Research Paper

Histopathological Evaluation of the Effects of *Pistacia atlantica* Extract on a Mouse Model of Diabetic Wound

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ABSTRACT

Introduction: Type 2 diabetes is the most common type of diabetes characterized by insulin resistance and beta cell dysfunction. The speed of wound healing is very important in the healing process. Research shows that the use of natural products and traditional medicine methods in treating many diseases and wounds has spread worldwide. Therefore, the present study aims to investigate the histopathological effectiveness of *Pistacia atlantica* extract (Beneh) in improving the experimental model of diabetic wounds.

Methods: This study was conducted on 48 BALB/c mice. To induce type 2 diabetes, the animals received a high-fat diet for two weeks, and then a single dose of 30 mg/kg was injected intraperitoneally. The wound was created by an excisional wound splinting model and biopsy punch. Glucose level was measured with a glucometer, and insulin level was measured using an ELISA kit. Histopathological examination was also done using hematoxylin/eosin staining and Masson trichrome staining.

Results: The macroscopic study showed that the wound size was reduced in both *P. atlantica* extract and silver sulfadiazine groups compared to the wound control group. The results of hematoxylin and eosin staining also showed a reduction in inflammation in the wound area in the treatment groups. The re-epithelialization occurred well in both treatment groups. However, its speed was higher in the *P. atlantica*-treated group than in the silver sulfadiazine group. Masson's trichrome staining results showed the collagen fibers in the *P. atlantica* group have a more regular arrangement than silver and wound control groups. The results of the serum analysis also showed that the gum extract of *P. atlantica* reduced the production of NO and MPO in the treatment groups compared to the control group.

Conclusion: The results of our study showed that the use of *P. atlantica* extract topically in the diabetic wound area can improve the rate of closure and re-epithelialization in the diabetic wound by reducing inflammation.

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Introduction

Diabetes mellitus is one of the most important risk factors for some disorders, such as nephropathy, retinopathy, neuropathy, and cardiovascular diseases [1]. Type 2 diabetes is the most common type of diabetes, characterized by insulin resistance and beta cell dysfunction. Insulin resistance occurs in the initial stages of the disease, and in this stage, beta cells maintain glucose homeostasis by increasing insulin secretion. After a long period of resistance to insulin, the function of beta cells is weakened and they cannot deal with insulin resistance, which leads to an increase in blood glucose and lipid metabolism disorder [2, 3]. Age, obesity, lack of physiological activity, and genetics are the factors that contribute to this type of diabetes [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. About 25% of diabetic patients face the problem of ulcers during their life. Disturbances in the wound-healing process in diabetic patients are mainly related to hyperglycemia, excessive expression of inflammatory cytokines, decreased angiogenesis, and microbial infections [5, 6]. Topical creams and dressings containing antibiotics and debridements are usually used in these patients to control disease and improve the healing process. However, some wounds do not respond to these treatments. Increasing antibiotic resistance is another concern when treating these patients with conventional methods [7].

In recent years, accelerating the wound-healing process has attracted the attention of researchers. Increasing the speed of wound healing in diabetic patients by using herbal and natural compounds can increase the survival rate in these patients [8]. *Pistacia atlantica* kurdica (*P. atlantica*) is a type of pistachio from the Anacardiaceae species. It is widely found in the Zagros mountain regions, specifically in the west and northwest of Iran. The species of this plant category are evergreen and durable. There are α-pinene, α-thogen, sabinene, limonene and beta-pinene in the gum of *P. atlantica* [9]. Pharmacologically, *P. atlantica* has antioxidant effects and antimicrobial activity [10]. The present study aims to investigate the histopathological effectiveness of the gum extract of *P. atlantica* in improving a mouse model of diabetic wound.

Materials and Methods

Animals

In this study, male BALB/c mice weighing approximately 22-25 g were purchased from the Laboratory Animal Breeding Centre of Baqiyatallah University of Medical Sciences. Mice were kept in standard conditions (temperature 22±2 ˚C, relative humidity 40–60 %, 12 h dark and 12 h light) with adequate water and food.

Induction of experimental type 2 diabetes

In this study, all mice were fed a high-fat diet (HFD) 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. After 21 days of dietary manipulation, mice fasted for 12-14 hours with access to water. To induce T2D, a single low dose of STZ (35 mg) was prepared in 0.1 mM citrate buffer (pH=4.5) and then injected intraperitoneally. After STZ injection, the blood glucose levels of each mouse were measured in tail-vein blood samples with a glucometer. Mice with fasting serum glucose levels >250 mg/dL were considered in the diabetic group.

Induction of diabetic wound and grouping

This study used an excisional wound splinting method to increase the similarity of the mic diabetic wound healing to human skin. For this purpose, after anesthesia, two full-thickness wounds were created on the back of each mouse with a 5-mm dermal biopsy punch. Then, the silicone ring was placed on the skin around the wound and fixed to the underlying tissue using 0-6 surgical sutures, preventing skin contraction.

Mice were randomly divided into three groups: The first group (wound control) included diabetic mice that induced wounds and did not receive any treatment. In the second group, after the induction of diabetic wound, the treatment with the extract of *P. atlantica* gum was used. Silver sulfadiazine ointment was used in the third group.

Macroscopic evaluation

To evaluate wound healing, wound area reductions were recorded using a digital camera on days 0, 3, 7, and 14. The wound area was calculated using ImageJ software, version 1.53. Wound closure rates were represented as a percentage of the original wound area. The

Table 1. Characteristics of normal and diabetic groups

Groups	Mean±SD	
	Normal	Diabetic
Serum insulin (pmol/L)	262±14	203±28
Serum glucose (mg/dL)	160±18	272±31

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percentage of wound contraction was determined using the Equation 1:

1. Wound contraction (%)=[(Wound area on day zero-Wound area on particular day)/wound area on day 0]×100.

Histopathological evaluation

On days 7, 14, and 21 after wounding, the samples were removed from the skin of all groups. After washing with physiological serum, they were immediately placed in 10% formalin to fix and prepare tissue sections. Then, tissue processing was done, and tissue sections with a thickness of 5 micrometers were prepared in the form of serial sections. The histopathological changes of the tissue were investigated by hematoxylin & eosin and Masson's trichrome staining.

Biochemical analysis

At the end of the study, blood samples were collected, and serum was separated from the cells by centrifugation at 3,000 rpm for 10 min. Serum samples were stored at -20 ˚C. The serum levels of MPO and NO were measured using nampox myeloperoxidase activity assay (NS-15063) and natrix nitric oxide assay (NS-15044) Kits according to the manufacturer's instructions.

Statistical analysis

Data analysis was done using SPSS software, version 21. Kolmogorov-Smirnov test was used to check the normality of data distribution. Repeated measurement variance analysis was used to check the difference between groups. The significance level of the study was considered $P<0.05$.

Results

Diabetes induction

The serum glucose level of normal mice was about 160 mg/dL, which increased to about 272 mg/dL in diabetic mice three days after STZ injection. Diabetic mic serum insulin level decreased from about 262 pmol/L to 203 pmol/L , indicating type 2 diabetes in these animals (Table 1).

Macroscopic evaluation

The macroscopic study showed that the wound size was reduced in the *P. atlantica* extract and silver sulfadiazine groups compared to the control group. The speed of wound healing in the group treated with Bene extract compared to the silver sulfadiazine and control groups showed a significant increase.

Histopathological evaluation

Figure 1 shows the results of hematoxylin and eosin staining. In the control group, the accumulation of inflammatory cells was seen. In the treatment groups, inflammation was reduced in the wound area. The re-epithelialization occurred well in both treatment groups. However, its speed was higher in the *P. atlantica*-treated group than in the silver sulfadiazine group.

Masson's trichrome staining showed that the collagen formation in the wound site in the early stages was very low. In the later stages of wound healing, collagen formation at the wound site increased, and this increase in the *P. atlantica* group was greater than in other groups. As seen in Figure 2, the collagen fibers in the *P. atlantica* group had a more regular arrangement.

NO and MPO serum level

The *P. atlantica* extract and silver sulfadiazine significantly reduced the production of NO and MPO compared to the control group (Figure 3). The NO production was lower in the *P. atlantica* group, and the MPO production was lower in the silver sulfadiazine group. However, the difference between these two groups was not significant.

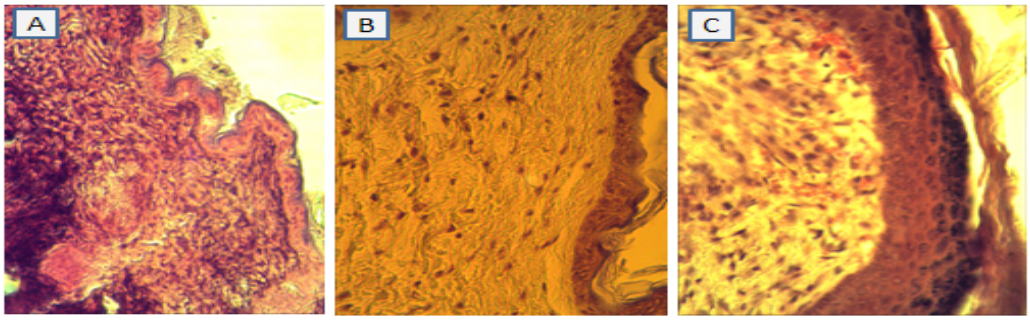

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Figure 1. Hematoxin & eosin staining of the diabetic wound in different groups
A) Control group, B) Group treated with silver sulfadiazine, C) Group treated with *P. atlantica* extract

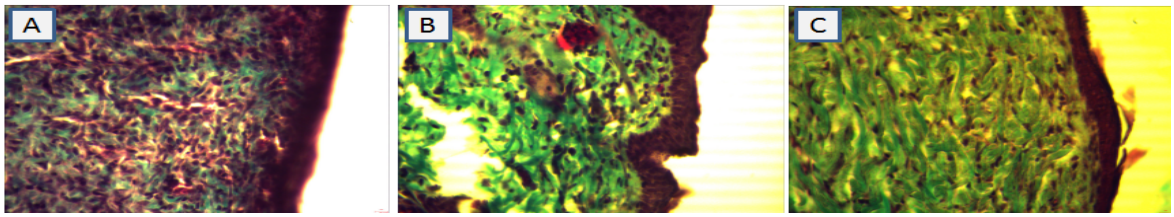

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Figure 2. Masson's trichrome staining of the diabetic wound in different groups
A) Control group, B) Group treated with silver sulfadiazine, C) Group treated with *P. atlantica* extract
Notes: The green colors indicated immature collagens.

Discussion

Previous studies have shown that streptozotocin has destructive effects on beta cells. The destructive impact of STZ has a direct relationship with its dose, such that high doses are used to induce type 1 diabetes. Using a high-fat diet with a low dose of STZ can induce type 2 diabetes in rodents [11-13].

In a study by Zhang et al. [14], the animals were fed a high-fat diet for four weeks and received STZ (30 mg/kg) intraperitoneally; the diabetes model was well established. In the present study, a low-dose STZ injection method was used along with a high-fat diet to induce type 2 diabetes. The results of the study showed that the gum extract of *P. atlantica* improved wound healing and reduced NO and MPO production in diabetic mice compared to the control group. Hamidi et al. designed a study on skin wound healing using topical application

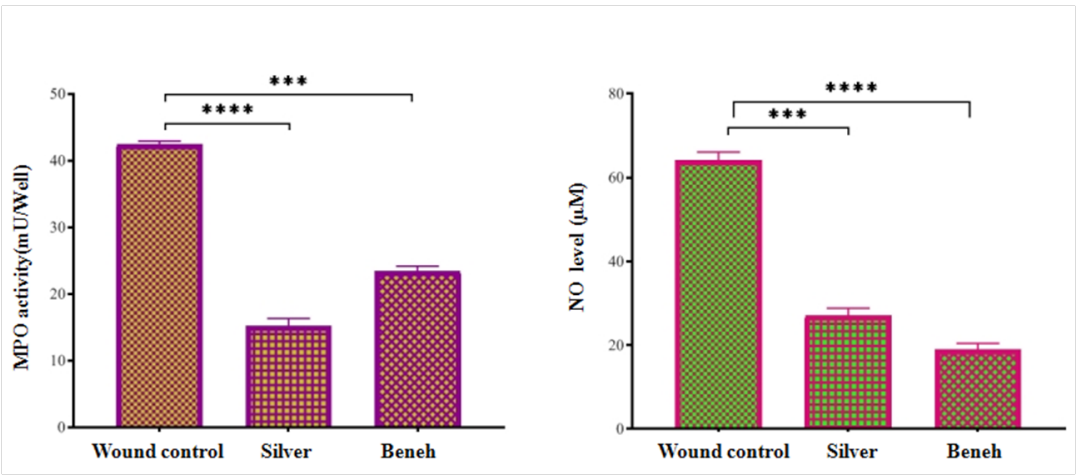

Figure 3. Serum level of NO and MPO in treatment and control groups

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of *P. atlantica* gel on rats. Their results, similar to our findings, showed that the topical application of *P. atlantica* oil gels improves re-epithelialization and mature granulation tissue compared to the control group. Collagen fibers showed a more organized pattern compared to the control group [15]. Esmaili et al. showed in their study that the aqueous extract of *P. atlantica* modulates the responses of the humoral and cellular immune system in Balb/C mice following immunization with SRBS [16]. Shahkarmi et al. showed that the aqueous extract of *P. atlantica* moderated the reactions of the immune system and reduced inflammatory cytokines in rats with experimental asthma [17]. Another study evaluated the dose-dependent effect of *P. atlantica* on angiogenesis and skin burn wound healing in rats. Their results, which were not consistent with our study, showed no statistically significant difference in wound closure speed. The concentration of NO on the fifth day after wounding was not significantly different between the groups. Previous studies have shown that *P. atlantica* extract has many compounds, and α-pinene (about 45-70%) is the main component, and α-pinene seems to be the active ingredient in the extract [19]. Memariani et al. showed that α-pinene is the main component of *P. atlantica* oil. They reported that *P. atlantica* oil had a protective effect against ethanol-induced gastric ulcers [20].

Collagen is an extracellular matrix protein that plays an important role in the strength of wound contraction and healing process. Collagen turnover is directly related to free hydroxyproline. *P. atlantica* extract can change the amount of collagen synthesis by changing the level of hydroxyproline, and accelerate the wound healing process. Farahpour et al. showed that *P. atlantic*a oil changed the collagen pattern and caused the formation of organized collagen fiber after three weeks, which is consistent with our study. They also found that the hydroalcoholic extract of *P. atlantica* increases the proliferation of fibroblasts and thus reduces inflammation [21]. Most likely, the α-pinene present in the *P. atlantica* extract reduces inflammation and stimulates the proliferation of fibroblasts, causing epidermal regeneration in the wound area and increasing the skin's strength by producing collagen fibers.

Conclusion

The results of this study showed that the topical use of *Pistacia atlantica* extract, by reducing the level of NO and increasing re-epithelialization, improve the rate of wound closure in diabetic mice.

Ethical Considerations

Compliance with ethical guidelines

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 (Code: IR.BMSU.REC.1400.002).

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Authors' contributions

Study design and Original draft preparation: Bahman Jalali Kondori; Data collection: Majid Mirzaee Nadoushan and Hadi Esmaeili Gouvarchin Ghaleh; Final approval: All authors.

Conflict of interest

The authors declared no conflict of interest.

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