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MACROSCOPIC EFFECT OF LIPOIC ACID IN SKIN INJURY IN THE MODEL OF DIABETES MELLITUS INDUCED BY ALLOXAN IN RATS

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ABSTRACT

Objective: Evaluate the macroscopic effect of lipoic acid on skin lesion in rats with alloxan-induced diabetes mellitus model. **Material and Methods:** For diabetes induction, Wistar albino rats received Alloxan at 50mg/kg. After 72 hours, the glycemia was verified to confirm diabetes; animals with values below 250mg/dl were discarded. After the diagnosis, surgical excision of a skin fragment was performed with a 7 mm punch. Following the procedure, the animals were divided into three groups containing five animals each, which received: oral distilled water and cellulose (control) or Alpha-Lipoic Acid (ALA) (100mg/kg or 200mg/kg) for 1, 7 or 14 days of treatment. At the end of each treatment period, a evaluation of the following parameters was performed: infection; hyperemic halo around the wound; crust formation; necrotic border, bleeding background and measurement of contraction percentage. **Results:** The ALA treatment reduced, in both doses, the crusts formation in the stipulated periods for evaluation. While ALA 100mg/kg increased the contraction percentage of the lesion at all times studied. A similar results was obtained with ALA 200mg/kg after 7 or 14 days of treatment. **Conclusion:** ALA presented satisfactory results in the healing process of wounds in diabetic rats.

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INTRODUCTION

Diabetes mellitus (DM) is characterized by a sequence of metabolic heterogeneous disorders. It is a degenerative condition, of chronic character and multifactorial etiology, associated with hyperglycemia, altered insulin excretion and/or insulin action, thus causing a decrease and/or inhibition of plasma glucose uptake by tissue (Care, 2018).Diabetes can lead to complications such as cardiovascular diseaseand premature death, and can damage eyes, kidneys and nerves. Globally, more than 400 million adults live with diabetes – a disease that caused 1.6 million deaths in 2015(World Health Organization, 2018). An important aggravating factor in this context is the large number of diabetics who are unaware of their disease condition, thus contributing to the increase of morbidity and mortality due to the early onset of pathological complications. Thus, it represents one of the main clinical causes of hospitalization in Brazil, causing high financial costs (Flor *et al*, 2017). The wound healing process is difficult in the diabetic individual, mainly as a result of compromised blood perfusion, which impairs the arrival of nutrients to the lesion, as well as an adequate oxygen supply and absorption of the used antibiotics, especially affecting the lower limbs. All these factors are responsible for disorganizing the initial repair

processes, delaying tissue regeneration (Sen *et al*, 2016). Thus, the diabetic patient hardly exhibits a normal process of cicatrization. As the healing stages undergo modifications, other actions that facilitate the repair process are required, which in turn are often impaired by the metabolic alterations of the diabetes itself or by the presence of infections in the wound (Sen *et al*, 2016). The difficulties with the repair process are a consequence of a sequence of molecular and cellular disorders. Some of them are: high concentration of metalloproteinases (MMPs), oxidative stress, neuropathy, exaggerated composition of AGEs (advanced glycoxidation products), high probability of infection, nonphysiological inflammatory response, inefficientneoangiogenesis, growth factors and regulators of gene expression in improper amounts, imbalance between metabolism and nutrient delivery and cellular abnormalities (Li *et al*, 2015). In diabetes there is a lot of glycosylation of proteins and that fibroblasts, endothelial cells and macrophages have special receptors for glycoproteins. These, when activated, induce proinflammatory cytokines and proteases, reducing the expression of anti-inflammatory molecules and natural antiproteases, thus promoting the increase of the inflammatory lesion in the cicatricial processes and complicating tissue repair. In addition, the high glycemic level creates an environment opportune for the multiplication of pathogens (Sonnenschein, 2015). The interaction of inflammatory agents drive the inflammatory response and promote the release of pro-inflammatory molecules, such as TNF-alpha and metalloproteinases (MMPs), which extinguish the matrix, therefore impairing wound cicatrization. Furthermore, problems with fibroblast activity can cause a fall in the necessary deposition of collagen, significantly interfering with the habitual process of cicatrisation (Medeiros, 2017).

In this context, it is worth noting that the generation of free radicals and oxidative stress plays an important role in the pathogenesis of diabetes and its late complications (Wietzycoski *et al*, 2016), a consequence of excessive production of reactive oxygen species (ROS) and a reduced antioxidant capacity associated with a decrease in circulating levels of antioxidants (Farajpour *et al*, 2017).Moreover, there is an intense formation of free radicals during the inflammatory process, which are also responsible for the delay in the healing process due to the damage these molecules cause to healthy and newly formed tissues(Yildirimturk *et al*, 2016). Hence the importance of antioxidant compounds in the context of tissue repair. Accordingly, there is a need for research on experimental models of diabetes mellitus that provide important information for the prevention and treatment of the disease (Lerco *et al*, 2003). The alloxan-induced diabetes mellitus model, generally performed in rats, is quite common in experimental studies. Alloxan is a hyperglycaemic agent that acts directly on the degradation of beta cells, inhibiting insulin secretion and consequently promoting a rise in blood's glycemic level. It is one of the most studied chemical agents, such as streptozotocin, with specific cytotoxicity for beta cells (Lerco *et al*, 2003). Alpha-lipoic acid (ALA), also known as 1,2-dithiolane-3-pentanoic acid, is a natural component of biological membranes, being found in plants and animals (De Araújo *et al*, 2011). ALA appears to be easily absorbed by oral route and acts as a powerful antioxidant, which makes it relevant for the prevention of diabetic complications associated with chronic hyperglycemia (Alpha-Lipoic Acid Monograph Alpha-lipoic Acid, 2006).

Considering the role of free radicals in impairing wound healing in diabetes and the possible antioxidant effect of ALA in these lesions, a more direct study about this product is necessary, in order to contribute to a better treatment of such wounds. In view of the above, the present study evaluated the macroscopic effect of ALA on cutaneous lesions in the alloxan-induced diabetes mellitus model in rats.

MATERIAL AND METHODS

Animals

The experiments were carried out at the Laboratory of Technologies and Pharmacological Innovations – LATIF (CNPq/URCA) of the Regional University of Cariri on male wistar albino rats weighing 200-300 grams obtained from the Central Animal House of Regional University of Cariri were used. The animals were kept at a controlled temperature (23 ± 1 ° C), with a light / dark cycle of 12h and free access to water and food.All experimental procedures were performed in accordance with the Ethics Committee on the Use of Animals (CEUA) ofRegional University of Caririunder the opinion of Nº. 00167/2018.1.

Induction of Experimental Diabetes

For induction of diabetes the animals were fasted for 24 hours and after this period were anesthetized with xylazine 10mg / kg and ketamine 100mg / kg for administration of alloxan at a dose of 50mg / kg by the dorsal penile vein.Six hours after alloxan injection glucose solution (10%) was available for 24 hours. To verify diabetes, glycemia was verified 72 hours after alloxan administration and on the day of euthanasia. Animals that did not present values equal to or greater than 250 milligrams per deciliter of blood were discarded.The checks were done by drawing blood from the tip of the anesthetized animal's tail and dropping it onto Accu-Chek Active® reagent tapes and then reading it on an Accu-Chek Active® apparatus.

Trycotomyand Generation of Skin Wound

Once the diabetes was confirmed and after anesthesia, the animals were maintained in dorsal decubitus, then a manual tricotomy of the back was performed, followed by antissepsis of the operative field with povidine-iodine and, subsequently, the surgical excision of a skin fragment, measuring around 7 mmin its total length, with the aid of a punch. Thus standardizing the wound and taking care that all layers were removed, leaving only the underlying musculature. After the surgery, the animals were re-housed in clean cages, 5 individuals per cage.

Treatment Protocol

After surgery, the animals were divided into three groups containing five animals each receiving oral distilled water and cellulose (control) or ALA (100mg / kg or 200mg / kg) for 1 or 7 or 14 days (Fig. 1).

Macroscopic assessment of lesions

After the treatment period, the lesions were assessed by two independent evaluators, previously calibrated.

Table 1. Macroscopic evaluation of the cutaneous lesions of rats in the Alloxan-induced Diabetes Mellitus model. Crato - CE, 2019

VARIABLES	GRUPS	1º DAY						7º DAY						14º DAY					
		PRESENT		AUSENT		TOTAL		PRESENT		AUSENT		TOTAL		PRESENT		AUSENT		TOTAL	
		N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%
HYPEREMIC HALO	CONTROL	0	0	5	100	5	100	0	0	5	100	5	100	0	0	5	100	5	100
	ALA 100	0	0	5	100	5	100	0	0	5	100	5	100	0	0	5	100	5	100
	ALA 200	0	0	5	100	5	100	0	0	5	100	5	100	0	0	5	100	5	100
NECROTIC BORDER	CONTROL	0	0	5	100	5	100	0	0	5	100	5	100	0	0	5	100	5	100
	ALA 100	0	0	5	100	5	100	0	0	5	100	5	100	0	0	5	100	5	100
	ALA 200	0	0	5	100	5	100	0	0	5	100	5	100	0	0	5	100	5	100
BLEEDING BACKGROUND	CONTROL	0	0	5	100	5	100	0	0	5	100	5	100	0	0	5	100	5	100
	ALA 100	0	0	5	100	5	100	0	0	5	100	5	100	0	0	5	100	5	100
	ALA 200	0	0	5	100	5	100	0	0	5	100	5	100	0	0	5	100	5	100
INFECTION	CONTROL	0	0	5	100	5	100	0	0	5	100	5	100	0	0	5	100	5	100
	ALA 100	0	0	5	100	5	100	0	0	5	100	5	100	0	0	5	100	5	100
	ALA 200	0	0	5	100	5	100	0	0	5	100	5	100	0	0	5	100	5	100
CRUST FORMATION	CONTROL	5	100	0	0	5	100	5	100	0	0	5	100	3	60	2	40	5	100
	ALA 100	4	80	1	20	5	100	5	100	0	0	5	100	0	0	5	100	5	100
	ALA 200	0	0	5	100	5	100	0	0	5	100	5	100	0	0	5	100	5	100

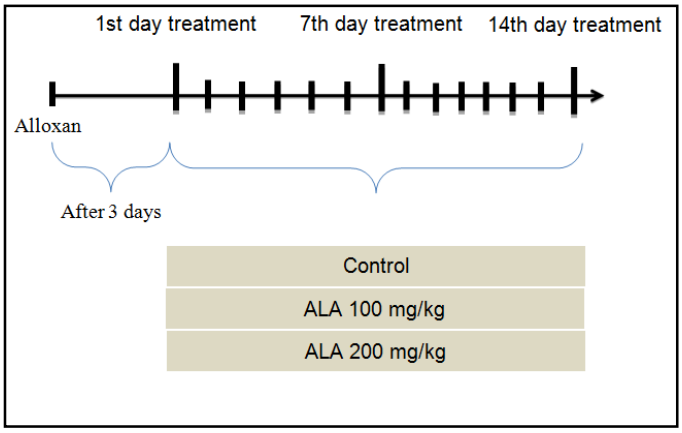

Figure 1. Treatment protocol with saline (Control), lipoic acid 100 mg/kg (ALA 100) or lipoic acid 200 mg/kg (ALA 200)

Lesion Evaluation

The following characteristics were evaluated: hyperemic halo, necrotic border, bleeding background, infection and crust formation. The contraction was evaluated by calculating the area of the wound, by measuring the major and minor diameters with the help of a digital caliper, applying the mathematical equation proposed by Prata *et al*. (1988): A = π. R. Where "A" represents the area, "R" is the largest radius and "r" is the smallest radius of the wound(Prata *et al*, 1988). The calculation of the contraction percentage was expressed through the mathematical equation suggested by Ramsey *et al*. (1995), where Wo represents the initial area of the wound, Wi area of the wound:% contraction = 100. [(Wo-Wi) / Wo] (Ramsey *et al*, 1995). The data were recorded in tables prepared for each period of analysis.

Data Analysis

Data analysis was done in a descriptive way, through absolute and relative frequencies. However, the evaluation of the percentage of wound contractionwas performed through analysis of variance (ANOVA) using Prism 5.0 software version for Windows, GraphPad Software (San Diego, CA, USA). For significance assessment, multiple comparisons were made with ANOVA and by Tukey as post hoc tests. The results were considered significant at $p < 0.05$ and presented as mean ± SEM.

RESULTS

Regarding the evaluation of the hyperemic halo, as can be seen in Table 1, it was observed that for treatment of 1, 7 or 14 days, 100% (5) of the animals belonging to the control group, as well as the ALA group at the dose of 100 or 200 Mg / kg showed no hyperemic halo formation within or in the perilesional tissue. As for the evaluation of the necrotic border, it was evidenced that for the treatment of 1, 7 or 14 days, 100% (5) of the animals belonging to the control group orALA group at the dose of 100 or 200 mg / kg did not present necrotic border formation, as indicated in Table 1. Similarly, when evaluating the formation of the bleeding fund, it was observed that for the treatment of 1, 7 or 14 days, 100% (5) of the animals belonging to the control group or the ALA group at dose of 100 or 200 mg / Kg did not present bleeding background, as indicated in Table 1. Table 1, in which the development of infection was evaluated, indicated that for the treatment of 1, 7 or 14 days 100% (5) of the animals belonging to the control group or ALA group at the dose of 100 or 200 mg / kg did not develop infection. When evaluating the crust formation inside the lesion, it was observed that for 1 day treatment, 100% (5) of the animals belonging to the control group and 80% (4) of the animals constituting the ALA 100 group presented crust formation, whereas 100% (5) of the ALA 200 treated rats had nocrust formation, as shown in table 1.Similar to the previous result, it was identified that after treatment in repeated doses for 7 days, 100% (5) of the animals belonging to the control group and 100% (5) of the ALA 100 group presented crust formation, while in 100% (5) of the ALA 200 treated mice the crust formation was absent.For treatment in repeated doses for 14 days, 60% (3) of the animals belonging to the control group presented crust formation, while 100% (5) of the animals treated with ALA 100 and 100% (5) of ALA 200 treated rats showed no presence of crust inside the lesion. Regarding the percentage of lesions contraction, it was observed that the acute treatment with ALA at the dose of 100mg / kg (49.6 ± 5.2) had an increase in the percentage of contraction of the lesion when compared to the control group (34.4 ± 1.1) (Fig. 2A).For treatment of 7 days, ALA at a dose of 100mg / kg (93.3 ± 0.9) or 200mg / kg (93.7 ± 0.0) presented an increase in the percentage of contraction of the lesion when compared to the control group (83.5 ± 8.0) (Fig. 2B).

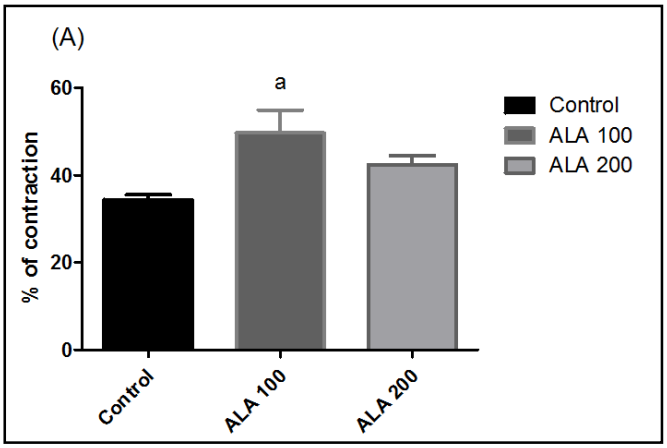

Figure 2A – Effects of Lipoic Acid on the contraction of lesions in diabetic rats induced by alloxan. The animals were treated for 1 day with vehicle (control) or lipoic acid (100 or 200mg / kg). Each bar represents mean ± SEM when compared to the control group. For all analyzes, p <0.05 was considered significant. ANOVA followed by Tukey as a post-hoc test.

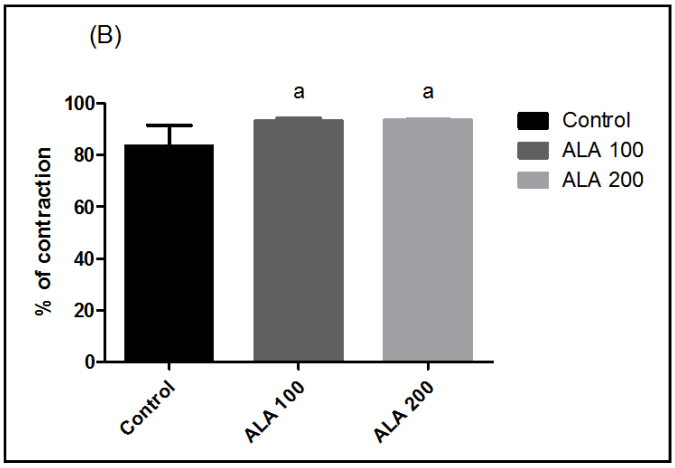

Figure 2B. Effects of Lipoic Acid on lesion contraction of alloxan-induced diabetic rats. The animals were treated for 7 days with vehicle (control) or lipoic acid (100 or 200mg / kg).Each bar represents mean ± SEM when compared to the control group. For all analyzes, p <0.05 was considered significant. ANOVA followed by Tukey as a post-hoc test

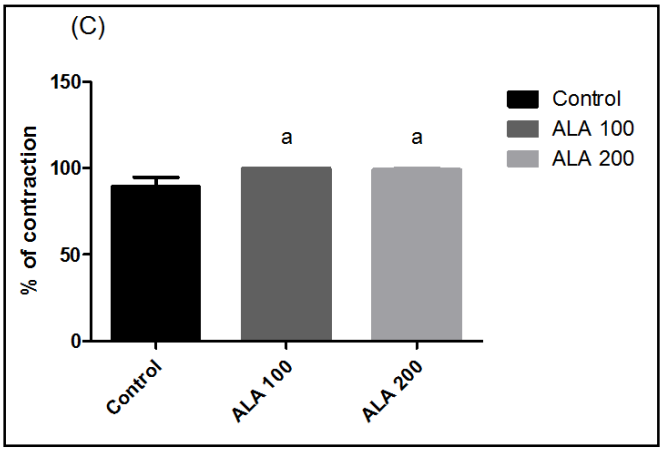

Figura 2C. Effects of Lipoic Acid on lesion contraction of alloxan-induced diabetic rats. The animals were treated for 14 days with vehicle (control) or lipoic acid (100 or 200mg / kg).Each bar represents mean ± SEM when compared to the control group. For all analyzes, p <0.05 was considered significant. ANOVA followed by Tukey as a post-hoc test

A similar result was obtained for the 14-days treatment, where ALA at the dose of 100mg / kg (99.8 ± 0.2) or at the dose of 200mg / kg (99.2 ± 0.8) were significantly different when compared to the control group (89.3 ± 5.5) (Fig. 2C).

DISCUSSION

The present study was conceived with the objective of evaluating the systemic effect of lipoic acid on the healing of cutaneous lesions in diabetic rats, taking into account the existing evidence that diabetes is a retarding factor for the cicatricial process of skin lesions(Lai *et al*, 2016; Singh *et al*, 2016). As an experimental model, we chose the Wistar rat, considering thatthey are easy to handle and have characteristics already known in diabetes induction(Zhang *et al*, 2017; Tsounapi *et al*, 2017). We also opted to use alloxan, since this substance induce an experimental diabetes which reproduce classical signs of human diabetes in animals, such as hyperglycemia, in addition to being a low-cost drug and being widely used in the induction of experimental diabetes(Dias Silva and Nogueira, 2015). An article discussing the effect of antioxidant substances on viability of dorsal cutaneous flap in ratsmentions the use of antioxidant substances as adjuvants in the healing of lesions, which are related to the lower formation of free radicals leading to less tissue damage(Pace, 2002).In this way,lipoic acid and its metabolite di-hydrolipoic (DHLA), exert potent antioxidant properties, not only by the elimination of reactive oxygen species (ROS), but also by metal chelation, regeneration of endogenous antioxidants and repair of oxidatively impaired biomolecules(Karakoyun *et al* , 2009).

In the present study, where the therapeutic properties of ALA were investigated,hypermoemic halo formation, necrotic border and bleeding background were not observed during the macroscopic evaluation of the experimental animals, regardless of whether they were treated with ALA or not, and in all times studied. This satisfactory result can be attributed to the good sanitary conditions to which the animals were subjected in the vivarium, as well as the care taken in relation to the antisepsis of the animals and the asepsis of the cages and the instruments used, which were rigorously fulfilled in all phases of the experiments, besides the nature of the lesion, that was clean and surgically performed. Regarding crust formation it was observed that for 1 and 7 days of treatment, the majority of animals belonging to the control and ALA 100 groups presented crust formation, while all rats treated with ALA 200 did not.For repeated dose treatment of 14 days, most animals belonging to the control group presented crust formation, while there was no presence of crust inside the lesion of diabetic rats treated with ALA 100 or 200.This effect suggests that lipoic acid action on crusting will depend on the dose of administration and the treatment time, since only on repeated treatment the lower dose of ALA has promoted the absence of crust formation. In a study investigating the action of another substance that helps in the cicatricial wound healing process, known as ipê-roxo, the popular name for *Handroanthus impetiginosus*, it was concluded that the crust was present in most of the control group animals, as well as theanimals treated on the 3rd and 10th days of postoperative. On the 14th postoperative day, the crusts were no longer noticed, with complete healing of the cutaneous wound in 100% of the animals in the treated group and in 80% of the control rats (Silva, 2006). However, it is worth noting that in the study described above, the animals did not present compromising healing factors, such as diabetes.

In another study using laser as a treatment of wounds, crust presence was observed in the first 3 days in both groups (control and laser) whereas in the seventh day the control group had an early loss of it, favoringcicatrization (Santos, 2010). The decrease in crust formation in the groups treated with lipoic acid, mainly in the dose group of 200mg / kg, regardless of the treatment time, 1, 7 or 14 days, is probably due to its anti-inflammatory effect, controlling the mediators of inflammation and oxidant, as well as controlling ROS, which are detrimental to tissue repair. The mechanism of wound contraction plays a key role in cicatrization by second intention.It is a mechanism mediated by cytokines, in which through contraction, the wound diminishes by the contractile action of myofibroblasts found in the granulation tissue.Thus, the period of greatest contraction activity occurs between six and fifteen days after the injury, that is, it corresponds to the peak of action of the myofibroblasts that compose the granulation tissue (Mott *et al*, 2003). When evaluating the percentage of lesions contraction in rats withalloxan induced diabetes, we observed that the acute treatment with ALA, only at the lowest dose, showed an increase in the percentage of contraction of the lesion when compared to the control group.A similar result was obtained for the treatment in repeated doses for 7 or 14 days with both doses of ALA studied. Therefore, from this observation of lipoic acid effect, at low and high doses, a reduction in lesion size was evidenced by the systemic use of ALA, which can be attributed to its antioxidant and anti-inflammatory properties. A similar result was observed in a model of diabetes induced by streptozotocin, where it was found that hyperglycemia in vitro or in vivo compromised angiogenesis, attenuated mitochondrial function and delayed wound healing. On the other hand, ALA in vivo or its metabolite, di-hydrolipoic acid, in vitro restored angiogenesis, cellular bioenergetics, and wound healing in hyperglycemia and diabetes (Coletta *et al*, 2015).

Thus, it is believed that alpha lipoic acid or its reduced form, di-hydrolipoicacid, have many biochemical functions acting as biological antioxidants, such as metal chelators, reducing the oxidized forms of other antioxidants such as vitamin C and E.These above-mentioned actions have been shown in experimental studies emphasizing the use of lipoic acid as a potential therapeutic agent for many chronic diseases such as obesity, non-alcoholic fatty liver disease, burning mouth syndrome, cardiovascular disease, hypertension, some cancers, glaucoma and osteoporosis (Gomes and Negrato, 2014). Accordingly, due to its strong antioxidant and anti-inflammatory effects, the therapeutic potential of lipoic acid has recently been studied for treatment of neuropsychiatric disorders (Sampaio *et al*., 2017; Araújo *et al*., 2011; Silva *et al*. 2013). Regarding the use of ALA in diabetes, a study evaluating the therapeutic effect of lipoic acid for diabetic nephropathy demonstrated that short-term ALA protects the kidney in initial diabetic nephropathy against general oxidative stress (Sun *et al*, 2017). Another study, which evaluated the effect of a dietary supplement, containing or α-lipoic acid or a placebo, on glyco-metabolic control and markers of oxidative stress in type 2 diabetics, found that the dietary supplement containing α-lipoic acid, L- Carnosine, zinc and B vitamins improved glycemic control, lipid profile and oxidative stress markers (Derosa G, D'Angelo A, Romano D, Maffioli P, 2016). Surprisingly, the effects of lipoic acid supplementation on cyclooxygenase activities, E2 prostaglandin levels and F2α metabolites in the offspring of rats with streptozotocin-induced diabetes showed that ALA did not completely prevent the occurrence of malformations. Thus, other factors may be involved in the pathogenesis of congenital malformations induced by diabetes (Al-Matubsi *et al*, 2016). The use of healing products generally contributes to the recovery of wounds. In a research involving propolis and aloe vera, it was proven that the use of these substances accelerated the healing process, presenting a greater contraction of the lesion when compared to the control group (Semenoff Segundo *et al*, 2007). In another study, Schirato *et al*. (2006) evaluated the effect of Anacardiumoccidentale L. (POLICAJU) polysaccharide on the inflammatory phase of wound healing in mice. No significant results were obtained in the reduction of contraction percentage because the lesions were evaluated only for six days, a period that still falls into the inflammatory phase of the cicatricial process, thus presenting a reduced number of myofibroblasts in the lesion (Schirato et, 2006). In view of the above, it was concluded that the use of the pharmacological model of diabetes induced by alloxan was important for the understanding of the macroscopic effects of lipoic acid on the lesions of diabetic rats and that these effects are probably related to antioxidant and anti-inflammatory activities. Finally, our research points to the possibility that lipoic acid, alone or associated with the use of other technology, may be used to improve the cicatricial process of chronic wounds in diabetic patients and probably reduce the time of treatment and provide quality of life for people living with diabetes lesions.

Conflict of interest: The authors declare no conflict of interest.

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