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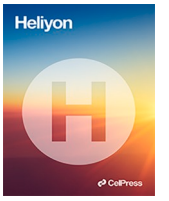

Research article

The effect of Iranian snake, *Naja naja oxiana* venom on the blood glucose concentration and some biochemical parameters of experimental diabetic rats

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ABSTRACT

Diabetes is a chronic disease resulting from impaired insulin production and function; leading to hyperglycaemia and long-term complications. The treatment for Type I diabetes treatment involves insulin injections while Type II diabetes treatments include drugs such as metformin and sulfonylureas, along with lifestyle changes. These medicines can be expensive and may have adverse effects. Therefore, the search for new therapeutic agents continues. Venoms from various animals yield numerous pharmacologically active compounds. In this study, we investigated the effects of the venom from an Iranian snake, *Naja naja oxiana*, on blood glucose concentration and certain serum biochemical parameters in male rats with induced diabetes.

Diabetes was induced in male rats using either a single injection of streptozotocin (STZ) alone (55 mg/kg i. p.) or STZ (65 mg/kg i. p.) preceded by nicotinamide (230/kg i. p.) administered 15 min earlier. The diabetic rats produced by either method received a single injection of either vehicle or venom (0.2 or 0.4 mg/kg i. p.). In the STZ rats, this was done 13 days after diabetes induction, while in the STZ-nicotinamide rats, venom was injected 3 days after diabetes induction.

The venom from *Naja naja oxiana* significantly reduced blood glucose levels in male rats with diabetes induced by either method. Additionally, the venom decreased serum cholesterol and triglycerides concentrations. However, the venom had no effect on the blood glucose levels of healthy male rats. Pretreatment with the venom did not prevent the induction of diabetes by STZ. These findings suggest that *Naja naja oxiana* venom exhibits an anti-diabetic effect and could be a potential candidate for effectively controlling diabetes.

1. Introduction

Diabetes is a major health challenge of the 21st century. In 2019 it was estimated that there were 463 million people with diabetes; this number is expected to increase to 578 million by 2030 and 700 million by 2045 [1]. It is a serious metabolic disorder characterized by hyperglycemia, due to a decrease in insulin production and/or insulin resistance. Diabetes can lead to long-term complications affecting the heart, blood vessels, eyes, kidneys, and nerves [2]. There are two major types of diabetes: type I (DT1) & type II (DT2), the latter accounting for 90 % of cases. Type I diabetes results from an autoimmune destruction of pancreatic beta cells, while type II

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diabetes results from both insulin resistance and impaired insulin secretion [2,3]. Currently, the only pharmacological treatment for type I diabetes is insulin. Treatment options for type II diabetes include sulfonylureas, metformin, glitazones, SGLT2 inhibitors and incretin-based therapies, as well as insulin [4–6]. Despite the currently available antidiabetic medications, many patients still experience inadequate metabolic control [7–9].

Furthermore, treatment may be limited by side effects such as weight gain and hypoglycemia. Consequently, there is an ongoing search for more effective drugs with fewer adverse effects [10]. Additionally, the management of type II diabetes, these medications may eventually prove insufficient in controlling blood glucose levels, necessitating the use of insulin [11]. As a result, there has been extensive exploration of natural products for the treatment of diabetes [12]. While numerous plants have been investigated, the search has expanded to include venoms from various creatures. Much research has focused on identifying novel therapeutic peptides from venoms that could potentially be utilized in the treatment of various diseases [13–17]. Scorpion venoms have been shown to reduce blood glucose levels in diabetic animals [18,19]. Additionally, glucagon-like peptide 1 (GLP-1) analog have been developed from the venom of the Gila monster [20]. However, there has been limited research conducted on the antidiabetic effects of snake venoms. Viper venoms have been found to contain insulinotropic compounds, including peptides such as serine proteinases and phospholipases A2 (PLA2) [21]. An insulinotropic peptide called cardiotoxin 1 was discovered in the venom of the cobra *Naja kaouthia* [22]. In this study, we examined the effects of the venom from the Iranian snake, *Naja naja oxiana,* on blood glucose levels and various plasma biochemical parameters in two male rat models of diabetes. This is the first time such an examination has been conducted.

2. Materials and methods

2.1. Animals

Fifty-six male adult albino Wistar rats weighing 200–250 g and aged 8–10 weeks were obtained from the animal house of Mashhad University of Medical Sciences for use in this study. The rats were housed in the animal center of the Faculty of Veterinary Medicine, in standard rodent cages maintained at a temperature of 24±2 °C, relative humidity of 55 ± 10 %, and a 12:12 h light-dark cycle. They were provided with a standard rodent pellet diet and had access to water ad libitum. The experimental protocols were conducted with the approval of the Animal Ethics Committee of the Faculty of Veterinary Medicine at Ferdowsi University of Mashhad, under the code IR. UM.REC.1398.019, ensuring efforts were made to minimize animal suffering during the study.

2.2. Venom and drugs

The lyophilized crude venom of *naja naja oxiana* was generously provided by the Razi Vaccine and Serum Research Institute in Karaj, Iran. The venom was stored at 4 °C and freshly prepared by dissolving it in a sterile saline solution prior to injection into the animals. Streptozotocin (STZ) were purchased from Sigma-Aldrich and nicotinamide (NA) were purchased from Samchun chemical. Co., Ltd of Korea [23].

2.3. Induction of diabetes in the experimental rats

Male rats were provided with free access to water and fasted for a minimum of 8

Hours prior to the induction of diabetes. Diabetes was induced using one of two methods: either a single intraperitoneal (i.p.) injection of STZ (55 mg/kg) freshly dissolved in 0.1 M cold citrate buffer (pH 4.5), or a single i. p. Injection of STZ (65 mg/kg) preceded by nicotinamide (230 mg/kg/) administered 15 min earlier. The male rats that developed diabetes through the second approach are referred to STZ-N diabetic rats. Blood glucose concentrations were measured using an Easy Glucometer (Iran) by obtaining a drop of blood from the tip of the animals' tails, 3 days after the STZ or STZ-N injections. Animals exhibiting hyperglycemia (>250 mg/dL) were considered diabetic and selected for this experiment. It is important to note that some animals did not develop diabetes or died before any tests could be conducted. These animals were excluded from the experiment and replaced with other male rats to maintain the same number of animals in each group [24–30]. The blood glucose concentration of normal male rats was below 120mg/dl.

2.4. Experimental design for evaluation of antidiabetic activity of venom

Male rats were randomly divided into seven experimental groups (I, II, III, IV, V, VI, and VII) consisting of 8 animals each, as follows:

Group I did not receive any materials and served as the normal control, with untreatedand healthy mice.

Group II received STZ-Nicotinamide at doses of 65 mg/kg and 230 mg/kg respectively, and served as the untreated STZ-Nicotinamide diabetes control group (venom was not injected).

Groups III and IV received STZ-Nicotinamide at doses of 65 mg/kg and 230 mg/kgrespectively, consisted of STZ-Nicotinamide diabetic rats treated with a single injection of 0.2 or 0.4 mg/kg venom, respectively.

Group V received STZ at doses of 55 mg/kg and served as the untreated STZ-diabetes control.

Group VI received STZ at doses of 55 mg/kg and consisted of STZ-diabetic rats that received a single injection of 0.2 mg/kg venom.

Group VII consisted of healthy rats that received 0.2 mg/kg venom for 7 consecutive days prior to the administration of a single injection of STZ (55 mg/kg). The intraperitoneal route (i. p.) was used for all injections.

2.5. Biochemical assay

To measure biochemical parameters, including plasma triglyceride, cholesterol, albumin, and total protein levels, blood samples were collected from the retro-orbital sinus under ether anesthesia to minimize stress to the animals. All steps were consistently performed at the same time of day on days 0, 15, and 30 of the study. All serum samples were transferred to clinical pathology laboratory of veterinary teaching hospital of university. Serum albumin was measured by bromocresol green method. Total protein levels were measured by using Biuret methods, total cholesterol levels were measured using the CHOD-PAP method, which involves the use of cholesterol oxidase (CHOD) and 4-aminoantipyrine (PAP) and triglyceride levels were measured by the GPO-PAP method utilizing glycerol 3 phosphate oxidase enzymatic reaction. They were assessed using the Auto Analyzer device (Pars Azmoon, Co., Tehran, Iran). For the collection of blood samples, a sterile hematocrit capillary tube was used to prevent any possible periorbital infection or potential long-term damage to the animals' eyes. After collecting the blood sample, direct pressure was applied to the eye using a piece of sterile gauze until the bleeding ceased. The blood collection interval was 15 days, allowing sufficient time for the animals to recover. They were monitored during this recovery period for any adverse effects.

2.6. Statistical analysis

Data analysis was performed using SPSS program, version 24 (SPSS Inc., Chicago, IL, USA) and Prism 8 software. All data are expressed as mean ± standard error of the mean. The normality of the variables was assessed using the Shapiro-Wilk test. For normally distributed variables, repeated measures ANOVA and Bonferroni post-hoc test were used for pairwise comparisons between days. Comparisons. For non-normally distributed variables Friedman test and Dunn's multiple comparisons test (Sample vs. control) were employed. A p-value less than 0.05 was considered as statistically significant for detecting differences in means.

3. Results

3.1. The effect of Iranian snake Naja naja oxiana venom on the concentration of blood glucose in STZ-nicotinamide rats

In group III, which was STZ-Nicotinamide male rats diabetic, after a single dose of venom (0.2 mg/kg i. p.), blood glucose concentrations of, significantly decreased from 500 mg/dL to approximately 111 mg/dL over a period of 34 days following the injection Fig. 1. Similarly, a single dose of venom (0.4 mg/kg i. p.) resulted in a significant decrease in blood glucose concentration, from over 500 mg/dL to less than 90 mg/dL within 28 days after the injection Fig. 1. It is worth noting that the higher dose led to a more rapid reduction in blood glucose concentrations.

3.2. The effect of Iranian snake Naja naja oxiana venom on the concentration of blood glucose in STZ-diabetic rats

In group VI which was STZ-diabetic male rats, the blood glucose concentration fluctuated during the first 20 days after diabetes induction, but it significantly decreased from approximately 300 mg/dL around 100 mg/dL over a period of 26 days following a single injection of venom (0.2/kg). In contrast, the control group experienced (group V) an increase in blood glucose concentration, reaching 600 mg/dL during the same time frame Fig. 2.

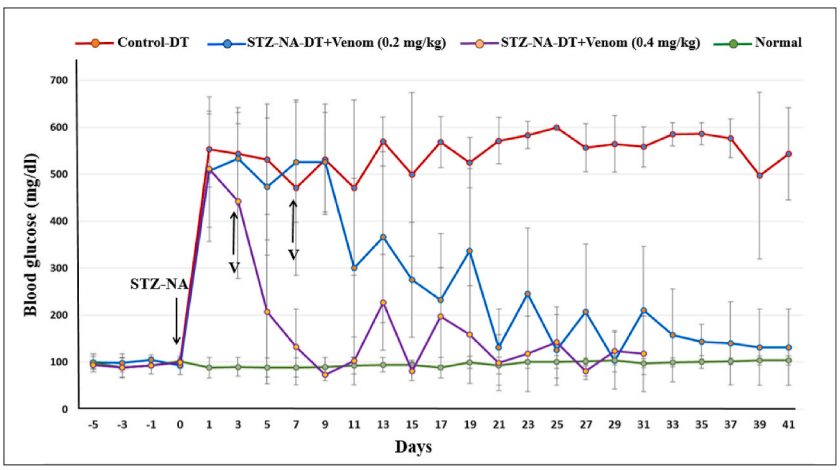

Fig. 1. The effect of the venom of Iranian snake *Naja naja oxiana* **(0.2 mg/kg/), group III** and **(0.4 mg/kg/), group IV** on the concentration of blood glucose in diabetic male rats (induced by streptozotocin (**65 mg/kg/**) and nicotinamide (**230 mg/kg/**), compared with the control diabetic group (**group II**) and control normal group **I**. (STZ: Streptozotocin, NA: Nicotinamide, DT: Diabetic, V: Venom).

3.3. The lack of prevention effect of the venom of Iranian snake Naja naja oxiana on induction of STZ-diabetes in male rats

Injection of venom (0.2 mg/kg) daily for seven consecutive days did not modify blood glucose concentrations in healthy male rats and did not prevent the effect of STZ (55 mg/kg) in inducing diabetes in male rats Fig. 3.

3.4. The effect of venom of Iranian snake Naja naja oxiana on the plasma

Biochemical parameters in diabetic male rats.

3.4.1. The effect of venom on the plasma cholesterol

The male rat plasma cholesterol concentrations in Group III and IV were significantly reduced compared to Group II (control STZ-Nicotinamide) and Group V (STZ) after a single injection of venom at doses of 0.2 or 0.4 mg/kg Fig. 4.

3.4.2. The effect of venom on the plasma triglyceride

The plasma triglyceride concentrations in group III and IV were significantly reduced compared to group II (control STZ-nicotinamide) and group V (STZ-diabetes) after a single injection of venom at doses of 0.2 and 0.4 mg/kg Fig. 5.

3.4.3. The effect of venom on the blood total protein and albumin

The concentrations of blood total protein and albumin on days 0, 15, and 30 in the Treatment groups did not show significant changes compared to the control group after a single injection of venom at doses of 0.2 and 0.4 mg/kg body weight (Data not shown).

3.5. The effect of 7 consecutive days injection of the venom of Iranian snake Naja naja oxiana on the plasma cholesterol, triglyceride in STZ-diabetic male rats

After seven days of repeated venom injection (0.2 mg/kg, group VI), there was no significant change in the plasma cholesterol concentration. However, compared to the healthy control group, the concentration of plasma triglycerides in diabetic male rats significantly increased after two weeks Fig. 6.

3.6. The effect of the venom of naja naja oxiana on the health conditions and behavior of diabetic male rats

The animal behaviors were observed for several days before and after the induction of diabetes by administration of streptozotocin (STZ) and STZ-ND and venom. The rats in the healthy control group exhibited normal behavior, including regular food and water uptake, clean fur, and high-quality haircoats. Their glucose levels remained within the normal range throughout the study, at approximately 100 ± 10 mg/dL. In the diabetic groups, the rats displayed hyperglycemia along with signs of fatigue, depression, immobility, isolation, increased thirst (polydipsia), decreased appetite and grooming, poor haircoat quality, and a relative decrease in weight. One of the most notable behavioral changes was the increased frequency of urination (polyuria), necessitating daily bedding changes.

Following treatment with *naja naja oxiana* venom, the rats gradually regained normal activity, appearance, well-being, clean fur, and good-quality haircoats and were indistinguishable from normal control. They also exhibited normal appetite, regular food and

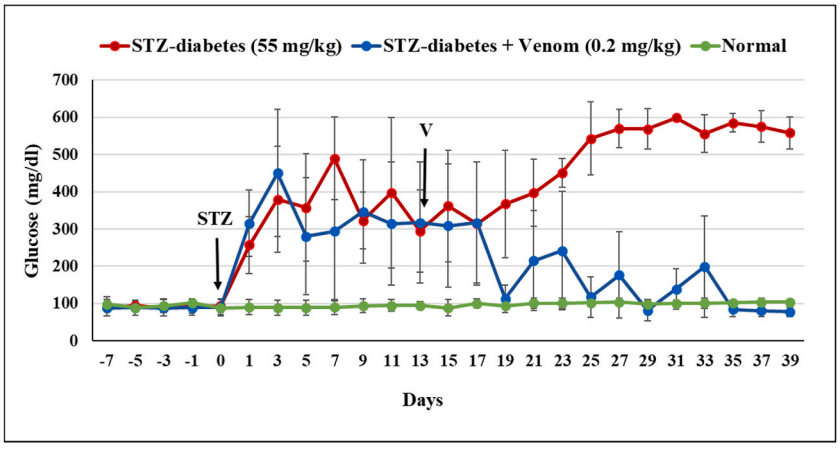

Fig. 2. The effect of the venom from the Iranian snake *Naja naja oxiana* (0.2 mg/kg) on blood glucose concentration in STZ-diabetic male rats (group VI). The rats were induced with a single injection of streptozotocin (55 mg) on day 0, and the venom was injected after 13 days compared with the control diabetic group V, and control normal group I. (STZ: Streptozotocin, V: Venom, STZ-diabetes: STZ induced diabetes) compared with the control diabetic group V, and control normal group I.

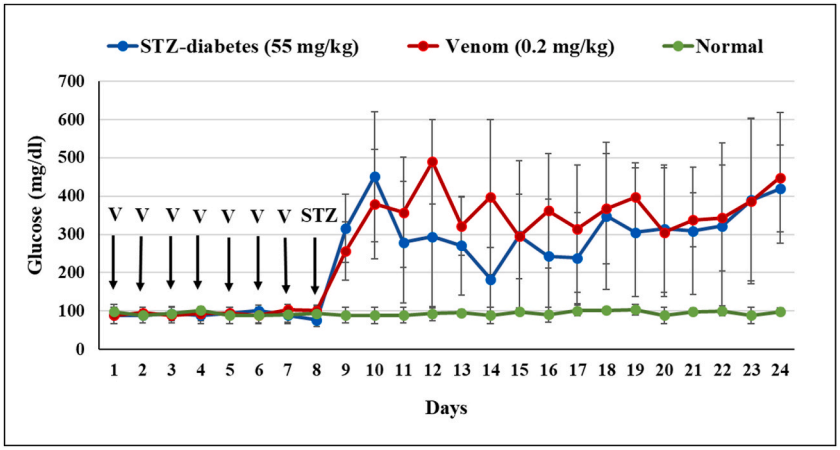

Fig. 3. Lack of prevention effect of venom of Iranian snake *Naja naja oxiana* (**0.2 mg/kg/**), (Injected for 7 consecutive days) on the induction of STZ-diabetes (Streptozotocin/**55 mg/kg/**) at day (8) group VII, in comparison with the control diabetic group V, induced with the same method and control normal group I. (STZ: Streptozotocin, V: Venom, STZ-diabetes: STZ induced diabetes).

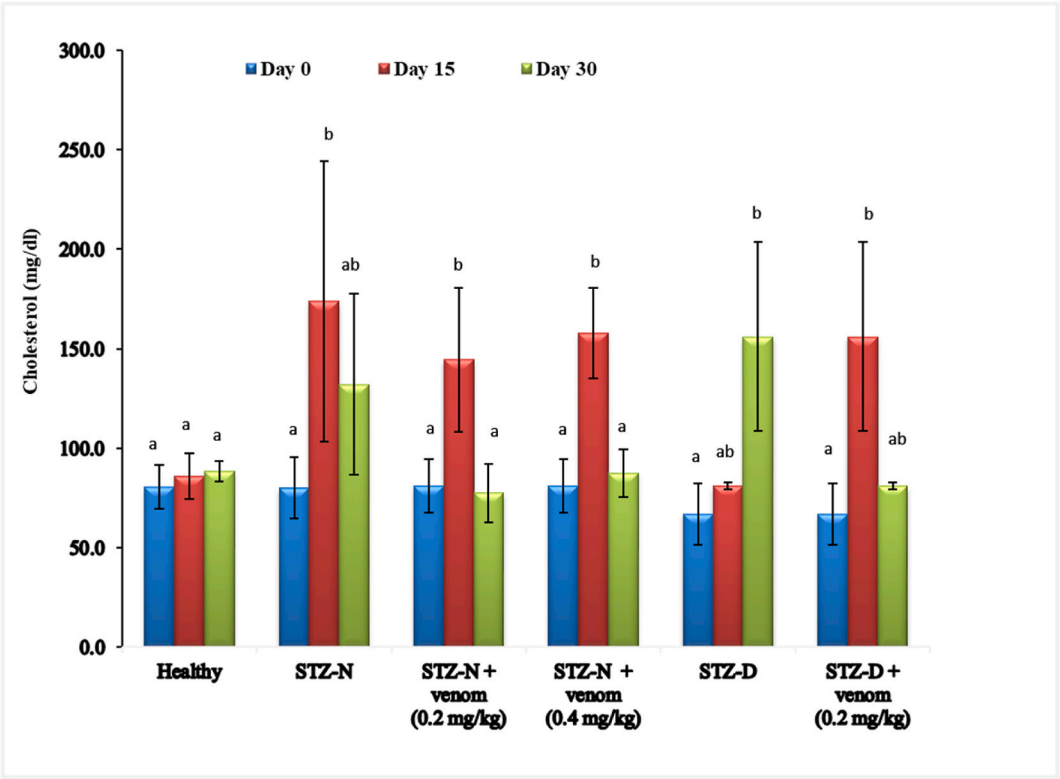

Fig. 4. The effect of venom of Iranian snake *Naja naja oxiana* on the level of cholesterol in diabetic male rats (n = 8) on days 0, 15 and 30, data in columns as Mid ± SEM (a, b). They show significant differences between different groups $p > 0.05$. (STZ-D: STZ diabetes/STZ induced diabetes; STZ-N: Streptozotocin-nicotinamide).

water intake, with no sign of polyuria. As a result, daily bedding changes were no longer required. It should be noted that some rats died or required euthanasia due to severe illness following STZ or STZ-ND administration. Consequently, these rats were replaced with healthy animals. None of the rats died after the injection of venom.

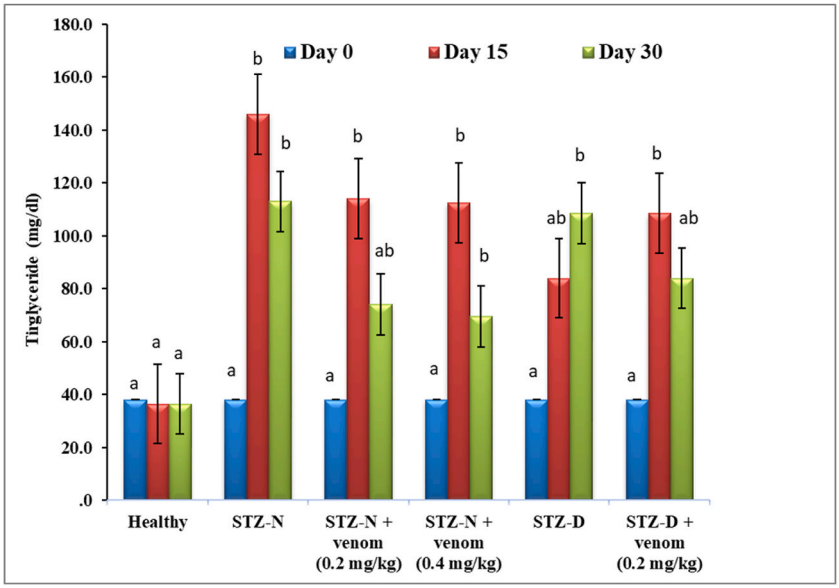

Fig. 5. The effect of venom of Iranian snake *Naja naja oxiana* on level of triglyceride in diabetic male rats (n = 8) on days 0, 15 and 30, data in columns as Mid ± SEM (a, b). They show significant differences between different groups $p > 0.05$. (STZ-D: STZ diabetes/STZ induced diabetes; STZ-N: Streptozotocin-nicotinamide).

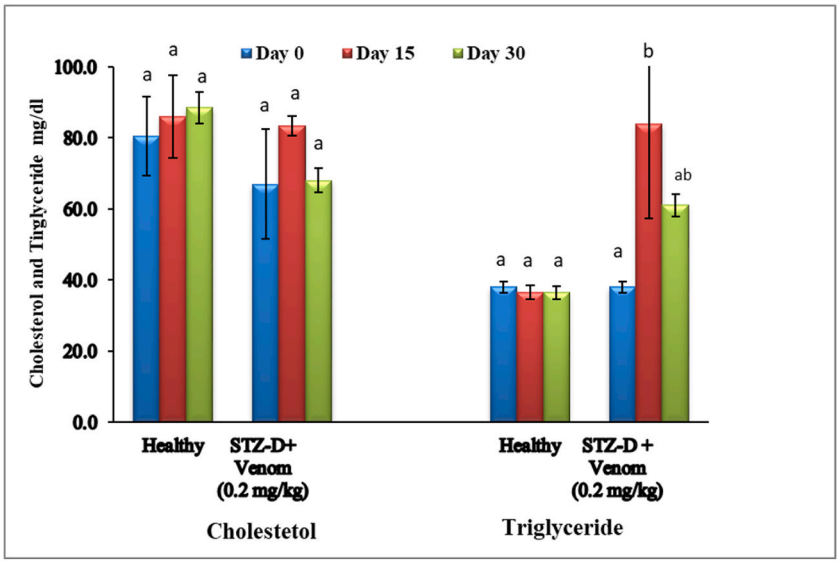

Fig. 6. The effect of 7 consecutive days injection of Iranian snake *Naja naja oxiana* venom on cholesterol and triglyceride levels in diabetic male rats (n = 8) on days 0, 15, and 30 , data in columns as Mean ± SEM (a, b). The venom showed no effect on the level of plasma cholesterol while significantly increasing triglyceride levels after two weeks in compare to healthy male rats ($p > 0.05$). (STZ-D: STZ-diabetes/STZ induced diabetes; STZ-N: Streptozotocin-nicotinamide).

4. Discussion

Venoms have provided a platform for the development of new drugs and treatments [15,31]. One venomous snake is *Naja naja oxiana*, known for its potent venom. Our novel finding was that a single injection of *Naja naja oxiana* venom significantly reduced blood glucose concentrations in STZ-diabetic male rats. Additionally, it decreased the diabetes induced elevations in plasma concentrations of cholesterol and triglycerides. Remarkably, these effects were observed within days after administration and appeared to be long lasting, with glucose, cholesterol and triglycerides being reduced to control levels by the end of the observation period. The doses used were determined based on previous studies in mice, several new pilot studies with male rats, and the LD50 of *naja oxiana* reported in

the literature. Single doses of 2, 3, and 4 mg/kg were found to be non-lethal to male rats. Furthermore, seven days of venom injection were also non-lethal and did not alter blood glucose concentrations. To the best of our knowledge, this study represents the first evaluation of the antihyperglycemic activity of *Naja oxiana* venom in experimental diabetes. We chose streptozotocin-induced diabetes as the model, as this is well-established, extensively used and closely mimics many aspects of human diabetes. It offers good face validity and has been instrumental in advancing our understanding of the disease and developing potential therapies [12]. Initially, we utilized STZ preceded by nicotinamide to establish a model of insulin-deficient type 2 diabetes [27]. However, we found that the diabetes induced using this approach was more severe than that induced by STZ alone, which is a well established model for type 1 diabetes. Consequently, due to insufficient evidence, we refrained from asserting any distinctions between the two methods in terms of the resulting diabetes. The effects of the venom were observed regardless of the method employed to induce diabetes.

The present studies have not provided any evidence for the mechanisms underlying the significant effects of the venom in reversing diabetes. However, it is important to note that the venom was administered after the establishment of diabetes, and considering the instability of STZ [32,33], it is highly unlikely that the venom modifies the direct effect of STZ in destroying the pancreatic islet β-cells [27]. Furthermore, administering the venom prior to the induction of diabetes failed to produce any protective effects. The failure of the venom to modify blood glucose in normal rats suggests that its action is somehow exerted on the STZ-induced pathology. *Naja naja oxiana* venom contains PLA2 enzyme activity [34], similar to other cobra venoms [35–38]. PLA2 has been implicated in the insulinotropic effect of various venoms [21,39,40]. Therefore, the stimulation of insulin secretion from remaining β-cells may contribute to the hypoglycemic effect of the venom observed in our study. A previous study [41] demonstrated that the hypoglycemic effect of black cobra venom in diabetic animals depended on the presence of the pancreas. However, the remarkably long-lasting effect and the absence of venom-induced hypoglycemia in normal rats in the present study argue against acute stimulation of insulin secretion as the primary mechanism.

Diabetes is associated with excessive production of free radicals, especially reactive oxygen species (ROS) [42,43], and reduced antioxidant activity due to the modification of antioxidant enzymes [44,45]. Oxidative stress plays a major role in impairing β-cell function in diabetes [46]. Several studies have shown that cobra venoms have strong antioxidant activity due to components such as cobra venom factor (CVF), cobratoxin, neurotoxins, cardiotoxin/cytotoxins, and nerve growth factor (NGF) [47,48]. These components are documented in most cobra venoms, including *Naja naja atra*, *Naja kaouthia*, *Naja nigricollis*, *Naja haje*, and *Naja naja oxiana*. Cobra Venom Factor (CVF) is an acidic glycoprotein that can reduce complement C3 and thus inhibit immune responses and inflammation [49,50]. Therefore, it may be hypothesized that the effects of the venom are mediated by protecting pancreatic β-cells from the damaging effects of oxidative stress, allowing for the preservation of regenerated β-cells in the islets, as suggested for other agents with antioxidant activity [32,33,51].

4.1. Limitation of study

This study had several limitations that need to be taken into account when interpreting the findings.

Firstly, the retention time of experimental animals following treatment with *Naja naja oxiana* snake venom could potentially be longer. However, due to the severe restrictions imposed during the COVID-19 pandemic and the subsequent closure of our university, we were unfortunately unable to accurately determine the duration and possible reversibility of the venom's effects.

Secondly, the quality of the pathology samples obtained from various tissues deteriorated due to the same reason, making them unsuitable for thorough examination. Conducting a pathological investigation with a specific focus on the pancreas before and after treatment could provide valuable insights into the mechanism of venom action.

Furthermore, our study aimed to assess the impact of crude venom on various aspects. In order to enhance future investigations, it would be beneficial to identify the specific constituents of the venom that contribute to the reduction of blood glucose levels and the facilitation of recovery in male rats with diabetes. Despite these limitations, this study has the merit of considering *Naja naja oxiana* snake venom as a potential candidate for the treatment of diabetes.

5. Conclusion

The venom of *Naja naja oxiana* has a significant effect in reversing the metabolic consequences STZ-induced diabetes. It reduces blood glucose, plasma triglycerides, and plasma cholesterol levels while improving the health of diabetic male rats. However, the specific components the venom responsible for these effects and the underlying mechanisms are yet to be determined.

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Data availability

Data will be made available on request. Repository [Behrooz Fathi; b-fathi@um.ac.ir].

CRediT authorship contribution statement

Shiva Shahdadi: Data curation, Investigation. **Farshid Hamidi:** Funding acquisition, Supervision. **Behrooz Fathi:** Supervision,

Visualization, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have **no** known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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