

Aminoguanidine cream ameliorates skin tissue microenvironment in diabetic rats

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Abstract

Introduction: The aim of the study was to explore the effect of aminoguanidine cream on the skin tissue microenvironment in diabetic rats.

Material and methods: A total of 51 healthy male Sprague Dawley (SD) rats were randomly divided into three groups: the diabetes group ($n = 18$), the aminoguanidine group ($n = 18$) and the control group ($n = 15$). Rats in the diabetes group and aminoguanidine group were injected with 65 mg/kg streptozotocin to induce the diabetes model, and in the control group with citrate buffer. After successful induction of diabetes, the back hair of all rats was stripped by barium sulfide, and the aminoguanidine group was treated with aminoguanidine cream using disinfected cotton swabs twice every day for 40 days, while the diabetes and control groups were treated with the cream matrix. The pathological changes of skin were observed by HE staining, while the content of inflammatory cytokines (TNF- α , IL-8, ICAM and IL-1 α) and the antioxidant indexes (T-AOC, GSH-PX, MPO MDA H₂O₂) were examined using commercial kits.

Results: After 40 days of treatment, the diabetes group manifested tissue lesions, whereas the aminoguanidine group seemed normal. Compared with the diabetes group, the content of inflammatory cytokines TNF- α , IL-8, ICAM and IL-1 α was dramatically lower in the aminoguanidine group. T-AOC in all groups underwent dramatic changes and returned to normal finally. The activities of GSH-PX and MPO and content of H₂O₂ in the diabetes group were all higher than those in the aminoguanidine group.

Conclusions: Aminoguanidine may have a good systemic effect on alleviating the pathological changes of skin tissue in diabetic rats, which may be attributed to the regulation of GSH-PX, TNF- α , IL-8, ICAM and IL-1 α .

Key words: aminoguanidine, diabetic, skin tissue, oxidative stress, proinflammatory cytokines, streptozotocin.

Introduction

Diabetes mellitus (DM), a group of metabolic diseases with high blood sugar, is due to either the pancreas not producing enough insulin, or because cells of the body do not respond properly to the produced insulin [1]. The prevalence of diabetes is increasing every year worldwide, and in China, the number of diabetes patients is growing rapidly and has reached more than 92 million in the last decade. Generally, diabetic patients always suffer from various complications, such as chronic lower extremity skin ulcer, which affects about 15% of patients. The skin ulcer on the lower limb is known as a common complication for diabetes pa-

tients and it greatly affects the quality of patients' life and works [2, 3]. The ulcer may even progress into lower limb ischemia, which is a serious event in diabetes patients and may lead to increased mortality and morbidity in this particular patient population [4]. However, there is still no effective treatment to fight against the complication. It has been reported that locally external application of a gentamicin-impregnated collagen sponge can shorten the wound healing duration in diabetic patients [5]. Therefore, the external application of a certain drug to prevent or treat lesions may be a simple, effective and safe approach for diabetic skin ulcer.

Aminoguanidine is a nucleophilic hydrazine compound, and the basic chemical structure is HN-NH-C-NH. Aminoguanidine has been indicated to have a protective effect on the passive avoidance learning and memory in streptozotocin-induced diabetic rats acting through regulation of apoptosis-related genes in the hippocampus [6]. Moreover, aminoguanidine has also been reported to influence the counter-torque force of bone implants in streptozotocin-induced diabetic rats [7]. Aminoguanidine is suggested to be a safe drug for the treatment of diabetes complications at a dosage of 1 g/l [8]. Research has shown that aminoguanidine can competitively bind to the early glycosylation products, generate an inactive substitute which would not cause protein cross-linking, and thereby inhibit the formation of advanced glycation end products (AGE) [9]. Aminoguanidine can promote wound healing in diabetes by blocking AGE deposition and improving the microenvironment [10]. Advanced glycation end products are a kind of irreversible glycation products, which can cause changes in cell function and structure, lesions and oxidative stress of skin tissue matrix, and further lead to delayed healing [11–13]. It has been reported that lower extremity ulcer is refractory, mainly due to the accumulation of AGE in skin tissue of diabetic patients [14, 15]. Therefore, we speculate that materials which can reduce or inhibit the generation of AGE may have a therapeutic effect on the refractory complication. Moreover, aminoguanidine has been successfully applied in the treatment of many other diabetic complications, including diabetic nephropathy, atherosclerosis and other complications [8, 16–18]. However, studies involving the effect of aminoguanidine on trauma by improving the skin tissue microenvironment are relatively rare.

In the current study, we aimed to evaluate the effect of aminoguanidine in models of induced trauma after diabetes by detecting the AGE-related proinflammatory cytokines (tumor necrosis factor α – TNF- α , interleukin 8 (IL-8), intercellular adhesion molecule (ICAM) and IL-1 α) and oxi-

dative stress factors (total antioxidant capacity (T-AOC), methane dicarboxylic aldehyde (MDA), myeloperoxidase (MPO), glutathione peroxidase (GSH-PX) and H₂O₂). We expect the study to supply new insights for the treatment of lower extremity skin ulcers in diabetic patients.

Material and methods

Preparation of aminoguanidine cream

The oil-in-water cream matrix was prepared by mixing a certain proportion reagents, including an oil phase (100 g/ml stearic acid, 12% liquid paraffin, 15 g/l Vaseline, 15 g/l lanolin, 50 g/l isopropyl myristate) and a water phase (0.8% glycerol, 0.1% nipagin alcohol, 0.2% triethanolamine). Then the aminoguanidine powder was gradually added to the cream matrix below 60°C to finish the aminoguanidine cream.

Diabetic rat model and aminoguanidine treatment

A total of 51 healthy male Sprague Dawley (SD) rats (weighing 240–270 g, about 7 weeks old) were provided by the experimental animal department of Fudan University in Shanghai, China. Then the SD rats were randomly divided into three groups: the diabetes group ($n = 18$), the aminoguanidine group ($n = 18$) and the control group ($n = 15$). The rats were fasted for about 16 h (5 p.m. – 9 a.m.) before induction of diabetes as in previous studies [19, 20]. The diabetes group and aminoguanidine group were intraperitoneally injected with 65 mg/kg streptozotocin (STZ, Sigma, USA) to induce diabetes. In detail, 4 mg/ml STZ was dissolved in 0.05 M citrate buffer at pH 4.0–4.5 [21, 22] to finally prepare the 10 ml injection. The control group was injected with the same volume of citrate buffer. Blood glucose levels were detected using blood samples collected from the caudal vein by the GLUCOTREND 2 blood glucose detector (Roche company, USA), and weights of rats were measured using electronic scales once a week after injection. In the aminoguanidine group and diabetes group, successful induction of diabetes was determined by slowly increased weight, withered and yellow hair and elevation of blood glucose. All animal studies have been approved by China Ethics Committee and performed in accordance with the ethical standards. Rats were housed individually at 20–24°C with 40–60% relative humidity and a 12 h (7:00–19:00) light/dark cycle. They were fed with a standard laboratory diet and distilled water *ad libitum* during the experiment.

Six weeks after successful induction of diabetes, back hair of rats in all groups was stripped by barium sulfide, prepared using barium sulfide (35 g), flour (3 g) and talcum powder (35 g) dis-

solved in 100 ml water and tuned into a paste. In the aminoguanidine group, rats were smeared with aminoguanidine cream using disinfected cotton swabs twice every day (at 9: 00 a.m. and 16:00 p.m.) for 6 weeks, while in the diabetes group and normal control group, rats were only smeared with the cream matrix without aminoguanidine.

Hematoxylin and eosin (HE) staining

After successful induction of diabetes in SD rats, pathological changes of skin tissue were observed by HE staining using 4 randomly selected rats every 10 days. Skin tissues were extracted and prepared as described previously [23]. In brief, on days 10, 20, 30 and 40 days after induction of diabetes, the animals were weighed and anesthetized with pentobarbital sodium (40 mg/kg, ip), followed by being affixed to the operating table. Then the wound was excised with a margin of 1 cm of healthy skin around the lesion, incising deeply into the fascia muscle. After collection of materials, the animals were sacrificed using an overdose of pentobarbital sodium. Therefore, after days 10, 20 and 30 days after induction of diabetes, there were respectively 39, 27 and 15 rats left.

The skin samples were then divided into 2 parts. One was taken for measurement of proinflammatory cytokines and oxidative stress factors after homogenate in 1 : 9 (V/V) normal saline; the other was fixed in 10% formalin solution for more than 24 h, and then embedded in paraffin, and cut into 4 μ m thick sections, followed by HE staining with a standard procedure (performed by SunteamBio, China). The changes and inflammatory cell infiltration in skin tissues were observed under an Olympus AX70 microscope (Olympus, Tokyo, Japan).

Liquid chip analysis of proinflammatory cytokines

The expression of inflammatory cytokines TNF- α , IL-8, ICAM and IL-1 α in skin tissue was detected by liquid chip every 10 days. The reagents in liquid chip were purchased from Panomics Company (USA). The skin samples were first homogenized in the neutral buffer following the instructions, then centrifuged for 10 min at 1000 g to remove debris in samples. After that, the supernatants were ready for the following detection. The washing buffer was prepared according to the instructions, and then used to wash the well before and after addition of the antibody beads. After the standard samples and test samples were added into the wells, the wells were sealed with aluminum foil and incubated at 4°C overnight, followed by being washed with washing buffer three

times. Then the detection antibody was added, and incubated for 30 min at room temperature, followed by washing with buffer 3 times again. Finally, the streptavidin-PE was added, incubated for 30 min, and also washed three times. After that, the samples were added with reading buffer, and detected by the BioPlex200 system (Bio-Rad Laboratories, Inc., Hercules, CA).

Analysis of oxidative stress

The skin samples were homogenized with 9 volumes of normal saline, and the homogenate was used to detect by colorimetry the expression of oxidative stress factors such as the T-AOC, MDA, MPO, GSH-PX and H₂O₂. Before detection, the skin homogenate was diluted with 99 volumes of normal saline and then measured with a spectrophotometer (DU-65, Beckman, Germany) at 595 nm with 1 cm optical path. All the procedures were conducted following the instructions, and normal saline was used in the blank wells. Finally, the protein content was obtained according to the following formula: protein content = (test OD – blank OD)/(standard OD – blank OD) \times standard protein concentration (0.563 mg protein/ml) \times dilution multiple.

Statistical analysis

All data were expressed as mean \pm SD and analyzed using SPSS 13.0 (Chicago, IL, USA). The comparison among experimental groups was conducted with one-way ANOVA, while the multiple comparisons were performed using the LSD method. Value of $p < 0.05$ was considered statistically significant.

Results

Morphological changes in aminoguanidine treated skin tissues in diabetic rats

As shown in Figure 1 A, scaly epithelium and collagen fiber of skin tissue were normal, interstitial inflammatory reaction was not obvious and cutaneous appendages were visible in the control group during the whole experiment. However, after 10 days of treatment, scaly epithelium of skin tissue was slightly thinned in the diabetes group (Figure 1 B), while it was mildly hyperplastic in the aminoguanidine group (Figure 1 C). After 20 days, scaly epithelium of skin tissue became thinner in the diabetes group (Figure 1 D), but it was mildly hyperplastic and became thick in the aminoguanidine group (Figure 1 E). As shown in Figure 1 F, after 30 days of treatment, scaly epithelium of skin tissue slightly thickened in the diabetes group compared with 20 days; while in the aminoguanidine group (Figure 1 G) the morphol-

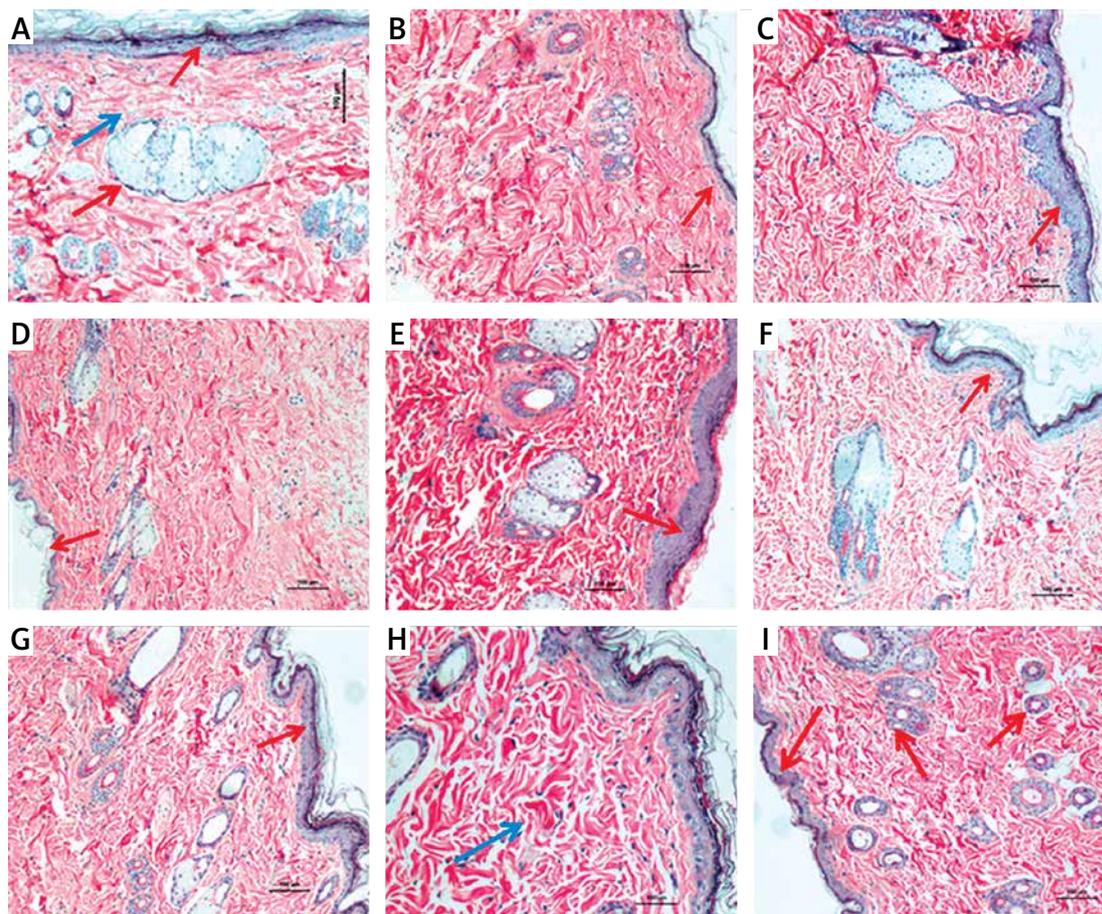


Figure 1. Pathological examination of rat skin tissues by HE staining (100 \times). During the experiment after diabetes induction, there were respectively 39, 27, 15 rats left after days 10, 20 and 30. **A** – Control group; **B, D, F, H** – diabetic rat skin at 10 (**B**), 20 (**D**), 30 (**F**), 40 (**H**) days; **C, E, G, I** – aminoguanidine treated diabetic rat skin at 10 (**C**), 20 (**E**), 30 (**G**), 40 (**I**) days. The red arrow points to the scaly epithelium, the blue arrow points to collagen fiber of skin tissue, and the dark red arrow points to the cutaneous appendages

ogy did not change compared with 20 days. After 40 days, scaly epithelium of skin tissue was still slightly thickened in the diabetes group and the collagen fiber became mildly hyaline, but the interstitial inflammatory response was negative and cutaneous appendages were visible (Figure 1 H). However, in the aminoguanidine group, scaly epithelium of skin tissue was mildly hyperplastic and became thick, collagen fiber was normal, interstitial inflammatory response was negative, cutaneous appendages were visible, and both hair follicles and sebaceous glands were proliferated (Figure 1 I).

Effect of inflammatory cytokine in skin tissue by using aminoguanidine in diabetic rats

As a result, the expression of IL-8 in the diabetes group and the aminoguanidine group were sustained at a higher level than the control group in the first 20 days ($p < 0.05$). After 20 days, the expression level of IL-8 in the diabetes group and the aminoguanidine group began to decrease, especially in the aminoguanidine group, in which the

IL-8 level on the 40th day was significantly lower than that on the 20th day ($p < 0.05$), almost the same as in the control (Figure 2 A).

As for TNF- α , there was no significant difference between the diabetes group and the control group in the first 30 days, but the diabetes group showed a sharp increase on the 40th day ($p < 0.05$). In the aminoguanidine group, the expression level of TNF- α was similar to the control group, and it decreased significantly compared with the diabetes group on the 40th day ($p < 0.05$) (Figure 2 B). The expression levels of IL-1 α in the diabetes group and aminoguanidine group were both higher than that in the control group on the 20th day and 30th day ($p < 0.05$, Figure 2 C).

Moreover, the IL-1 α level on the 40th day in the diabetes group was higher than that in the control group and aminoguanidine group; also, the IL-1 α level on the 30th day in the aminoguanidine group was significantly higher than that on the 40th day ($p < 0.05$, Figure 2 C).

Finally, the expression level of ICAM in the aminoguanidine group showed dramatic fluctuations during the 40 days, and was significantly lower

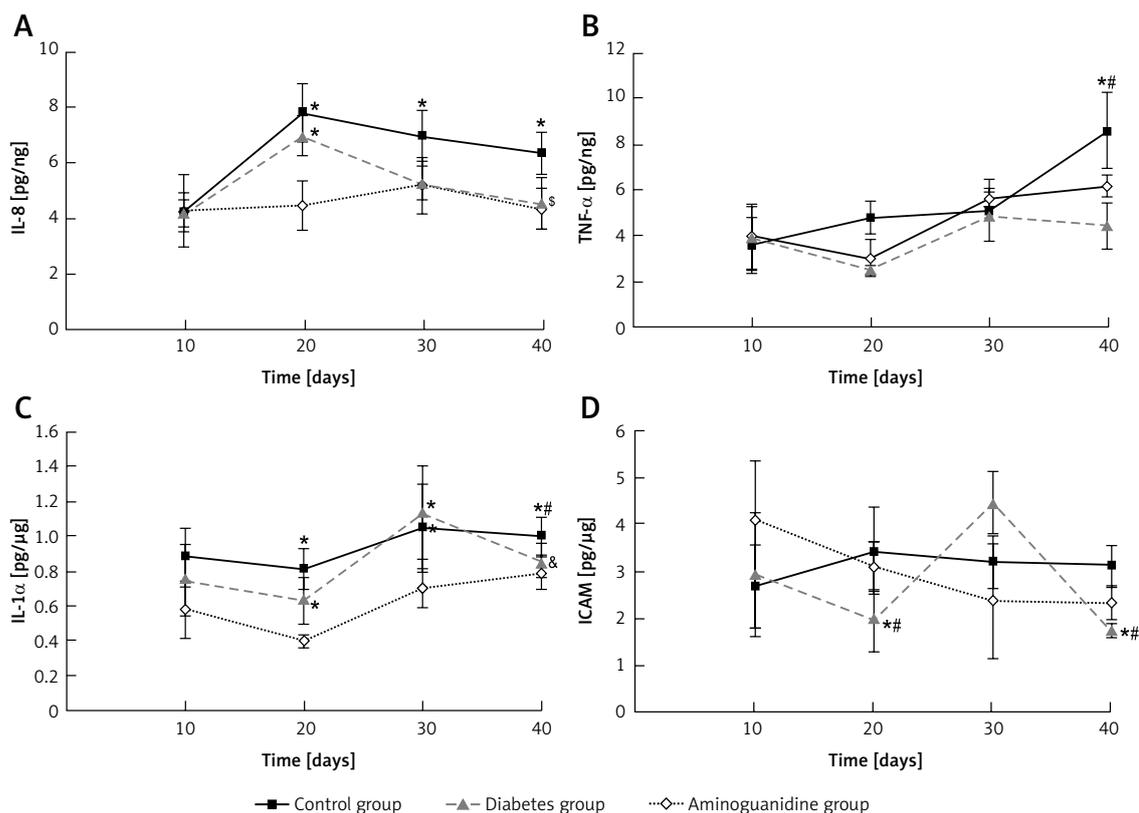


Figure 2. Expression levels of inflammatory cytokines in three groups of rats at different times (10, 20, 30, 40 days). **A** – IL-8, **B** – TNF-α, **C** – IL-12, **D** – ICAM

**P* < 0.05 vs. control group; #*p* < 0.05 vs. aminoguanidine group; ^s*p* < 0.05, IL-8 level on 40th day vs. that on 20th day; ^a*p* < 0.05, IL-1α level on 40th day vs. that on 30th day.

than that in normal and diabetes groups on both the 20th and 40th days (*p* < 0.05, Figure 2 D).

Effect of oxidative stress in skin tissue by using aminoguanidine in diabetic rats

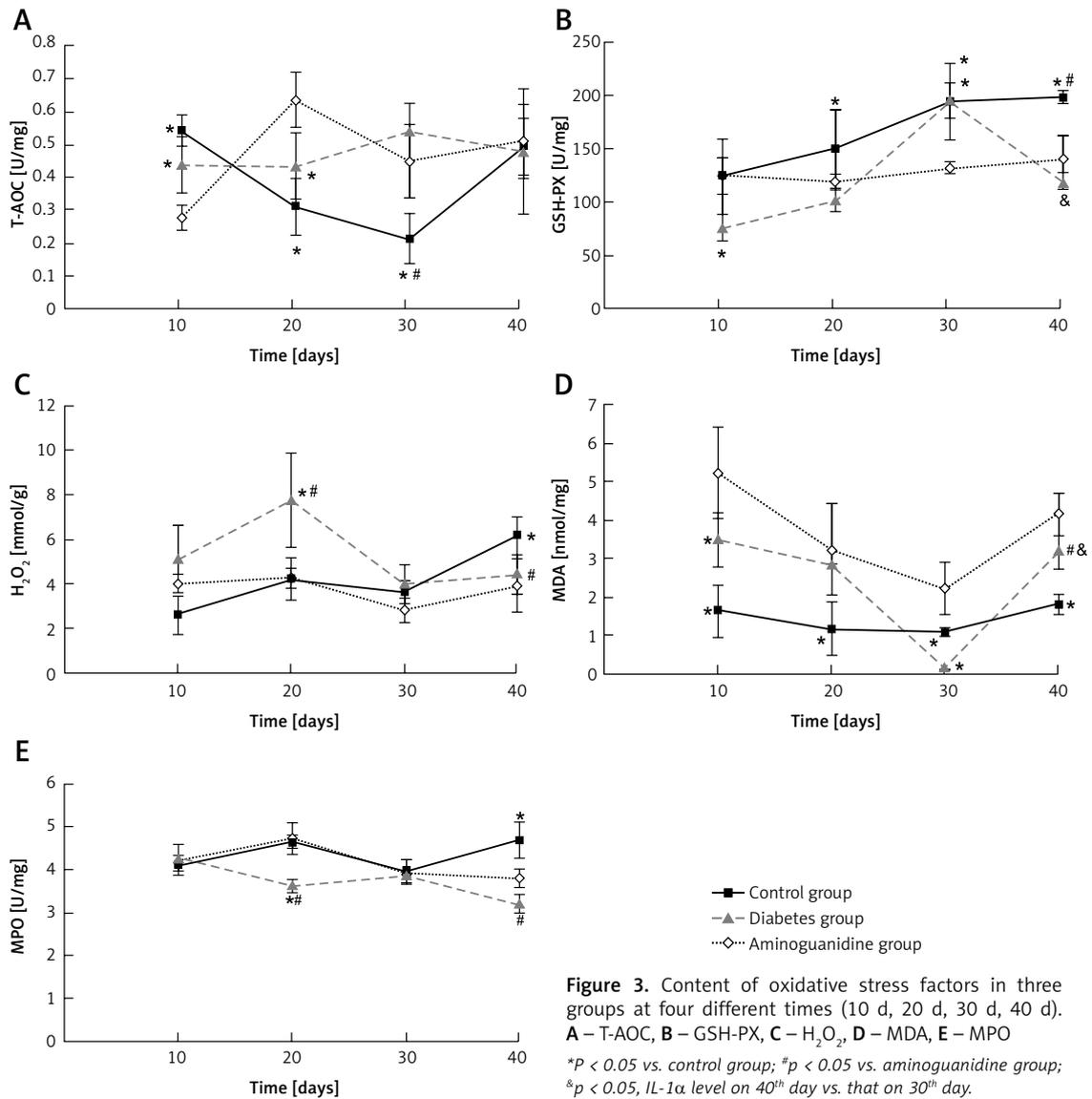
The expression of oxidative stress factors (T-AOC, MDA, MPO, GSH-PX, H₂O₂) was also detected in this study. As a result, the T-AOC in both the diabetes group and the aminoguanidine group was in a higher state on the 10th day, while on the 20th day, both were significantly lower than the control group (*p* < 0.05, Figure 3 A). On the 30th day, the T-AOC in the diabetes group decreased to the nadir during the whole experiment, which was significantly lower than that in the control and aminoguanidine groups (*p* < 0.05, Figure 3 A).

The content of GSH-PX in the diabetes group remained in a stage of growth and was obviously higher than that in the control group from the 20th day (*p* < 0.05), while in the aminoguanidine group, the GSH-PX level was markedly lower on the 10th day and significantly higher on the 30th day than that in the control group (*p* < 0.05, Figure 3 B). Another factor, H₂O₂, began to increase after 30 days in the diabetes group, while it started to decline in the aminoguanidine group, which had a peak on

the 20th day (Figure 3 C). The MDA expression level in the diabetes group was significant lower than the control group (*p* < 0.05). The level of MDA in the aminoguanidine group rapidly decreased after 20 days, but it apparently rose after 30 days and the difference was evident compared with the diabetes group (*p* < 0.05) (Figure 3 D). According to MPO, it reached a peak on the 40th day in the diabetes group. However, it kept at a relatively stable state in the aminoguanidine group, which was similar to the control group on the 40th day (Figure 3 E).

Discussion

Diabetes patients are easily hurt on account of the dull feeling caused by peripheral neuropathy. The peripheral neuropathy in diabetes has been reported to have an association with the APOE gene polymorphisms, although there is no robust evidence for the association [4]. Moreover, the trauma of diabetes patients is usually hard to heal because the high glucose environment of the related cells is a better substrate for bacterial reproduction. Numerous studies have demonstrated that the intractable trauma of diabetes is related to the deposition of AGE. Thus, we speculate that the trauma will heal more quickly if the for-



mation of AGE is reduced or hindered [24]. Moreover, protein glycation and AGE usually result in increased amounts of reactive oxygen free radicals and activity that then contributes towards the biomolecular damage and imbalanced oxidative-antioxidant defense system in diabetes [13]. As aminoguanidine is a nucleophilic hydrazine compound, it has an anti-oxidative stress function and can inhibit the formation of AGE [25]. If the status of the skin tissue microenvironment can be improved before trauma in diabetes, it can not only avoid spontaneous ulcer formation, but also accelerate the healing of wounds after they occurred. Therefore in this study, we tried to use aminoguanidine cream to improve the microenvironment of skin tissue in diabetes by external use, and evaluate the protective effect of aminoguanidine on wound healing through the anti-oxidative factors and proinflammatory cytokines.

A previous report on the induced diabetic rat model in 8 weeks stated that the thickness of the skin tissue epidermis and dermis layer of rats was significantly thinner, epidermal cell layers were unclear, part of the epidermis lacked multi-layer arrangement and the number of prickle cells was obviously reduced in the skin of diabetic rats. In addition, the dermal collagen was disorganized, degenerated or fractured, and the degeneration area of collagen presented focal infiltration of chronic inflammatory cells [25]. All the results were consistent with those observed in rats after 6 weeks of successful induction of diabetes in our study. But in the aminoguanidine group, the epidermis was slightly thickened, and the collagen and inflammatory cell infiltration were rarely normal. Thus, aminoguanidine cream can effectively alleviate the pathological changes of skin tissue in diabetes at the system level.

As aminoguanidine has acknowledged antioxidant activity, thus, we conducted several tests to decide whether the protective effect is related to the antioxidant activity of aminoguanidine. H_2O_2 is an important indicator to assess neutrophils' oxygen-dependent bactericidal capacity, and MPO can reflect the number of neutrophils in skin tissue. It has been reported that MPO in injured skin tissue of diabetes is significantly higher than that of the control [26], which was consistent with the present results in our study. However, the content of both MPO and H_2O_2 was kept at a higher level in the diabetes group, while it was significantly lower in the aminoguanidine group at the end of the experiment. Therefore, MPO and H_2O_2 in diabetes group may perform their bactericidal capacity during the experiment period, while in the aminoguanidine group they may be only partly activated or still in an inactive state. Thus the protective effect of aminoguanidine may not act through the function of MPO and H_2O_2 . It has been indicated that neutrophils dispersed in skin tissue could be activated by AGE, and the activated neutrophils can then release H_2O_2 to damage normal tissue [27, 28]. Furthermore, T-AOC reflects the overall cellular endogenous antioxidative capability including both enzymatic and non-enzymatic antioxidants [29], and GSH-PX is a kind of secreted selenium-dependent enzyme that can reduce hydroperoxides and organic hydroperoxides in the human body [30]. The reduction in activity of T-AOC and GSH-PX will lead to a decrease in antioxidant ability. The activity of T-AOC and GSH-PX in our study was in a state of dramatic change during the treatment period, and finally on the 40th day the activity of aminoguanidine was tending towards stability (control group), while the T-AOC activity of the diabetes group also reached the normal level. So the lower content of H_2O_2 and higher level of GSH-PX in the aminoguanidine group compared with the diabetes group indicate that aminoguanidine interference may have an effect on inhibiting AGE generation, and further reducing the burst of neutrophils through the antioxidant capacity of GSH-PX.

Studies have shown that TNF- α secretion from microglia could be stimulated by high glucose condition [31–34]. Thus, the release of inflammatory cytokines in the early stage does not necessarily depend on the generation of oxidative stress. In addition, the high glucose condition may also promote the secretion of inflammatory cytokines. In this study, the contents of TNF- α in the diabetes group increased significantly after 30 days, but the expression level of TNF- α was similar to the control group in the aminoguanidine group. Therefore, the increase of TNF- α in skin tissue of the diabetes group may be closely related to the high

glucose condition, and the increased TNF- α would then further lead to the increase of IL-8 and ICAM [35], as observed in the current study. TNF- α , IL-8 and ICAM all play important roles in regulating neutrophil migration and the inflammatory response [36]. Low content of TNF- α , IL-8 and ICAM in the aminoguanidine group may indicate that aminoguanidine inhibits the inflammatory response induced by diabetes. The IL-1 α is considered to be an important part of the skin protective barrier and is also a co-factor of other inflammatory mediators which can promote the formation of collagen, cell proliferation as well as cytokine release [37–39]. The low content of IL-1 α also contributes to the inhibitory effect of aminoguanidine on the inflammatory response. Therefore, aminoguanidine cream could alleviate the local inflammatory response by regulating the generation of inflammatory cytokines TNF- α , IL-8, ICAM and IL-1 α .

The observation by HE staining is only a simple and cursory evaluation for the damage and infiltration of immune cells. The method cannot provide the accurate number and position of infiltrating cells. Further study using a more accurate method, such as the immunofluorescence method, will be useful to discover the accurate changes in the skin tissue. Moreover, there were in total 51 rats used to evaluate the protective effect of aminoguanidine cream on the skin of diabetes rats. The sample was small, and many related indexes have not been completely studied here. In addition, MDA, as a main index of cell membrane lipid peroxidation, should be abundantly expressed in the case of AGE expression. Nevertheless, in the current study, MDA content was lower compared with the control group both in the aminoguanidine group and the diabetes group, which was puzzling. Therefore, a further study to evaluate the detailed mechanism for aminoguanidine in diabetes should be conducted with accurate examinations and a big sample, and to ensure the changes of MDA in the treatment.

In conclusion, aminoguanidine may have a good systemic effect on alleviating the pathological changes of skin tissue in diabetes rats, which may be attributed to the regulation of the antioxidant capacity of GSH-PX and the generation of inflammatory cytokines TNF- α , IL-8, ICAM and IL-1 α .

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Conflict of interest

The authors declare no conflict of interest.

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