The Effect of L-Arginine on Dural Healing After Experimentally Induced Dural Defect in a Rat Model

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BACKGROUND: **Incomplete repair of the dura mater may result in numerous complications such as cerebrospinal fluid leakage and meningitis. For this reason, accurate repair of the dura mater is essential. In this study, the effect of systemic and local supplementation of L-arginine on dural healing was evaluated.**

METHODS: **Thirty male Wistar rats were used and divided into control, local, and systemic L-arginine groups, with 10 rats in each. In each group, a 5-mm experimental incision was made at the lumbar segment of the dura mater and cerebrospinal fluid leakage was induced. Each group was divided into 2 subgroups and at the end of the first and sixth weeks, the rats were killed and the damaged segments of the dura were separated, histologically evaluated and the dural healing indicators including cell types, granulation tissue formation, collagen deposit, and vascularization were compared between groups.**

RESULTS: **The systematic supplementation of L-arginine showed a significant effect in dural healing compared with the control group. After the first week, granulation formation increased considerably ($P < 0.031$), and after 6 weeks, collagen deposition and neovascularization were significantly different compared with the control group ($P < 0.030$; $P < 0.009$). In comparison between different groups at the end of the first and sixth weeks, maximum changes in healing indicators were observed in the systemic group and the least variations were related to the control group.**

CONCLUSIONS: **The systemic supplementation of L-arginine may accelerate dural healing by increasing the level of granulation tissue formation, collagen deposition, and vascularization.**

INTRODUCTION

Cerebrospinal fluid (CSF) leakage is a complication of inefficient repair of the dura after trauma or intradural surgery and associated with poor wound healing, wound infection, encephalitis, meningitis, chronic subdural hematoma, and pseudomeningocele formation. Although the primary suture is the choice method for dural tearing,[1] in 5%–10% of cases the dura did not heal completely.[2] On the other hand, the improvement of minimally invasive spinal surgery increases the importance of suture methods. Several methods and materials for dural repair have been developed, such as different suture techniques, duraplasty materials, fibrin glues, absorbable gelatin substitutes, and synthetic surgical materials. However, none of these methods provides a satisfactory method for repair of the dura mater.[1,3]

L-arginine is a semiessential amino acid, which is essential for rapid growth or severe catabolic stress or injuries.[4] Arginine is a precursor in the synthesis of many biologically active components such as NO, creatine phosphate, agmatine, polyamines, and ornithine.[5] The effect of L-arginine in wound healing was introduced by Seifter et al. in 1978[6] in a rat model. Healing of the incisional wound was impaired in rats with an arginine-free diet. In 2001, Yu et al.[7] showed that in severely burned patients, concentration of L-arginine in the plasma is reduced, because of the increased ratio of arginine degradation to synthesis and these patients need supplementary L-arginine for wound healing. Several studies have shown that this healing effect of arginine moderated with different mechanisms.

Key words
- Arginine
- CSF leakage
- Dura mater
- Dural healing

Abbreviations and Acronyms
CSF: Cerebrospinal fluid

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L-arginine enhances cell migration,[8] stimulates fibroblast proliferation, and decreases apoptosis in fibroblasts.[9] It also increases collagen synthesis and deposition,[10,11] and contributes to granulation tissue formation and angiogenesis mediated by NO synthesis.[12]

Several studies have shown the beneficial effects of L-arginine in the healing of different ulcers such as diabetic foot,[13,14] pressure ulcers,[15,16] burned patients,[7] and healing of random skin flaps.[17]

In this study, the effects of L-arginine on dural healing after experimental dural opening in rats were histologically investigated. This is the first time that the effect of L-arginine supplementation on dural repair has been evaluated.

METHODS

Animals

Thirty adults male Wistar rats weighing 250–300 g were used for this study on approval of the ethics committee of Arak University of Medical Sciences and handled using protocols approved by the institutional animal care and use committee at the study institution. The animals were housed at the same room temperature and humidity conditions with a 12-hour-light/12-hour-dark cycle and unrestricted access to standard rat chow and water.

Anesthesia and Surgical Procedure

Anesthesia was induced via intraperitoneal administration of 50 mg/kg ketamine hydrochloride (Ketalar) and 5 mg/kg xylazine (Rompun) and repeated with half of this dosage as maintenance each 30 minutes or based on the changes in the vital signs. The rats were labeled with ear tags, placed in the prone position, and their backs were shaved, and the surgical site was disinfected with 10% polyvinylpyrrolidone/iodine. Using an aseptic technique and a surgical microscope, a midline incision was made along the spinous processes of the lumbar area. After dissecting the fascia and the paraspinal muscles, the spinous processes of the L-1 and L-2 vertebrae were removed. A laminectomy was performed with a high-speed diamond drill. The dura mater was opened longitudinally for 5 mm with a number 15 scalpel, and CSF leakage was observed with an operating microscope. All the surgeries were performed by the same surgeon.

The 30 rats were randomly assigned to 3 groups, each with 10 rats: group I, the control group, in which the dural defect was rinsed with 3 mL of normal saline solution; group II, the local arginine group, in which L-arginine (reagent grade, ≥98% [Sigma-Aldrich Co., St. Louis, Missouri, USA]) was located topically on the dural defect with a 40-mg/kg dose; and group III, the systemic arginine group, in which a 500-mg/kg/day dose of L-arginine was given via gavage (as a solution of 50 mg/mL in water) 1 hour before the operation and continuously once a day for 14 days. At the end of the operation, the wounds were closed in different layers, except for the dura mater. All rats were evaluated postoperatively, and mobility status and evidence of neurologic deficits were recorded.

Histologic Examination

Subsets of 5 rats from each group underwent a histologic examination of the durotomy site on the seventh day (designated groups IA, IIA, and IIIA) and sixth week postoperatively (designated groups IB, IIB, and IIIB). For this evaluation, the rats were killed with 100 mg/kg pentobarbital (Sigma, Aldrich, Arak University of Medical Sciences) intraperitoneally. After, the spinal column corresponding to the laminectomy-durotomy site, including the surrounding muscle tissue, was removed en bloc. The specimens were fixed in 10% buffered formalin solution for 1 week and then placed in EDTA until complete decalcification had occurred. The samples were embedded in paraffin, and 20-µm-thick sections from the laminectomy-durotomy site were then obtained. Each section was stained with hematoxylin-eosin. All specimens were evaluated by the same histopathologist, who was blinded to the groups.

The grading system used for histologic evaluation was based on quantifying histopathologic criteria published by Ozisik et al.[18] (Table 1). The existing cell types, granulation tissue formation, collagen deposition, and vascularization characteristics were recorded to determine the extent of the healing process at the durotomy site.

Statistical Analysis

All the data were analyzed and compared with Kruskal-Wallis and Mann-Whitney U tests. A P value of <0.05 was considered statistically significant.

RESULTS

All of the 30 rats survived until the end of the study. None of the rats developed neurologic deficits during the experiment. There was no observable CSF collection or leakage from the wounds, and none of the animals developed wound infection.

Histologic Evaluation

One of the samples of group IIB was not prepared properly during the decalcification process and was excluded from the experiment. All the other 29 samples were evaluated and scored based on a

Table 1. Grading System for Quantifying Histopathologic Findings[18]

Criteria/Score	+1	+2	+3	+4	+5
Cell types	No cells/few inflammatory cells	Inflammatory cells and few fibroblasts	Moderate numbers of fibroblasts and inflammatory cells	Fibroblast dominancy	Few fibroblasts
Granulation formation	None	Thin layer	Moderate thickness	Thick	Thick
Collagen deposit	None	Few fibers	Moderate number of fibers	Intensive fibers	Densely organized fibers
Vascularization	None	Few new capillaries	Moderate number of capillaries	Dense capillaries	Dense capillary network

Table 2. Histopathologic Grading Scores of All Samples

Groups	Number of Cases in Each Subgroup	Cell Type	Granulation	Collagen	Vascularization
IA	5	2	1	2	2
IB	5	2	2	2.5	2
IIA	5	2	2	2	2
IIB	4	2.5	2	3	2
IIIA	5	3	2	2	3
IIIB	5	3	2	4	3

Data are shown as the median for each subgroup.
IA, control group after 1 week; IB, control group after 6 weeks; IIA, local group after 1 week; IIB, local group after 6 weeks; IIIA, systemic group after 1 week; IIIB, systemic group after 6 weeks.

grading system introduced by Ozisik et al. The results are summarized in Table 2.

Histopathologic evaluation of the cases at the early phase (1 week after the operation) showed few cellularity and collagen deposits, a thin layer of granulation tissue formation, and few capillary formations in the local group. In the systemic group, there were moderate fibroblasts with thin layer granulation tissue formation and few fibers of collagen in the background with a moderate number of capillaries.

The comparisons of the criteria for wound healing between groups after 1 week by Kruskal-Wallis test showed no significant differences for cellularity, collagen, and vascularization ($P = 0.219$; $P = 0.373$; $P = 0.129$, respectively). The score of granulation tissue formation was significantly increased ($P < 0.031$), and for this variable, the Mann-Whitney U test showed that the difference was significant between control and systemic groups ($P < 0.032$).

Histopathologic evaluation of the cases at the late phase (6 weeks after the operation) showed few to moderate fibroblasts, a moderate number of collagen fibers, a thin layer of granulation formation, and little vascularization in the local group. In the systemic group, a moderate number of fibroblasts, intensive collagen deposit, thin layer of granulation formation, and moderate number of vascularization were evident (Figure 1).

The comparisons of the criteria for wound healing between groups after 6 weeks by Kruskal-Wallis test showed no significant differences for cellularity and granulation formation ($P = 0.403$; $P = 0.098$, respectively). There was a statistically significant difference for collagen deposits and vascularization criteria between groups ($P < 0.033$; $P < 0.011$, respectively), and analysis with Mann-Whitney U test showed that the differences were significant only between control and systemic groups ($P < 0.030$; $P < 0.009$, respectively).

In comparisons between subjects among each group in the early and late phase (after 1 week and 6 weeks) for wound healing indicators by Mann-Whitney U test, there were no significant differences for all the indicators in the control group as well as in the local group. In the systemic group, there was a significant difference in early and late phases just for collagen deposit criterion ($P < 0.032$) (Figure 2).

DISCUSSION

The wound healing mechanism is a complex process with 4 interferential phases: first, the coagulation phase, which happens a few minutes after injury and consists of hemostasis and platelet aggregation; second, the inflammatory phase, which is shown by migration of neutrophils and monocytes in the first few days after injury; third, the proliferation phase, which is shown by fibroblast migration, collagen deposit, granulation tissue formation, and neovascularization days to weeks after injury, depending on the size of the damage; and fourth, the wound remodeling phase, which consists of reorganization of collagen fibers and lasts for months.[19,20] The dura mater structure is generally composed of fibroblasts, collagen layers, and elastic fibers. Vandenabeele et al.[21] evaluated the spinal dura mater under an electron

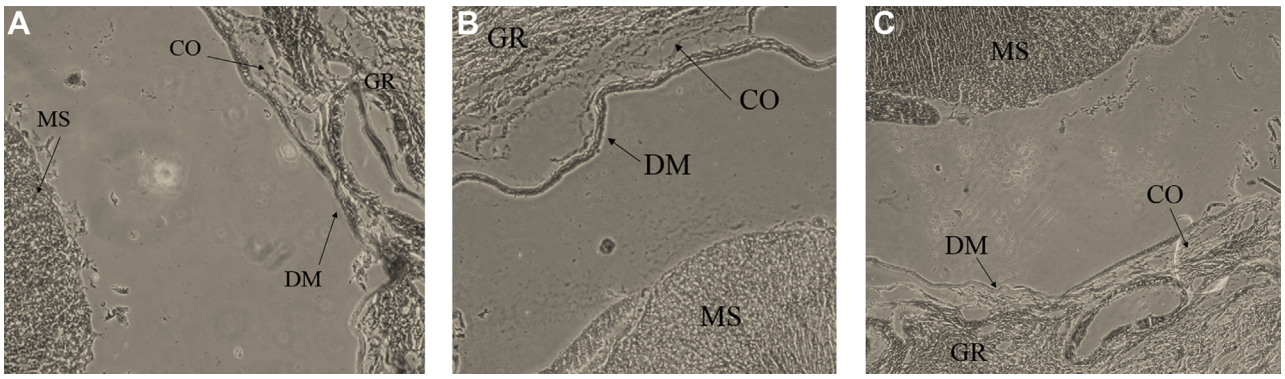

Figure 1. Histologic photographs of rat tissue samples obtained 6 weeks after laminectomy-durotomy (hematoxylin-eosin staining, original magnification ×40): (**A**) control group (IB), (**B**) local group (IIB), (**C**) systemic group (IIIB). CO, collagen deposit, DM, dura mater, GR, granulation tissue, MS, medulla spinalis.

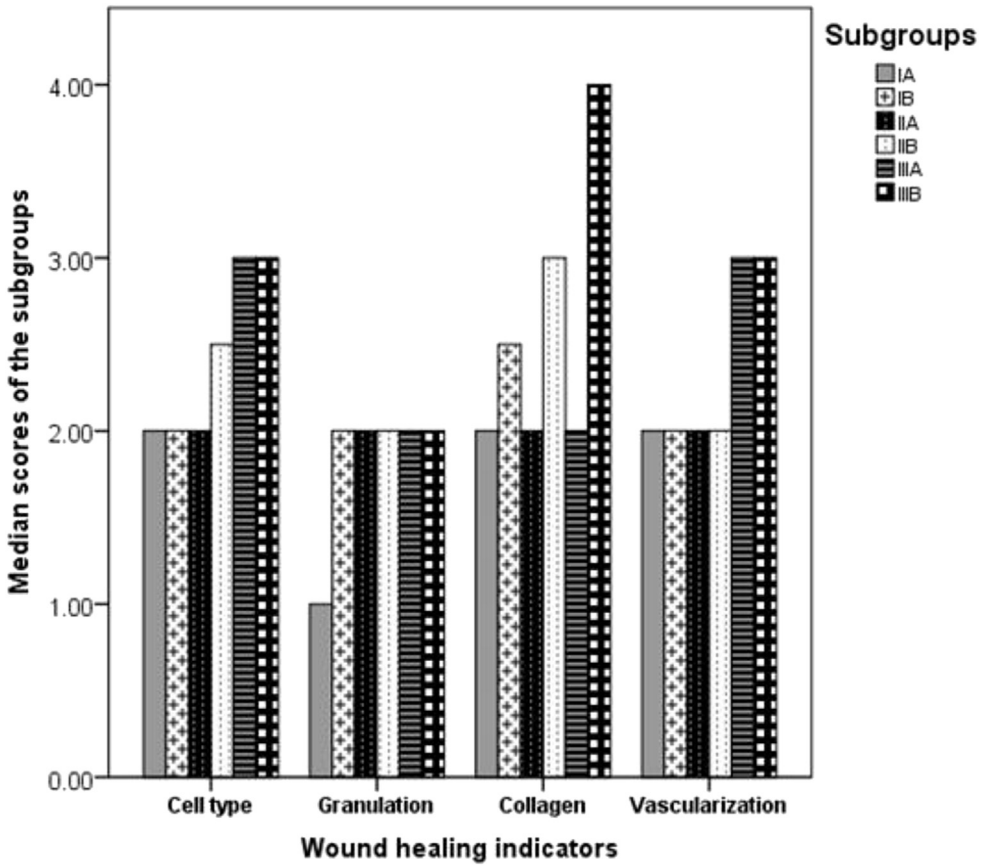

Figure 2. Graph representing the median values of wound healing indicators (cell type, granulation tissue formation, collagen deposit, and vascularization) based on the grading system adapted from Ozisik et al.[18] IA, control group after first week; IB, control group after sixth week; IIA, local group after first week; IIB, local group after sixth week; IIIA, systemic group after first week; IIIB, systemic group after sixth week.

microscope and defined 3 layers: the outermost fibroblastic layer; the middle collagenous layer, which is richly vascularized; and the innermost cellular layer with multiple interdigitating cell processes. Based on this structure, pharmacologic agents that stimulate collagen and fibroblast proliferation may affect dural healing. In this study, the effect of L-arginine supplementation on experimentally induced dural tearing has been evaluated, especially the dura of the brain because the scar formation is mainly beneficial when in the brain but not in the spinal cord because of further risk of canal stenosis.

Arbós et al. reported the effect of L-arginine supplementation in fibroblast proliferation in rat models. These investigators implanted a polypropylene mesh in the abdominal wall of 48 rats and then divided them into 2 groups: 1 receiving high values of L-arginine via infusion, and the same volume of saline in the control group. Histologic evaluation of the abdominal wall around the implanted mesh showed that cellular infiltration and fibroblast proliferation were significantly higher in the L-arginine group after 48 hours.[22] Fujiwara et al.[9] evaluated the effect of L-arginine on fibroblast stimulation in embryonic mouse cells and human dermal fibroblasts. These investigators observed a significant dose-dependent proliferation of fibroblasts by increasing the L-arginine concentrations, and also embryonic mouse cells and human dermal fibroblasts deprived of L-arginine showed a significant increase in DNA fragmentation as a marker of apoptosis in the first 24 hours of the experiment. In this study, there was no significant difference in cell types between cases with and without L-arginine supplementation. Because the cell migration and proliferation occur hours to a few days after injuries, a 7-day interval (when the first histologic evaluation has been performed) may be enough to achieve balance in the cell types in all groups, and because of that, no difference in cell types was detected in this study.

Pollock et al.[23] evaluated the effect of arginine through NO synthesis in granulation tissue formation after experimentally induced abdominal wall herniation and repair with silicone sheeting in a porcine model and showed that arginine may have a role in granulation tissue formation. Akçay et al.[24] reported the effect of arginine in granulation tissue formation in experimentally induced burn wounds in a mouse model. These

investigators indicated that granulation tissue formation was increased in cases with NO synthesis inhibitor compared with a control group. In our study, there was a significant difference in granulation tissue formation in systemic L-arginine supplementation group after 1 week compared with the control group.

In 1978, Seifter et al.[6] reported that collagen deposition in the site of cutaneous incisions after 10 days is significantly reduced in rats with a diet lacking arginine compared with rats with a laboratory chow containing arginine. In 1990, Barbul et al.[11] evaluated 36 healthy volunteers for the effect of arginine supplementation on acute wound healing. These investigators inserted a small segment of expanded polytetrafluoroethyelene catheters into the deltoid region of the patients subcutaneously, and then volunteers were given 2 different doses of arginine and placebo. After 2 weeks, the amount of hydroxyproline was determined as an index of collagen deposition. Arginine supplementation showed a significant increase in collagen deposition compared with placebo. In our study, a remarkable difference in collagen deposition was detected between systemic L-arginine supplementation and the control group in the chronic phase (after 6 weeks) ($P < 0.03$).

Arginine also affects angiogenesis through the NO pathway. Lee et al.[25] evaluated angiogenesis in endothelial nitric oxide synthase knockout mice. These investigators reported that endothelial nitric oxide synthase is required for endothelial cell migration, proliferation, and differentiation. Arbós et al.[22] evaluated the early effect of intravenous L-arginine perfusion after implantation of a mesh graft into the abdominal wall of the rats. Forty-eight hours after implantation, angiogenesis was significantly higher compared with rats without arginine perfusion. The results of our study showed a significant difference in vascularization in the systemic arginine supplementation group and control cases after 6 weeks ($P < 0.009$).

Heffernan et al.[26] evaluated the effect of local L-arginine supplementation in the healing of the abdominal wall in porcine cases. These investigators induced experimental abdominal hernia defect in 12 porcine cases and, then, wound closure was performed by silicone sheet and suturing. The investigators inserted and fixed osmotic minipumps in the field of surgery, which were filled with normal saline in 6 animals (as a control group) and L-arginine in the other 6 animals. The investigators evaluated the histologic changes in the site of surgery after 14 days in both groups. They reported that continuous local supplementation of L-arginine increased granulation tissue formation and vascularization. In our study, there was no significant difference in wound healing indicators between local L-arginine and the control group in the acute or chronic phases, perhaps because there was single exposure of the wound with local L-arginine instead of continuous infusion.

In this study, systematic application of L-arginine had a significant effect in dural healing, compared with the control group. In the acute phase (after the first week), granulation formation increased considerably ($P < 0.031$), and in the chronic phase (after 6 weeks), collagen deposition and vascularization represented a significant increase ($P < 0.030$; $P < 0.009$, subsequently). There was no remarkable difference between the local application of L-arginine and the control group in dural healing.

Comparing different groups at the end of the first and sixth weeks, maximum changes in healing indicators were obvious in the systemic group and the least variations were related to the control group. This is the first study to show the effect of L-arginine supplementation in dural healing. and it has some drawbacks: the small sample size in each group and the limited period of the follow-up. Therefore, a trial with a larger sample size with a long-term follow-up may provide more attributable results for evaluation of the efficacy of L-arginine in dural healing.

Systemic supplementation of L-arginine has positive effects on dural healing by increasing collagen deposition and vascularization in the field of trauma, which can reduce the possibility of CSF leakage and complications. Further studies with a larger sample size and long-term follow-up in animal and human models are required.

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