Original Article

# The Effects of Acidified Nitrite on Wound Healing in Streptozotocin-Induced Diabetic Mice

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## Abstract

**Background:** Diabetes mellitus (DM) is one of the most common metabolic diseases in the world. Studies have shown that nitric oxide (NO) promotes re-epithelialization and stimulates angiogenesis and neovascularization. This study aimed to investigate the effect of exogenous NO on diabetic wound healing.

**Materials and Methods:** This study was performed on 63 male BALB/c mice. For type 2 diabetes induction, the animals were fed a high-fat diet followed by a single dose of streptozotocin (STZ) (35 mg/kg) injection intraperitoneally. Acidified nitrite cream was prepared with 3.0% (w/v) sodium nitrite (SN) and 4.5% (w/v) citric acid monohydrate, respectively, in the aqueous cream base. Histopathological examinations were performed using hematoxylin and eosin and Masson's trichrome staining.

**Results:** The results showed that in the silver sulfadiazine-treated group, the size of the wound surface on the 7$^{th}$ day was significantly ($P < 0.05$) reduced compared to the control group. There was a significant ($P < 0.005$) decrease in the size of the wound in the SN-treated group on days 7 and 14 compared to the control group. The results of histopathological studies also showed that re-epithelialization and granulation in the diabetic wound site increased in the groups treated with acidified nitrite cream compared to other groups.

**Conclusion:** The use of topical acidified nitrite cream increases the speed of wound healing and it accelerates the healing of diabetic wounds in mice by causing a delay in the inflammation process and increasing the speed of re-epithelialization.

**Keywords:** Diabetes mellitus, granulation, re-epithelialization, sodium nitrite, streptozotocin

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**Submitted:** 04-Mar-2024; **Revised:** 01-May-2024; **Accepted:** 05-May-2024; **Published:** 28-Oct-2024

## Introduction

Diabetes mellitus is one of the most common and fastest-growing diseases which threaten global health.[1] According to data from the International Diabetes Federation (IDF), 415 million adults around the world suffer from DM, which is forecasted to affect 693 million adults by 2045.[1,2] Diabetes is a chronic metabolic disorder determined by high blood glucose levels that result from total or relative insulin deficiency in the context of pancreatic β-cell dysfunction, insulin resistance, and alterations in lipid metabolism.[2]Among non-communicable diseases, type 2 diabetes (T2D), which is known as non-insulin dependent diabetes, is responsible for over 90-95% of all diabetes.[1,3] Obesity is the most common risk factor for the progression of T2D. This may lead to a rise in serum triglycerides, hypertension, and insulin resistance.[4] Diabetes is a multifaceted metabolic disease that people are suffering from; it withstands long-term complications such as cardiovascular diseases, nephropathy, retinopathy, neuropathy, and diabetic foot ulcers (DFUs).[5] DFU is a severe chronic

Access this article online

Quick Response Code:

Website: www.advbiores.net

DOI: 10.4103/abr.abr_115_24



**How to cite this article:** Ghorbani M, Ghajari G, Jalali Kondori B. The effects of acidified nitrite on wound healing in streptozotocin-induced diabetic mice. Adv Biomed Res 2024;13:97.

diabetic complication of patients who have DM, which is not well controlled and comprises lesions in the deep tissues related to neurological disorders and peripheral vascular disease in the lower limbs.[6] It is estimated that the incidence of DFUs increased between 15% and 25% due to the worldwide outbreak of DM.[5] Nearly, it is reported that DFUs are responsible for limb amputation every 30 s, while DFU and amputation are the main reasons for morbidity and mortality.[7] DFUs display the most severe form of diabetic wounds.[8] In fact, the 5-year mortality rate related to DFUs requiring amputation ranges from 39 to 80%; this is very close to some progressive cancers.[9] Nowadays, the treatment of diabetic ulcers largely depends on the patient's education, prevention, and timely diagnosis.[10] Management of infection, offloading (especially in DFU complex with neuropathy), correcting systemic factors to augment healing, and negative pressure wound therapy is the gold standard care for DFUs.[11] However, when a lesion occurs, invasive therapies are costly, and non-invasive treatments are less efficient. Therefore, the investigation continuously focuses on discovering novel strategies to promote healing in the DFUs and decrease morbidity and mortality. Wound healing is a dynamic and complex physiological process that involves a series of coordinated and overlapping phases that are required to come together for skin entirety to be recovered.[12] These multi-stage restructuring mechanisms involve sophisticated interactions between different cell lineages such as fibroblasts, endothelial cells, macrophages, and platelets that cross-talk to each other via the secretion of cytokines and growth factors for restoring a tissue after damage.[13] Different stages of wound healing homeostasis, inflammation, proliferation, and remodeling of the extracellular matrix are destroyed in T2D, which can postpone the healing process.[14] Impaired growth factor production, dysregulated angiogenesis, chronic inflammation, diabetic hyperglycemia, hypoxia-induced oxidative stress, defective collagen accumulation, keratinocyte, and fibroblast migration and proliferation play fundamental roles in the pathophysiology of DFUs.[8,10] NO is the endogenous regulator of inflammation and an antibacterial agent produced by L-arginine by a group of three isozymes called NO synthase (NOS), which has become an attractive option for wound healing therapy.[15] This group of isozymes is composed of neuronal (nNOS/NOS1), endothelial (eNOS/NOS3), and inducible (iNOS/NOS2).[15] NO has a pivotal function in many aspects, from inflammation to tissue remodeling. NO plays a critical role in the adjustment of vasodilatation, stimulates angiogenesis, cell proliferation, and inflammation, and has antibacterial effects.[16] Unusual production of NO, which could be a feature of T2D, has been at once related to slowing wound healing and the development of chronic ulcers.[17] Current proofs suggest that diabetic wound fluid has substantially decreased levels of NO compared to healthful wound fluid, owing to down-regulated eNOS.[15] Because of the critical function of NO in wound healing and its safety for human treatment, NO-based therapies have gained growing interest in recent years.[15] Topical administration of exogenous NO has been applied for skin wound healing or as an antibacterial agent.[18] The evidence shows that the production of NO from the combination of 3.0% (w/v) sodium nitrite (SN) and 4.5% (w/v) citric acid has beneficial effects on infected wound healing.[18] Since the use of gaseous NO is restricted by factors such as its cost, long processing time, and potential host cytotoxicity, it seems that topical application of these two compounds separately produces NO.[19] Since nitric oxide can be absorbed through the skin, the topical route is the most convenient and easiest for patients and allows the direct treatment of damaged tissues. Therefore, the aim of this study was to appraise the effects of acidified nitrite on wound healing in type 2 diabetic mic.

## Materials and Methods

### Animal care and induction of experimental type 2 diabetes

In this study, 63 male BALB/c mice with approximately weight 18-20 g were purchased from Laboratory Animal Breeding Centre of Baqiyatallah University of Medical Sciences. All animal procedures were conducted in accordance with the animal care and use protocol approved by the ethics committee of the Baqiyatallah University of Medical Sciences (IR.BMSU.REC.1400.042). Mice were kept in standard conditions (temperature 22 ± 2°C, 12 h dark and 12 h light) with adequate water and food. In this study, all mice were fed a high-fat diet (HFD) for 21 days with a total calorie value of ~4900 kcal/kg (58.8% lipids, 14.2% proteins, and 27% carbohydrates) to induce type 2 diabetes and insulin resistance.[1] Mice were kept on HFD until the end of the study. After 21 days of dietary manipulation, to induce T2D, a single low dose of STZ (Sigma Aldrich, Germany) was prepared in 0.1 mM citrate buffer (Merck, Germany), then injected intraperitoneally.[20] After STZ injection, the blood glucose levels of each mouse were measured in tail-vein blood samples with an Accu-Check glucometer (Roche Diagnostics). Mice with fasting serum glucose levels >200 mg/dL were considered in the diabetic group. We used the HFD-STZ model of type 2 diabetes in which 3 weeks after HFD utilization, insulin resistance is developed, and the injection of low-dose STZ creates partial β-cell dysfunction. This model has characteristics similar to those of human T2D. Mice were divided into a diabetic control group ($n$ = 21), an acidified nitrite-treated group ($n$ = 21), and a silver sulfadiazine-treated group ($n$ = 21). Mic in subgroups was assessed at 3 time points on days 3, 7, and 14 after wounding.

### Induction of diabetic wound

For induction of diabetic wounds, mice were administered anesthesia with ketamine and xylazine (70/10 mg/kg) injection.[16] Their dorsal fur between the shoulders was shaved, and the skin was cleaned with 70% ethanol. Two circular, 5-mm, full-thickness wounds were created on the dorsum of each mouse with a dermal biopsy punch.[21] In this study, we used 7 mg/kg bupivacaine (Eugia US LLC) to reduce postoperative pain.

### Cream composition and application

The acidified nitrite cream consists of SN and citric acid, which were prepared separately and combined with each other at the wound area.[22] The reaction between nitrite and acid produces NO exogenously. This convenient method of NO production permits localized treatment, with salts like SN easily inserted into creams and ointments. Acidified nitrite cream was prepared with 3.0% (w/v) SN (Sigma Aldrich, Germany) and 4.5% (w/v) citric acid monohydrate (Merck, Germany), respectively, in the aqueous cream base (Farabi Pharmaceutical Company, Iran).[16,22] For topical use of cream, the mice were completely limited, and after immobilizing, the wound surface was covered with a thin layer of SN and citric acid creams and gently mixed together, then the surface of the wound was dressed. The creams were used once a day at the wound site until the last day of the study.

### Wound closure analysis

To evaluate wound healing, reductions in wound area have been recorded by using a digital camera (Euromex DC5000, Holland) at days 0, 3, 7, and 14. Wound areas on days 7 and 14 are considered as a main indicator of wound healing. The wound area was calculated using ImageJ software (National Institute of Health, Bethesda, MD). Wound closure rates were represented as a percentage of the original wound area.[23]

### Histological study of wound

Histological analyses were performed on days 3, 7, and 14 post-wounding. Before the animal was sacrificed and wound tissue was collected, mice were euthanized by cervical dislocation under anesthesia using isoflurane (Piramal Critical Care, USA). Standard specimens were cut off along with 1–2 mm from the surrounding normal skin. The wound samples were fixed in neutral-buffered formalin for 72 h. Samples were then placed in a series of increasing ethanol concentrations at the processing machine for the dehydration process. After the paraffin wax was embedded, samples were sectioned using a histological microtome. For the observation of cellular infiltration, formation of new blood vessels, the neuroepithelium length, and the degree of collagen maturity, sections of 5 µm thick tissue were mounted on a coated glass slide and stained using hematoxylin and eosin (H and E) and Masson's trichrome respectively. ImageJ software was used to measure the mean thickness of epidermis and dermis of H and E histological stained and the extent of regions staining positive for collagen (blue) in Masson's trichrome-stained histological sections of all treatment and control groups of mice.[24-26]

### Statistical analysis

The results were presented as the mean ± standard error mean (mean ± SEM). Continuous data with a normal distribution were analyzed using a *t*-test and one-way analysis of variance followed by Tukey's test. Statistical analyses were performed using SPSS software version 15.0 (Chicago, IL, USA). Significant difference at the level of $P < 0.05$ was considered for all the tests.

## Results

The blood glucose levels of mic were measured in tail-vein blood samples with a glucometer. Serum glucose levels higher than 250 mg/dL were considered as diabetic mic. Our results indicated that serum glucose concentrations in the diabetic rats were significantly ($P < 0.01$) higher than in the control rats.

The results of the macroscopic evaluation showed that the size of the wound in both treatment groups with SN and silver sulfadiazine decreased compared to the diabetic control group [Figure 1]. The speed of wound healing in the group treated with SN compared to the silver sulfadiazine and diabetic control groups showed a significant increase on day 3 post-wounding.

The results showed that the average size of the wound in the silver sulfadiazine treatment group decreased on days 3, 7, and 14 compared to the diabetic control group, but this decrease was significant only on days 7 post-wounding. In the group

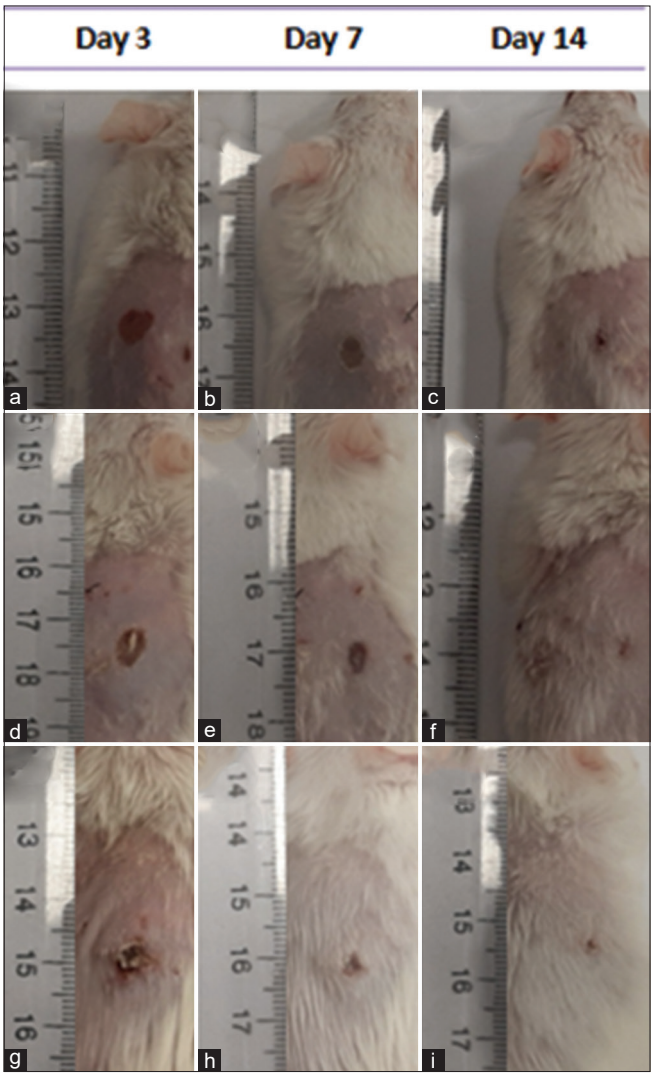


**Figure 1:** Macroscopic evaluation of wound closure rate in the diabetic control group (a-c), treatment groups with silver sulfadiazine (d-f) and sodium nitrite (g-i) on days 3, 7, and 14 post-wounding

treated with SN, 3 days after wounding, the size of the wound on the 7th and 14th days compared to the diabetic control group significantly [Figure 2]. These results indicated that the size of the wound in the SN ointment did not decrease in the first days post-wounding compared to other groups.

The accumulation of inflammatory cells can be seen in the diabetic control group [Figure 3]. In the SN and silver sulfadiazine treated groups, inflammation decreased in the wound area at 7 and 14 days after wounding. The re-epithelialization occurred well in both treatment groups. However, its speed was higher in the SN-treated group than in the silver sulfadiazine group. The amount of granulation in the SN-treated group has increased compared to the control and silver sulfadiazine groups, which is significant compared to the control group.

The results of Masson's trichrome staining [Figure 4] showed that there was an increase in collagen synthesis in both treatment groups, but collagen synthesis was more in the group treated with SN than the silver sulfadiazine group.

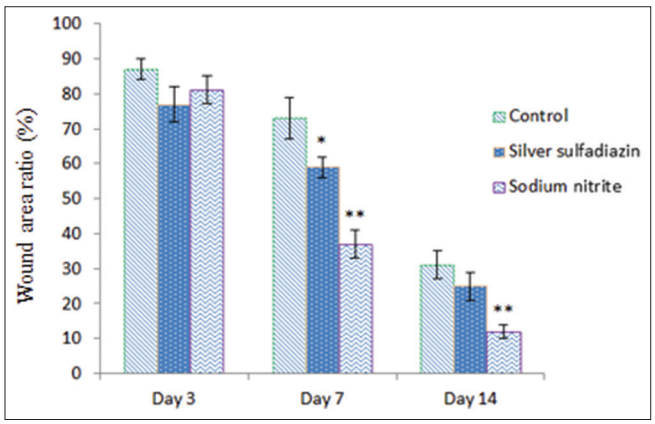


**Figure 2:** Wound healing ratio (%) in diabetic control, silver sulfadiazine, and sodium nitrite groups at 3, 7, and 14 days after wounding. $*P < 0.05$ and $**P < 0.01$ indicate significant differences in comparison with the respective control group

## Discussion

Our study results indicated that acidified nitrite cream accelerates the treatment of type 2 diabetic wound healing. Also, based on the histopathological results, the use of this cream can accelerate re-epithelialization and collagen formation at the wound site. Re-epithelialization and speed-up wound healing can prevent the adverse effects of diabetic wounds such as infection. The results showed that the speed of wound healing in the diabetic control group was low. In the silver sulfadiazine treated group, the size of the wound surface (percentage of the wound surface compared to day 0) decreased significantly compared to the control group on the 7th day postwounding, but it did not show any significant difference on the 3rd and 14th day postwounding. There was a significant decrease in the size of the wound surface in the SN treatment group on days 7 and 14 compared to the control group. The results collected indicate that NO improves wound repair in diabetic rats. NO is generated internally via the enzyme NOS, which gives rise to three distinct NOS isoforms. The nNOS is present in keratinocytes, while eNOS has been seen in the basal epidermal layer of keratinocytes and dermal fibroblasts, and iNOS is recognized in fibroblasts, keratinocytes, and endothelial cells.[27,28] Numerous studies indicate that decreased levels of NO result in poorer wound healing in individuals with diabetes.[29,30] Research indicates that the expression of skin eNOS or the levels of NO have been significantly diminished in mice with type 1 diabetes caused by STZ, leading to a decline in the healing process. Nevertheless, the implementation of skin gene therapy successfully reinstated eNOS expression and NO levels, hence expediting the process of wound healing in animals with type 1 diabetes produced by STZ. In an attempt to heal diabetic wounds, NO donors have been prescribed to deliver exogenous NO to the wound site.[31,32] Stallmeyer *et al*.[33] provided evidence that a modest concentration of NO promotes the growth of keratinocytes in a laboratory setting and observed increased proliferation of fibroblasts. Additional research indicates that dressings

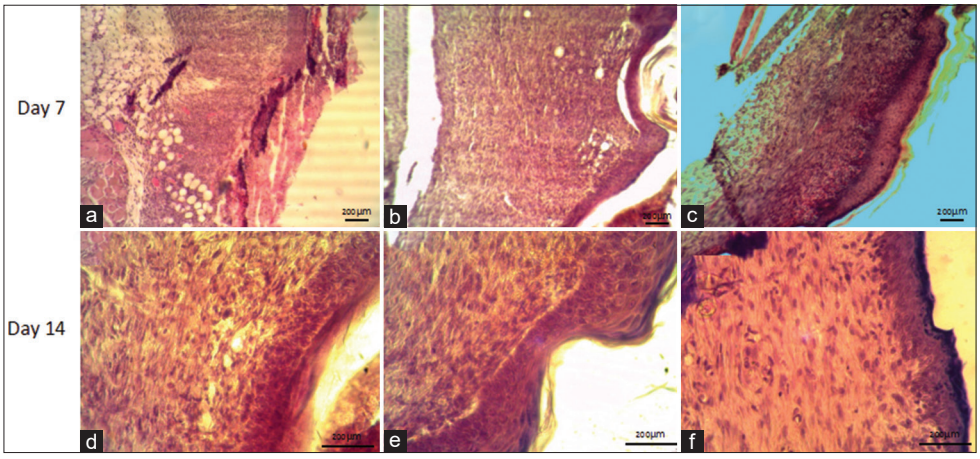


**Figure 3:** Hematoxylin–eosin stained histological sections at days 7 and 14 after wounding. (a) and (d) self-healing in the untreated diabetic wound. (b) and (e) diabetic wound treated with silver sulfadiazine. (c) and (f) diabetic wounds treated with sodium nitrite. (ie) immature epidermis; (gt) granulation tissue; (e) epithelium. Scale bar: 200 μm

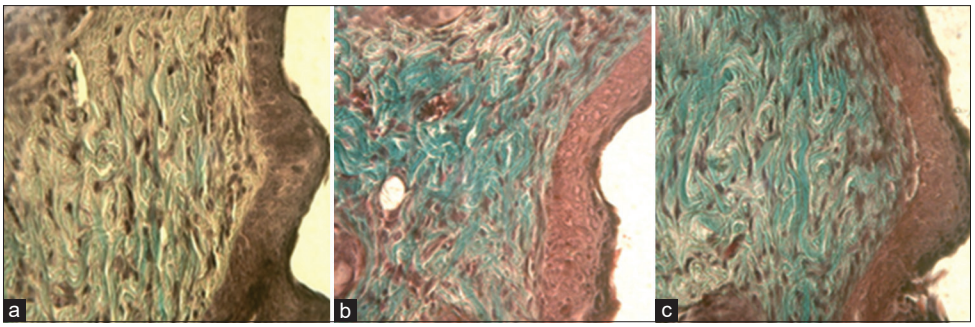


**Figure 4:** Masson's trichrome stained histological sections. (a) Self-healing in the untreated diabetic wound. (b) Diabetic wound treated with silver sulfadiazine. (c) Diabetic wound treated with sodium nitrite

made from PVA, which releases NO, can enhance the healing of wounds in individuals with diabetes.[34] Furthermore, in accordance with the findings derived from this investigation, Afzal *et al*.[16] demonstrated that acidified nitrite expedites the process of wound healing in rats with type 2 diabetes by swiftly regenerating the dermis, enhancing the formation of new blood vessels, and hastening the accumulation of collagen in the injured tissues.

The results of histopathological investigations showed that re-epithelialization and granulation in the diabetic wound site increased in the groups treated with SN ointment compared to other groups. In previous studies, the wounds were induced using a biopsy punch in animals. New studies show that according to the differences in the structure of the layers under the skin of mice compared to humans, the skin in these animals has a much greater range of motion, and the speed of wound healing is very different from that of humans.[35,36] In this research, the excisional wound-splitting model was used to solve this problem and increase the similarity of the diabetic wound model. Considering the basic differences in the structure of mouse and human skin, the use of this method to create a wound in this study made the diabetic wound more similar to the human wound model. Generally, the healing rate of a diabetic wound depends on the time of using SN and the amount of its use in the wound. Previous studies have shown that the use of NO on the first day of wound formation delays wound healing.[37] Here, the speed of wound healing in the group treated with SN was low at first, but gradually, from the 7$^{th}$ day onwards, it increased significantly compared to other groups. NO is a potentially effective agent for wound healing due to its beneficial effect on angiogenesis. In another study, a set of multifunctional hydrogels with carboxymethyl chitosan (CMCS), 2,3,4-trihydroxybenzaldehyde (THB), copper chloride (CuCl2) and graphene oxide (GO)-N, N'-di-sec-butyl- were developed by He *et al*. N, N'-dinitroso-1,4-phenylenediamine (BNN6) (abbreviated as GB). These hydrogels have antibacterial, antioxidant, self-healing, and conductive properties and can release NO upon near-infrared (NIR) laser irradiation. The purpose of producing these hydrogels is to increase wound healing in people with TID. Overall, these hydrogels, which include many functions such as photothermal conductivity, antioxidant properties, and self-healing abilities, along with the ability to release nitric oxide, demonstrate significant promise in the treatment of type I diabetic wounds.[38] The negative effect of using SN on the first day of wound occurrence has been attributed to inhibiting the accumulation of platelets in the wound area by NO donors. However, recent studies have shown that the use of SN ointment can reduce inflammation and delay the wound inflammation process by reducing the expression of TNF-α at day 7 after wounding.[16] In addition, NO hinders infection by regulating bacterial growth when applied externally, hence facilitating wound healing.[39]

## Conclusion

Inadequate control of chronic lesions and DFUs may require amputation or preservation of the limb, thus reducing the quality of life of the affected person. To overcome this challenge, in this study, a wound-healing ointment composed of acidic SN that releases nitric oxide was synthesized. NO, which is naturally generated by endothelial cells in normal physiological states, promotes cell proliferation, cell migration, and stem cell differentiation. This ultimately results in improved vascularization and accelerated wound healing. In diabetic wounds, the natural production of NO is decreased or delayed, resulting in an inadequate supply of NO. This insufficient supply of NO fails to address important difficulties, including microbial infections, chronic inflammation, and nutritional deficits in developing cells, mostly due to a lack of angiogenesis. Our results show that the use of topical acidified nitrite in the treatment of type 2 diabetic wounds in mice accelerates wound healing by delaying the inflammation process and increasing the speed of re-epithelialization and collagen synthesis. Therefore, the use of acidified SN as a NO donor is a promising strategy in the treatment of diabetic wounds. Despite significant progress previously achieved, future research should prioritize efforts aimed at minimizing potential detrimental effects, regulating the release of NO, and enhancing the stability of NO donors.

### Acknowledgments

The authors are grateful to the Baqiyatullah University of Medical Sciences for the financial support.

## Financial support and sponsorship

This study was approved by Baqiyatallah University of Medical Sciences (400000112), and this university was responsible for funding provision.


## Conflicts of interest

There are no conflicts of interest.

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