



The effect of two Iranian viper snake; *vipera albicornuta* (zanjani) and *vipera latifii* (lattifii) venoms on the viability of rat bone marrow mesenchymal stem cells *in vitro* and *in vivo*

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ABSTRACT

Snake venom is a complex mixture of different compounds which have potential pharmacological properties and may affect mesenchymal stem cells (MSCs). In this study we investigate the effects of two Iranian vipers; *vipera albicornuta* and *vipera latifii* crude venoms on the viability of MSCs *in vivo* and *in vitro*. The cells in *in vitro* tests were treated with different concentrations (1, 2, 3, 4 and 5 µg/100µl) of mentioned venoms for 24, 48 and 72 hours. The cells in *in vivo* experiment only were treated with *v. Latifii* venom at the concentration of 1µg/100µl and time intervals as *in vitro* tests. The cell viability in *in vitro* experiment was assessed using MTT assay. The results of *in vitro* experiments showed that maximum cell viability was observed at concentrations of 1 and 2 µg/100µl of *v. albicornuta* and *v. latifii* venoms after 48 and 72 hours, respectively. The results of *in vivo* experiment showed that the cells treated with *v. Latifii* venom for 72 hours *in situ* have the highest proliferation rate after passages three, four and five in comparison to control. The results of this study showed that the *v. albicornuta* and *v. latifii* venoms can affect the confluence and viability of the MSCs.

Keywords

Mesenchymal stem cells, Venom, Viper snake, Cell culture, Bone marrow

Abbreviations

MSCs: Mesenchymal stem cells
NGF: Nerve growth factor
rBM-MSCs: Rat bone marrow-mesenchymal stem cells
V: vipera
EVs: Extracellular vehicles

Introduction

MSCs are known as self-renewing, multipotent progenitor cells, capable of differentiating into mesodermal lineages, including adipocytes, osteocytes, and chondrocytes (1). They were initially discovered in bone marrow (2) and then were isolated from almost every type of tissue (3,4) and because of their ease of isolation and *ex vivo* expansion, MSCs are known as promising therapeutic tools for the treatment of various diseases (5). In human medicine, MSCs are used for their anti-inflammatory properties and ability to aid in tissue and bone regeneration (6). Due to the advantages of MSCs compared to other cells for therapeutic applications, the techniques for MSC proliferation and differentiation are needed. In stem cell therapy, extensive use of synthetic substances, recombinant cytokines and growth factors could be a contributing factor in increased side effects and toxicity. Therefore, search for alternative natural products to be used as growth factors and other effective components in stem cell therapy is important (7). Venomous animals possess a novel source of pharmacologically effective substances including metallic cations, carbohydrates, nucleosides, biogenic amines and lipids and other components in their venoms (8,9). Among them, snake venoms are a cocktail of diverse molecules, biologically active compounds, and a combination of many different proteins and enzymes with a variety of pharmacological effects and valuable therapeutic potential (8,10,11). A number of studies have revealed the ability for snake venom toxins to be used as a diagnostic tool, and that they possess possible therapeutic properties and great potential for the development of lead compounds for new drugs

(12,13). Previous studies have reported that nerve growth factors (NGFs) isolated from Chinese cobra snake venom, exert potential effects on the chondrogenic differentiation of bone marrow MSCs (BMSCs) and cartilage regeneration *in vitro* and *in vivo* (14,15). Direct addition of the snake venom phospholipase A2 is effective in the enhancement of the PMA-induced HL-60 cell differentiation to macrophages (16). Surveys such as that conducted by Liu et al., showed that vascular endothelial growth factor (VEGF) dependent mechanisms, stimulate the balance between osteoblast and adipocyte differentiation in BMSCs (17).

Gasparotto and colleagues used a new fibrin sealant (FS), derived from *Crotalus durissus terrificus* snake venom to evaluate the *in vitro* growth and cell viability of MSCs (25). The new fibrin sealant was a three-dimensional scaffold that maintained cells survival without promoting their differentiation (18). Kouchesfahani et al., showed that applying honey bee venom (BV), which consisted of mellitin, phospholipase A2, apamin and other bioactive ingredients, with retinoic acid has an effect on mouse P19 cell proliferation and differentiation to neurons (19).

The objective of this research is to determine the possible effects of two Iranian snakes, *v. albicornuta (zanjani)* and *v. latifii (latifii)* venoms on the viability rate of MSCs derived from rat bone marrow *in vitro* and *in vivo*.

Results

Vipera Latifii venom effect on viability of BMSCs *in vitro*

BMSCs were treated with various concentrations of *v. latifii* venom (1, 2, 3, 4, 5 $\mu\text{g}/100 \mu\text{l}$) *in vitro*, and

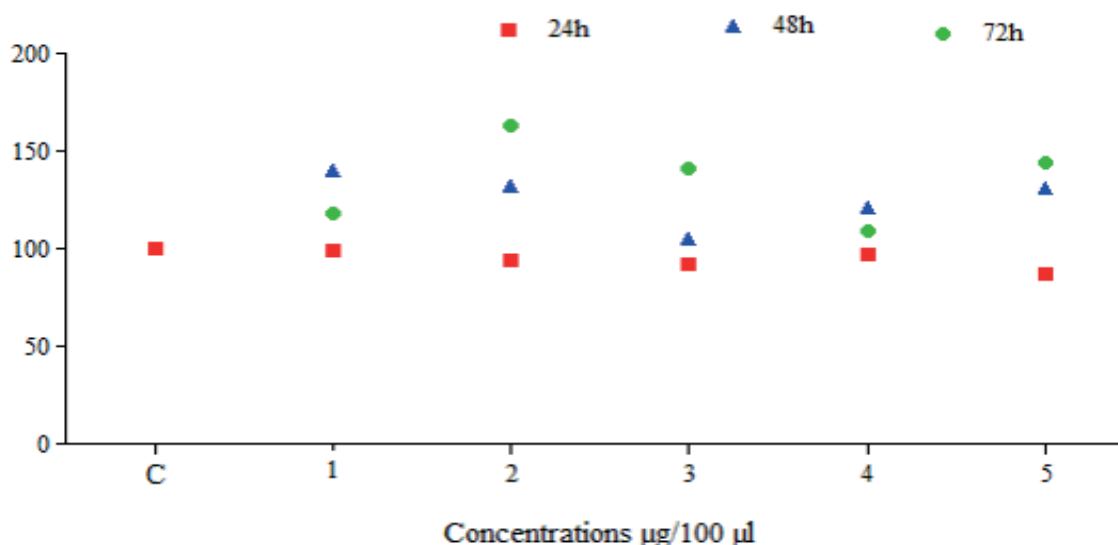


Figure 1
Viability (%) by *vipera latifii* snake venom at different concentrations (1, 2, 3, 4, 5 $\mu\text{g}/100 \mu\text{l}$), at 24, 48 and 72 hours

the effect on cell viability was analyzed using the MTT assay. The maximum cell viability and proliferation at different concentrations was observed after 72 hours (Figure 1). The viability differences between 24 and 72 hours was significant ($p= 0.015$) at concentration of $2 \mu\text{g}/100\mu\text{l}$, while it was not as significant between 48 and 72 hours. In addition, viability differences between different concentrations was not significant (Figure 1).

Vipera albicornuta (zanjani) venom effect on viability of BMSCs in vitro

BMSCs were treated with various concentrations of *v. albicornuta (zanjani)* venom (1, 2, 3, 4, 5 $\mu\text{g}/100\mu\text{l}$) *in vitro*, and MTT assay and Post Hoc test were used to evaluate the viability of BMS cells. The maximum cell viability at different concentrations was observed after 48 hours (Figure 2). At concentration of $1 \mu\text{g}/100\mu\text{l}$ the viability differences between 24 and 48 and also between 48 and 72 hours was significant ($p=$

0.046) and ($p= 0.014$), respectively. At concentration of $3 \mu\text{g}/100\mu\text{l}$ the viability differences only between 48 and 72 hours was significant ($p= 0.039$) (Figure 2).

The measured viability showed that among different concentrations there is no significant differences at 24, 48 and 72 hours (Figure 2).

Comparison between vipera latifii and vipera albicornuta (zanjani) snake venom effects on viability of BMSCs at 72 hours

At concentration of 2 and $3 \mu\text{g}/100\mu\text{l}$ the viability differences between *v. latifii* and *v. albicornuta* venoms was significant ($p= 0.004$) and ($p= 0.014$), respectively (Figure 3).

Preliminary results of the effect of Vipera Latifii venom on rat bone marrow

The quality of extracted MSCs were evaluated by the doubling time in cell culture. Cells were grown to

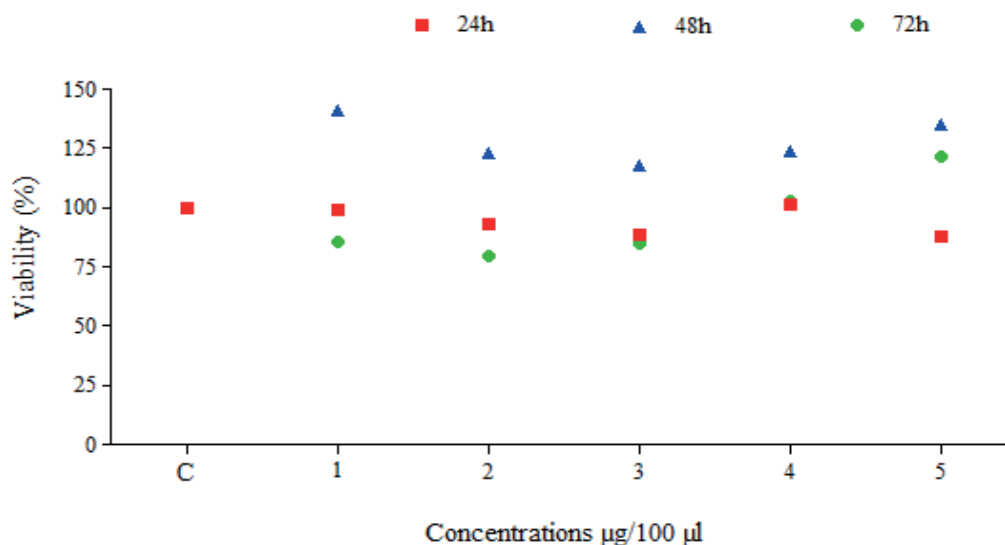


Figure 2
Viability (%) by *vipera albicornuta (zanjani)* snake venom at different concentrations (1, 2, 3, 4, 5 $\mu\text{g}/100 \mu\text{l}$) at 24, 48 and 72 hours

80 to 90% confluency as determined by visual assessment using phase contrast microscopy every 24 hours. Cells were harvested and subcultured at 1:2 ratio. The results showed that the cells treated with venom for 72 hours *in situ* have the highest proliferation rate after passages three, four and five in comparison to control, while cells treated for 24 hours with venom *in situ* were growing more slowly in comparison to control (Figure 4).

The cells at different passages in control group were reached to confluency in relatively the same time interval, while the cells in treatment groups showed

higher rate of proliferation and growth. As shown in figure 4, the rate of cell proliferation increased after third passage at 48 and 72 hours.

Discussion

We sought to evaluate the effects of two Iranian viper snake venoms on MSCs and this study demonstrated that these venoms are able to affect cell viability *in vitro* and *in vivo*. The results of this study showed that MSCs cells viability increased after incubation

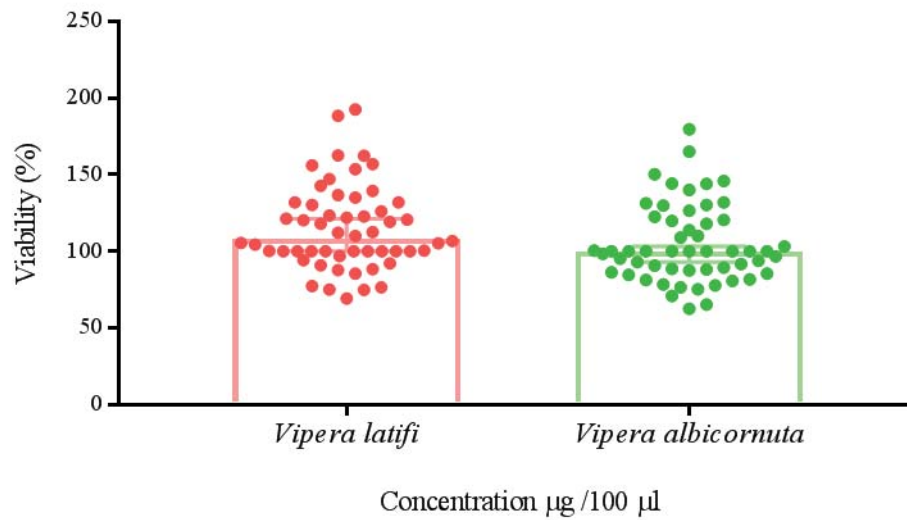


Figure 3
Viability (%) by vipera latifi and vipera albicornuta (zanjani) snake

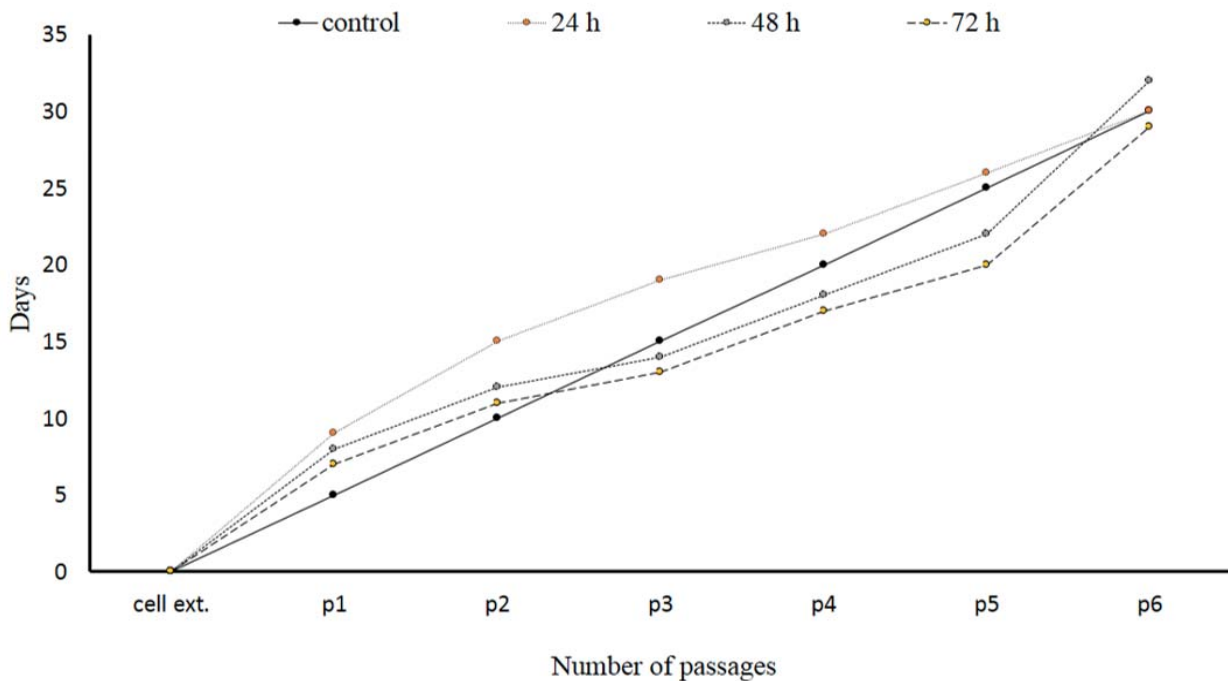


Figure 4
The time interval between passages of bone marrow derived MSCs treated with *vipera latifi* venom at 1µg/100 µl.

in vitro with media containing venoms. For *v. latifi* snake venom, the highest viability was observed after 72 hours incubation at concentration of 2 µg/100 µl venom with 63% increased compare to control, and the lowest viability was after 24 hours (Figure 1). For *v. albicornuta* (*zanjani*) snake venom, the maximum

viability occurred at 48 hours of treatment at concentration of 1 µg/100 µl venom with 40% increased compared to control (Figure 2). The observed effects seems to be more dependent to time than concentration as there was not significant differences among different concentrations at 24, 48 and 72 hours.

It was found that *in vivo*, the doubling rate of the cells after being treated with *latifii* venom was the highest in the 72 hours treatment group (Figure 4). Considering the effects of endogenous factors and injected venom interactions, MSC viability may differ from that of *in vitro*. It is important to recognize that this study is the first study of its kind to investigate the effects of snake venom on stem cells *in situ*. Although the exact mechanisms by which these venoms affected the viability of MSCs are not clear, several points can be considered in an effort to explain this phenomenon. For example, in 2009, Chen and colleagues showed that initial expansion of MSC cells is influenced by the culture parameters such as media, cell density and even culture flasks (20). Therefore one possible conclusion is that any factors which change the media composition like venoms, may can affect cell viability. It has been shown that MSCs behavior such as tissue repair, immunomodulation, differentiation and proliferation may be influenced by their secretion products (paracrine factors) including soluble factors and extracellular vehicles (EVs) which participate in formation of a favorable microenvironment (21). Some of EV's are secreted after cell apoptosis, some during stress or metabolic changes, and some are secreted continuously (22–26). It is possible that after dissolving within this specific microenvironment, the individual components of the complex venoms undergo a biochemical change which could result in an alteration of MSC viability. Furthermore, growth factors are often added to stimulate the differentiation and improve cell proliferation. Several reports indicate that all snake venoms are a rich source of growth factors such as NGF (27). In 2017, Lu and colleagues isolated NGF from venom of Chinese cobra snake which induced the BMSCs to differentiate into the chondrogenic lineage *in vitro* and *in vivo* (14). Therefore, another possibility can be that these snake venoms possess several growth factors that stimulate MSCs proliferation and viability.

In 2002 Liu et al., reported that bee venom (BV) inhibited cell proliferation and induced K1735M2 mouse melanoma cells differentiation *in vitro*. In 2015, Jung et al. reported that bee venom has neuroprotective effects against rotenone-induced cell death in NSC34 motor neuron cells and pre-treatment of these cells with bee venom significantly enhanced cell viability. In 2010, Kouchesfahani and colleagues reported that honey bee venom induce differentiation of cholinergic neurons. They also showed that applying a combination of bee venom with retinoic acid (RA) has an additive effect on cell differentiation and proliferation. They concluded that phospholipase A2 (PLA2), the most abundant component of bee venom play an important role in the differentiation of cholinergic

neuron in P19 cell line. Previously Nakashima et al. have suggested that PLA2 of BV plays an important role in cell differentiation and induced neurite outgrowth in PC12 cells (28). Also in 2005, Mora and colleagues reported that snake venom Lys49 PLA2 homologues at lower concentrations, caused cell proliferation (29). To the best of authors' knowledge, no systematic publication exists to address the exact presence of PLA2 in *vipera latifii* and *vipera albicornuta* snake venoms. Although, it has been explored in prior studies that (PLA2) is one of the most abundant protein families found in vipera venoms (8,30). This suggest that possible (PLA2) of *vipera latifii* and *vipera albicornuta* venom treated the viability of rat MSCs the way the bee venom (PLA2) treated.

This is the first study investigating the effect of vipera snake venoms on MSCs *in vitro* and *in vivo*. In this study, direct injection of venom into bone marrow raised this possibility that venom may react to bone marrow composition *in situ*, that in turn affected the rate of cell growth and viability. The present results showed that the cells that were treated with *vipera latifii* venom in bone marrow for 72 hours can grow noticeably faster when they transfer to normal culture media.

It is beyond the scope of this study to examine the effect of venom peptide and enzyme compounds separately on MSCs. It is undisputable that venoms have varieties of pharmacologically active compounds and therefore various biological effects. The venoms somehow affect the viability of MSC and altered the survival and proliferation rate of rat bone marrow MSCs *in vitro* and *in vivo*. However, their fundamental and detailed molecular mechanisms is not clear. In addition, the bone marrow can be considered as a suitable model to investigate venom and similar compound effects on viability of stem cells *in situ*, however more research on this topic needs to be undertaken to explore the effect of individual components of these venoms and their mechanisms.

Material and methods

Chemicals

All chemicals including: Dulbecco's Modified Eagle's Medium (DMEM), penicillin/streptomycin and amphotericin B, phosphate-buffered saline (PBS), fetal bovine serum (FBS), chloroform, ketamine, xylazine and, MTT powder were obtained from Sigma (Germany) unless otherwise stated. Reagents were prepared and stored according to the manufacturer's guidelines. Non chemical material were; insulin syringes, 75 cm² culture flasks and, 96-well plates.

Venoms

The *vipera albicornuta* and *vipera latifii* venoms were generously provided by Exhibition of Animal Sciences Development,

Tehran, Iran.

Animals

Five Sprague-Dawley female rats (1-2 month old) were purchased from Animal House of Mashhad University of Medical Sciences. The rats were housed in the Animal Center of Faculty of Veterinary Medicine in a standard animal facility and maintained on a 12-hour light/dark cycle and were allowed food and water ad libitum. It should be noted that all animal experiments were approved by Animal Care and Use Committee of Ferdowsi University of Mashhad.

Isolation and culture of MSCs

One rat was succumbed with chloroform, its legs cleaned with warm water and shaved, afterwards the skin was swabbed with 70% alcohol. With two small surgical incisions, its two femurs and tibias were removed and transferred to the laminar hood. The muscles around the bones were removed, and washed 3-5 times with 1x phosphate-buffered saline (PBS). Bones were sterilized with 70% ethanol, washed by Dulbecco's Modified Eagle's Medium (DMEM) and epiphysis of the bone was separated by scissors. The bone marrows were then flushed out using DMEM, supplemented with 10% (v/v) FBS, 1% penicillin/streptomycin and 0.1% Amphotericin B. The medium was flushed into the bone using an insulin syringe. Cell suspension was collected and centrifugated at 200g for 5 minutes. Supernatant was removed and cell pellet was re-suspended with 1ml of culture medium. Finally, the released cells were collected into 75 cm² culture flasks with 10 ml DMEM culture medium. The cells cultures were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO₂. Cells were allowed to attach for 4-5 days, the non-adherent cell population was removed and the culture medium was replaced with fresh pre-warmed culture medium. MSCs were attached to the bottom of the flask, due to their adhesion properties.

In-vitro study design

The cells were used after three passages, seeded into 96-well plates at a density of 0.05×10^6 cells/well. The cells were divided into six groups that included one control and five test groups. 150 µl of fresh pre-warmed culture media was added to each well of all groups. After 24 hours, the medium was aspirated and the test groups were treated with 200 µl phosphate-buffered saline (PBS) containing different concentrations (1, 2, 3, 4, and 5 µg/100 µl) of *v. albicornuta* and *v. latifii* venoms while only 200 µl PBS was added to the control group. After one hour venom/PBS solutions were removed and all groups prepared for MTT test. It should be noted that, the selected venom concentrations were based on our previous research (unpublished results), on determination of the LD₀, LD₅₀ and LD₁₀₀ of these venoms. Based on that experiment we chose the concentrations lower than LD₅₀.

MTT Assay

Cell viability was assessed via a quantitative colorimetric assay using MTT (27). Five mg MTT powder was dissolved in 1ml PBS and the solution was sterilized using 0.2 µm filter. After 24, 48 and 72 hours, 20 µl MTT solution was added to each of 96-well containing the cultured cells in the culture medium and incubated for 4 hours at 37°C. The culture medium was carefully aspirated and 150 µl DMSO solution was added to each well. Finally, the optical absorption of each well was measured using an ELISA reader at 570 nm.

In-vivo experiments

Three rats were anesthetized with a combination of 60 mg/kg ketamine and 6 mg/kg xylazine (IP route) and one rat used as control. The animals were kept in a dorsal recumbent position, the anterior face of their thighs shaved, and the area disinfected with 70% ethanol. For intra-femoral injection, a minor surgery under anesthesia was performed. By opening the femur skin, the greater trochanter of the proximal femur was exposed. Five hundred microliters of *v. latifii* venom at concentration of 1 µg/100 µl was injected into the bone marrow of the right femur by using a 22 gauge metallic needle and then the animals returned to their cages. Their food and water consumption were normal and they were in healthy condition. MSCs were extracted after 24, 48, and 72 hours from the bone marrow of these rats. The cells were counted in each passage. To determine doubling time, when the cells reached to 90% confluency, they were cultured to sixth passages.

Statistical analysis

The Kruskal Wallis nonparametric test was used to compare medians among groups (n=3 per group) and Dunn's multiple comparison test was used to compare two groups in terms of viability. Values of *p* lower than 0.05 were considered statistically significant.

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Author Contributions

All authors contributed to the design of study, data analysis and manuscript preparation.

Conflict of Interest

The authors declare that there is no conflict of interest.

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اثر زهر مارهای افعی ایرانی، زنجانی و لطیفی بر زنده ماننی سلول های بنیادی مزانشیمی استخراج شده از مغز استخوان رت بصورت داخل و برون تنی

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چکیده

زهر مار یک ترکیب پیچیده با مواد مختلف با خصوصیات فارماکولوژیک است. هدف از این تحقیق، بررسی اثر زهرمارهای افعی آلبيکورنوتا (*vipera albicornuta*) (زنجانی) و افعی لطیفی (*vipera latifii*) (لطیفی) بر رفتار سلول های بنیادی مزانشیمی در محیط کشت بود. سلول ها با غلظت های مختلف (۱، ۲، ۳، ۴، ۵، ۱۰۰ $\mu\text{g}/\mu\text{l}$) از زهر های نام برده به مدت ۲۴، ۴۸ و ۷۲ ساعت *in vitro* و *in vivo* تیمار شدند. سلول ها در شرایط *in vivo* تنها با زهرمار افعی لطیفی در غلظت ۱۰۰ $\mu\text{g}/\mu\text{l}$ و فواصل زمانی مشابه آزمایش *in vitro* تیمار شدند. زنده ماننی سلولی با استفاده از تست MTT با الیزاریدر در ۵۷۰ nm برآورد شد. بیشترین زنده ماننی سلولی در غلظت ۱ $\mu\text{g}/\mu\text{l}$ و ۲ برای زهر مار زنجانی و لطیفی به ترتیب بعد از ۴۸ و ۷۲ ساعت بود. نتایج آزمایش *in vivo* نشان داد که سلول های تیمار شده با زهر افعی لطیفی برای ۷۲ ساعت در شرایط *in situ* بالاترین نرخ تکثیر را بعد از پاساژهای ۳ و ۴ در مقایسه با کنترل داشتند. زهر مارهای زنجانی و لطیفی می توانند بر تراکم و رفتار سلول های بنیادی مزانشیمی اثر بگذارند.

واژگان کلیدی

سلول های بنیادی مزانشیمی، زهر، مار افعی، کشت سلول، مغز استخوان