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# EFFECT OF L-ARGININE ON ORAL MUCOSA REPARATION IN RATS WITH CHEMICALLY INDUCED DIABETES

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## ABSTRACT

This research was designed to study the effect of L-arginine on wound healing in oral mucosa of diabetic rats. Four groups of rats were studied ($n$ = 8 adult males per group). Group I was the control group (saline solution *i. p.*). Group II, normal rats were treated with 1.0 mL of 10 mM L-arginine *i. p.* Group III, diabetic control treated with saline solution and Group IV, diabetic rats were treated with 1.0 mL 10 mM L-arginine. Under anesthesia, a wound (1.0 cm) was produced in the oral mucosa of all animals. Blood glucose was measured for 12 days, at the end of this time, the animals were sacrificed. A biopsy of tissue around the wound was taken and arginase activity, dry weight, total proteins, L-hydroxyproline and deoxyribonucleic acid were measured. The results show a constant increase in glycemia in group II with values of 400 mg/dL that persisted until day 12. While hyperglycemia diminished near day 8, arginase activity and the concentration of total proteins, L-hydroxyproline and deoxyribonucleic acid were significantly increased ($P < 0.05$) with L-arginine. The highest values of all biochemical parameters were observed in group II. These results suggest that L-arginine prevents hyperglycemia and reduces the time of oral mucosa reparation.

**KEYWORDS:** Oral Mucosa, Alloxan, Rats, Diabetes Mellitus, L-Arginine, Arginase Activity, Wound Healing.

## 1. INTRODUCTION

Diabetes mellitus is a metabolic disorder characterized by hyperglycemia resulting from the depletion of insulin secretion, insulin action, or both.[1] Hyperglycemia affects organs and tissues. The oral cavity does not escape of this condition, several systemic factors such as hormonal and metabolic status are involved in oral mucosa inflammatory process.[2] Previous evidence proved that when oral mucosa inflammation occurred in diabetes mellitus, it was more difficult to heal.[3] Besides the control of glucose levels, the prevention of chronic complications presented in connective tissues including oral mucosa improves the life quality of diabetic patients. Effects of diabetes mellitus on oral mucosa include dehydration[4], diffused erythema or paresthesia, cyanosis and dental caries.[5] Wound healing is one of the major problems in diabetes mellitus. Studies had been carried out with streptozotocin-induced diabetic rats using incisional wounds, a decrease of tissue tension and collagen production in these animals was observed, indicative of complications on wound reparation.[6]

To protect and/or reverse damage to the oral mucosa our group has been studying the effect of several substances among them the effect of L-arginine. This amino acid plays a key role in several biochemical and metabolic processes such as those in the urea cycle in the liver[7] or in DNA compaction through arginine-rich histones in eukaryotic cells (histones H3 and H4).[8,9] L-Arginine stimulates growth hormone secretion[10] and is also a powerful insulin secretagogue.[11]

Previous studies carried out by our group showed that hyperglycemia and dyslipidemia are regulated by L-arginine in diabetic rats.[12] The functional relevance of L-Arginine in diabetes mellitus has been evaluated both in animal models and in human subjects.[13]

In extrahepatic tissues L-arginine leads to the formation of connective tissue through L-ornithine, a precursor in the L-proline and L-hydroxyproline formation, both amino acids involved in the collagen synthesis in fibroblasts.[14] L-arginine increases the concentration of L-hydroxyproline at specific points of wounds[15], but this amino acid also increases the collagen levels when it is administered to wounded animals.[16] It is also known that increased arginase activity in macrophages results in increased collagen synthesis in injured animals, leading to the proliferation of fibroblasts.[17]

Although the effect of L-arginine on the repair processes of injured animals is known, the effect of this amino acid on the repair of the oral mucosa in diabetes is still unknown.

In this study, we inform about the beneficial actions of L-arginine administration on oral mucosa reparation in rats under diabetic conditions. Several biochemical parameters including dry weight, total proteins, L-hydroxyproline, arginase activity, and deoxyribonucleic acid content were measured in oral mucosa of diabetic rats.

## 2. MATERIAL AND METHODS

### *2.1.Chemicals*

Alloxan, Droperidol, Ketamine, Tris-HCl, Mannitol, Sodium Carbonate, Potassium Sodium Tartrate, Sodium Hydroxide, Copper Sulphate (Pentahydrate), Folin-Ciocalteu Reagent, Bovine Serum Albumin, Thiosemicarbazide, Diacetyl monoxime, Acetone, Ninhydrin, L-Hydroxyproline, Acetaldehyde, Diphenylamine and Deoxyribonucleic Acid were purchased from Sigma (Sigma Chemical Co., St. Louis, MO, USA). L-arginine, Potassium Dichromate, Phosphoric Acid and Glacial Acetic Acid were purchased from Merck & Co. (E. Merck, D-6100 Darmstadt, Germany). Sulfuric Acid, Ferric Chloride and Ethanol were purchased from J.T. Baker (Mallinckrodt Baker, Xalostoc, Mexico). Urea, Hydrocloric Acid and Perchloric Acid were purchased from Monterrey (Monterrey, N. L., Mexico).

### *2.2.Animals*

Adult male Sprague-Dawley rats weighing 270 ± 30 g were used in this study. Animals fed *ad libitum* were grouped in four groups ($n$ = 8 adult males per group) that were studied for 12 days. Group I was the control group treated with 1.0 mL 0.154 M NaCl (*i.p.*). Group II were normal rats administered 10 mM L-arginine in 1.0 mL 0.154 M NaCl (*i.p.*). Group III rats were treated with 120 mg/kg alloxan (*i.p.*) and served as diabetic controls. Group IV rats were also diabetized as in Group III, these animals received 10 mM L-arginine in 1.0 mL 0.154 M NaCl (*i.p.*) as did those in Group II. On day 0, the animals from Group I and II were anesthetized by 0.4 mL droperidol and 0.6 mL ketamine (*i.m.*); a 1 cm incision wound was produced in the oral mucosa of all animals, Groups III and IV were also anesthetized, but the 1 cm wound was produced after being diabetized. At the end of the 12-day period the animals were sacrificed. A biopsy of tissue around the wound was taken, the tissue was kept in 40 mM Tris-HCl buffer. Then tissue was homogenized in the same buffer and used for biochemical determinations. Special care was taken in the handling of the animals following all the recommendations and rules established for these purposes in the Mexican Official Standard: NOM-062-ZOO-1999.[18]

### 2.3. Biochemical Determinations

A DU-64 Beckman spectrophotometer was used to register the absorbance when it was necessary.

#### *2.3.1. Glucose Determination*

Blood glucose was measured for 12 days using the Bayer Glucometer GX.

#### *2.3.2. Dry Weight*

Dry weight was determined as described previously.[19] It was as follows: 50 µL of homogenized tissue (10 % P/V) were diluted 20 times in distilled water, then 2 mL of 20 g/L potassium dichromate in sulfuric acid (dissolved at 70 °C with slight agitation) were added and mixed vigorously for 1 minute in a Vortex mixer, after 15 minutes the absorbance was registered at 630 nm. To establish the standard curve, solutions were prepared by diluting 2 mg/mL of mannitol.

#### *2.3.3. Protein Measurement*

The protein determination was carried out according to the Lowry *et al.*[20], with slight modifications. Briefly, 50 µL of homogenized tissue (10 % P/V) were diluted 1:10 in 0.1 N NaOH, the mixture was incubated at 37 °C for 30 minutes, then it was centrifuged for 10 minutes at 3,000 rpm. 500 µL of the supernatants were diluted 1:5 in a solution containing 50 volumes of a solution containing 2% sodium carbonate, 0.02 % potassium sodium tartrate and 0.1 N NaOH with 1 volume of 0.05 % copper sulphate (pentahydrate). After 10 minutes, 200 µL of diluted Folin-Ciocalteu reagent (diluted 1:2 in distilled water) were added to each sample. The absorbance was registered at 530 nm after 20 minutes. Standard curve was prepared with solutions diluted from 0.2 mg/mL bovine serum albumin.

### *2.4. Arginase activity*

Arginase activity was performed by measuring the urea concentrations as described previously.[21] 50 µL of the homogenized tissue (10 % P/V) were diluted 1:10 in a buffer solution containing 0.002 M $MgCl_2$ in 40 mM Tris-HCl and 0.9 % sodium chloride, at pH 7.5. The diluted samples were incubated at 55 °C for 1 hour. Then 100 µL of 0.4 M L-arginine-0.15 M glycine solution were added to 400 µL of the supernatants. After incubating at 37 °C for 15 minutes, the mixture was immediately heated in boiling water for 7 minutes. 300 µL of the heated sample were used to complete a volume of 1 mL with distilled water, 1 mL of a solution containing 5 mg thiosemicarbazide plus 3.5 mL of 4.155 g/L diacetylmonoxime and distilled water in a total volume of 25 ml. After this, 2 mL of a

solution containing a combination of 25 uL of a solution 0.12 M $FeCl_3$ in 56.7 % $H_3PO_4$ plus 25 ml of 20 % $H_2SO_4$. The total mixture was heated at 92 °C for 20 minutes, then cooled at room temperature and the absorbance registered at 530 nm. Solutions for the standard curve were prepared from 0.015 mg/mL urea.

### *2.5. L-Hydroxyproline*

The Chinard method[22] with slight modifications was performed in the determination of L-hydroxyproline. Briefly, 100 µL of the homogenized tissue (10 % P/V) were acidified by the same volume of 6 N HCl and then incubated at 70 °C for 16 hours. After that, it was centrifuged at 4,500 rpm for 15 minutes. 100 µL were taken from each centrifuged sample and 50 µL of acetone were added to eliminate the presence of water. After the acetone was evaporated, the residue was washed 3 times with 200 µL ethanol every time, the ethanolic extract was collected with 1 mL of the ninhydrin reagent (0.031 g of ninhydrin plus 4 ml of 6.0 M $H_3PO_4$ in glacial acetic acid). Then the samples were heated in boiling water for 30 minutes and cooled. The absorbance was registered at 530 nm. Standard curve was prepared with solutions diluted from 1 mg/mL L-hydroxyproline in acetic acid.

### *2.6. Deoxyribonucleic acid*

Deoxyribonucleic acid determination was performed as previously described.[23] 100 µL of 0.5 M $HClO_4$ were added to the same volume of homogenized tissue (10 % P/V), the mixture was centrifuged at 3,500 rpm for 15 minutes. After the elimination of supernatants, 100 µL of 2 M $HClO_4$ were added, the samples were then incubated at 70 °C for 30 minutes. Another centrifugation was carried out at 4,500 rpm for 20 minutes after the incubation. 100 µL of the acid fraction were diluted 5 times with 2 M $HClO_4$, and then 50 µL of a solution of 1.6 mg/mL acetaldehyde in distilled water (this solution must be kept under refrigeration) and 500 µL of a solution containing 8.4 % diphenylamine in glacial acetic acid were added to the diluted sample. The mixture was incubated at 37°C for 24 hours. The absorbance was read at 600 nm. Dilutions from 0.1 mg/mL of deoxyribonucleic acid were used to establish the standard curve.

### *2.7. Statistical analysis*

For data obtained from the 4 groups, the mean ± S.D. were calculated. Significance among experimental groups was calculated by one-way ANOVA test and Tukey-Kramer test for multiple comparisons using program R version 2.2.0, $P < 0.05$ denoted statistical significance.

## RESULTS

Diabetic state was produced with the concentration of alloxan administered and it was well characterized by measuring the glucose levels. Hyperglycemia was produced 24 hours after the injection of alloxan in Group III and IV. Serum glucose levels increased gradually until day 11. Normal rats treated (Group II) or non-treated (Group I) with 10 mM L-arginine did not present significant variations on glucose concentrations. Data registered daily from the beginning of the experiment showed that L-arginine increased glucose serum concentration until day 8 but significantly decreased the levels of hyperglycemia from day 9 to day 12 (Figure 1).

Significant differences in protein concentrations between normal and diabetic control rats were observed; diabetic rats (Group III) had a decreased capacity of protein synthesis however, the protein levels of diabetic rats were back to normal after administered L-arginine (Group IV), it was proved by the result Group I and Group IV showed no statistical difference ($P > 0.05$). L-arginine helped serum protein concentrations reach even higher levels than normal in Group II (Figure 2). A similar situation was observed in the determination of urea (Figure 3) and L-hydroxyproline (Figure 4).

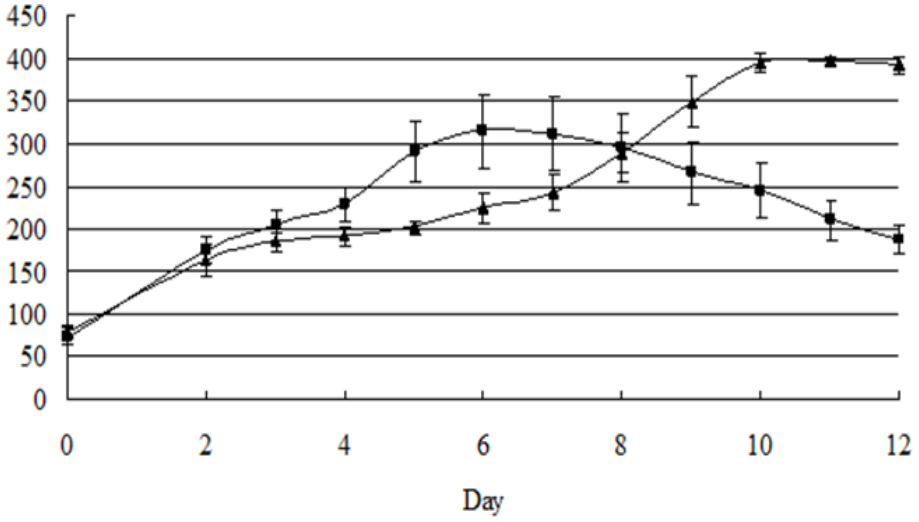


**Figure 1: Mean ± S.D. (N = 8) of glucose in serum of diabetic rats treated and non-treated with 10 mM L-arginine. Glucose concentration is expressed as mg/dL and appears on the y-axis. The day that the blood samples were taken is on the x-axis. Each value is the mean of eight determinations. Group III or diabetic control = black triangles; Group IV or diabetic treated with L-arginine = black squares.**

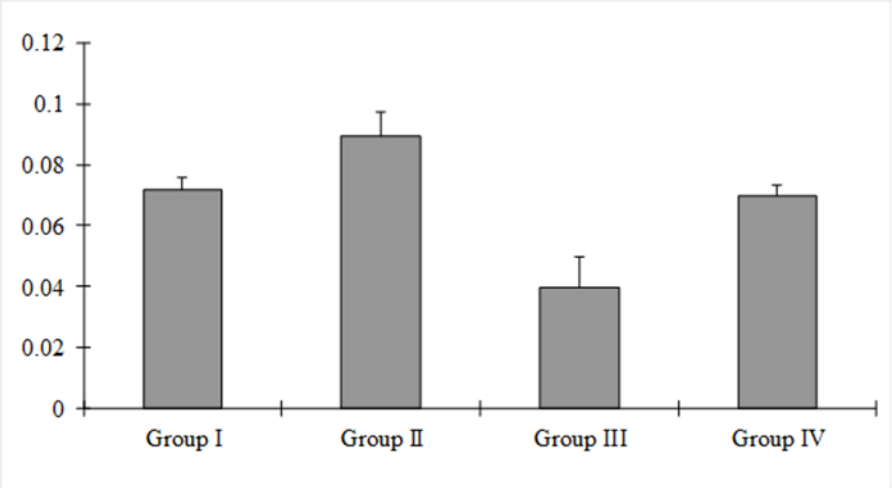


**Figure 2: Mean ± S.D. (N = 8) of protein in serum of normal and diabetic rats treated and non-treated with 10 mM L-arginine. Protein concentration is expressed as µg of protein/µg of dry weight and appears on the y-axis. Group I vs. Group II (P < 0.05), Group I vs. Group III (P < 0.05), Group I vs. Group IV (P > 0.05), Group II vs. Group III (P < 0.05), Group II vs. Group IV (P < 0.05), Group III vs. Group IV (P < 0.05).**

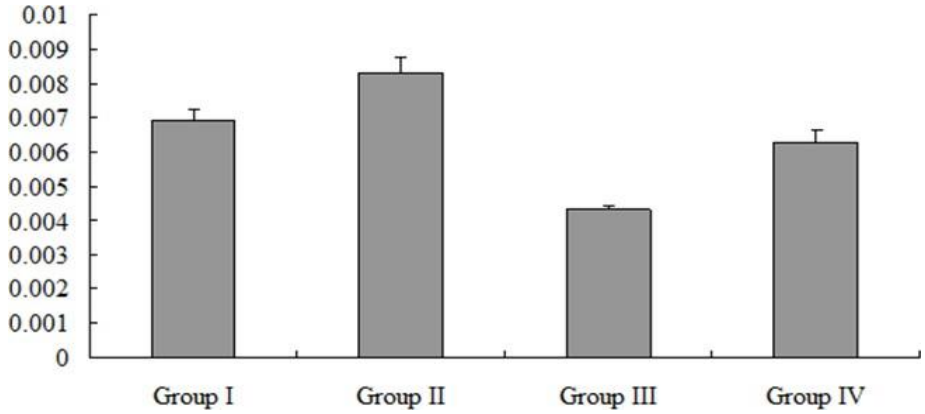


**Figure 3: Mean ± S.D. (N = 8) of urea in serum of normal and diabetic rats treated and non-treated with 10 mM L-arginine. Urea concentration is expressed as µg of urea/µg of dry weight and appears on the y-axis. Group I vs. Group II (P < 0.05), Group I vs. Group III (P < 0.05), Group I vs. Group IV (P < 0.05), Group II vs. Group III (P < 0.05), Group II vs Group IV (P < 0.05), Group III vs. Group IV (P < 0.05).**

High levels of urea synthesis on L-arginine administered samples (Group II and IV) means L-arginine increased the arginase activity in connective tissue. The increase of L-hydroxyproline synthesis was also arrested when the rats were treated with L-arginine.

No significant difference was detected in the concentrations of deoxyribonucleic acid in normal rats treated (Group I) or not (Group II) with L-arginine (P > 0.05),

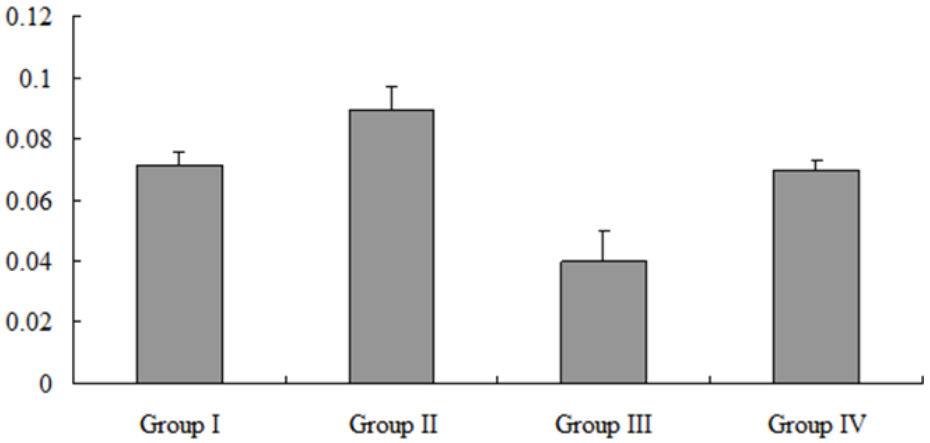


**Figure 4: Mean ± S.D. (N = 8) of L-hydroxyproline in serum of normal and diabetic rats treated and non-treated with 10 mM L-arginine. L-hydroxyproline concentration is expressed as µg of L-hydroxyproline/µg of dry weight and appears on the y-axis. Group I vs. Group II (P < 0.05), Group I vs. Group III (P < 0.05), Group I vs. Group IV (P < 0.05), Group II vs. Group III (P < 0.05), Group II vs. Group IV (P < 0.05), Group III vs Group IV (P < 0.05).**

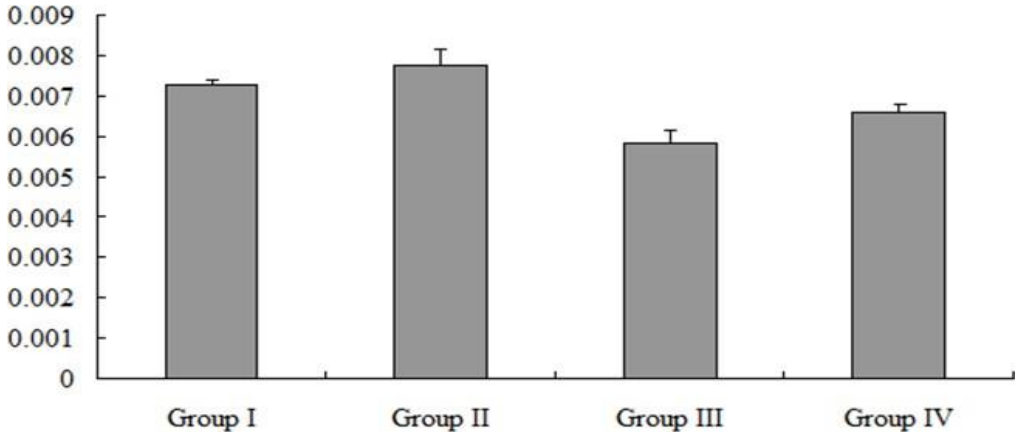


**Figure 5: Mean ± S.D. (N = 8) of deoxyribonucleic acid in serum of normal and diabetic rats treated and non-treated with 10 mM L-arginine. Deoxyribonucleic acid concentration is expressed as µg of deoxyribonucleic acid/µg of dry weight and appears on the y-axis. Group I vs. Group II (P > 0.05), Group I vs. Group III (P < 0.05), Group I vs. Group IV (P < 0.05), Group II vs. Group III (P < 0.05), Group II vs. Group IV (P < 0.05), Group III vs. Group IV (P < 0.05).**

## 3. DISCUSSION

L-arginine is a semi-essential amino acid in animal and human cells, serving as a precursor for the synthesis of a great number of biochemical molecules such as proteins, proline, urea and others, it plays an important role in regulating cellular homeostasis.[24]

Previously, it had been demonstrated that L-arginine prevented hyperglycemia by reducing the glucose serum concentrations[25], In Group III we observed a constant increase of glucose levels during the first 6 days, but a significant decrease started at the day 8 and reached the bottom at the day 12 (under 200 mg/mL); however, the glucose levels rose up constantly in Group IV and peaked at the day 11. The increase of glucose levels on both groups was caused by alloxan, and the L-arginine was responsible for the drop of Group IV after day 6. These results were congruent with our previous studies.[26]

From the determinations of biochemical parameters including proteins, arginase activity, L-hydroxyproline and deoxyribonucleic acid, we observed that most pairs of groups demonstrated significant differences in mean ± S.D., but less significant differences were detected between Group I and IV, which means that L-arginine helped diabetic animals maintain the normal levels of important biomolecules to wound healing.

The top levels of protein concentrations in Group II rats can be explained by the stimulation of protein synthesis due to the participation of L-arginine. The lowest protein levels in Group III were probably caused by the depletion of insulin biosynthesis, a decrease in mRNA and ribosome activity. There was no statistical difference between Group I and IV, because two of the major effects of L-arginine are: accelerates the protein synthesis and prevents hyperglycemia.[26]

It had been demonstrated that L-arginine levels decreased in wounds due to the activation of arginase. The high concentrations of urea in Group II and IV were caused by the increase of substrate levels (L-arginine), which stimulated the arginase activity. Hyperglycemia reduced the arginase activity in Group III. Under diabetic conditions, body arginine is focused on pancreas in order to stimulate the biosynthesis and release of insulin, so there is not enough L-arginine for wound reparation. The administration of L-arginine in Group IV increased insulin synthesis and therefore the arginase activity in cells around the wound returned to normal.

The formation of L-hydroxyproline peaked in Group II because L-arginine increased the biosynthesis of amino acids to form collagen. Therefore L-arginine participated directly in the incisional skin wound reparation. The lowest levels of L-hydroxyproline were detected in Group III, because diabetic conditions provoked insufficient collagen formation in fibroblasts.[27] However, the administration of L-arginine stimulated insulin and collagen

synthesis by the production of L-hydroxyproline in Group IV, which means L-arginine effectively accelerates the wound healing.

L-arginine also participates in the formation of histones providing them with a positive electric charge at neutral pH[28]; however, no statistical difference was detected between the normal groups (Group I and II). In diabetic rats, the rate of deoxyribonucleic acid biosynthesis and transcription was reduced because of the chronic complications, probably causing less insulin synthesis. The levels of deoxyribonucleic acid were increased in diabetic rats when they were treated with L-arginine (Group IV) in comparison with diabetic control (Group III).

## 4. CONCLUSIONS

In conclusion, L-arginine plays several roles in oral mucosa wound reparation, stimulating the proteins and deoxyribonucleic acid synthesis, it also increases the arginase activity. In addition to previous findings that L-arginine prevents hyperglycemia[13], these results provide evidence that it also increases the collagen production, reducing the time of oral mucosa wound healing.

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