 mg/kg) that is not able to kill any mouse is equal to LD100 of viper Latifii (2.43 mg/kg) and is able to kill all animals.

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Ethical Considerations

Compliance with ethical guidelines

The study was approved by the Ethics Committee Guidelines of Ferdowsi University of Mashhad (Code: IR.UM.REC.1400.173).

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Authors' contributions

Methodology, Writing – original draft, and Supervision: Behrooz Fathi; Conceptualization, Investigation,

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and Data collection: Fatemeh Younesi and Fatemeh Salami. All authors read and approved the final manuscript.

Conflict of interest

The authors declared no conflict of interest.

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References


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