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Original article

Local Administration of L-Arginine Accelerates Wound Closure

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Abstract

Objective(s)
The process of wound healing involves tightly integrated events including inflammation, granulation tissue formation and remodeling. Systemic administration of L-arginine promotes wound healing but its global side effects are undesirable. To confine the action of L-arginine at the site of injury, we tested the effects of local administration of L-arginine on the healing of excisional wound in the rat.
Materials and Methods
Full thickness excisional wounds were generated on the dorsum of adult male rats. The test wounds received 200 µm or 400 µm of L-arginine on day 3 and 5 post-wounding. Normal saline was injected into the sham wounds which were otherwise treated as the test wounds. Control wounds remained unmanipulated. The wound size was monitored daily by imaging. To determine the rate of wound closure, wound images were scanned and the rate of size reduction was analyzed and quantified by ScnImage software. The repaired tissues were harvested on day 12 post-wounding. The tissue sections were prepared and stained for microscopic examination.
Results
Wounds treated with L-arginine showed a significant increase in the rate of wound closure. The morphology of basal keratinocytes was altered, and the thickness of neoepidermis was markedly reduced in the wounds treated with L-arginine. Both tested dose of L-arginine were equally effective.
Conclusion
Local administration of L-arginine accelerates wound closure and has profound effects on keratinocytes performance during the process of healing. Therefore, it can be potentially used for treatment of skin disorders, in particular, those characterized by hyperkeratosis.

Keywords: Keratinocyte, L-arginine, Rat, Skin, Wound healing

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Introduction

The process of wound healing involves tightly orchestrated events including inflammation, granulation tissue formation and remodeling. Soon after injury, activated cells from surrounding intact tissue migrate to the site of injury, and begin to deposit a provisional matrix, reconstruct blood vessels and epithelial layer and eventually close the wound. The quality of healing depends on the on time resolution of inflammation, rate of wound closure and myofibroblasts fading that determines restoration of normal tissue function (1-4).

Previous studies show that systemic administration of L-arginine promotes tissue repair and increases wound breaking strength (5-7). Although L-arginine increases the serum level of growth hormone- a repair promoting factor- and acts as a building block provider for protein synthesis, its positive effect on wound healing is mainly attributed to the nitric oxide (NO), a multifunctional free radical molecule (8-11). Cells involved in wound healing produce NO from its sole precursor: L-arginine. L-arginine can be metabolized by two major pathways: The arginase pathway produces proline; and the oxidative deamination pathway, catalyzed by nitric oxide synthase (NOS), leads to production of citrulline and NO. The products of both pathways are potentially beneficial to wound healing. Proline is an important amino acid for synthesis of collagen, a major dermal component which provides tissue tensile strength, and nitric oxide is a potent bioactive molecule with positive effects on wound healing (12-15). Wound healing studies using nitric oxide synthase of knock out mice show that production of NO is necessary for normal wound healing and that topical adenoviral-mediated NOS gene transfer reverses the impaired wound repair in these animals (16-18). Moreover, NO is shown to be essential for a switch from stationary to migratory phenotype in epithelial cells, indicating that this gas can also modulates the rate of re-epithelialization (19). Because L-arginine is the sole substrate for NO production, the production of NO depends on the availability of this amino acid. The level of L-arginine is critically low in the wound microenvironment due to the high concentration of released arginase by wound macrophages (12-15). Overactivity of arginase pathway, therefore, leads to substrate insufficiency for NO production at the wound site. Indeed, *in vitro* studies show that L-arginine utilization and breakdown by arginase can impair NO production because of substrate limitation (20).

To increase the level of L-arginine in the wound site and to confine its metabolites to the injured area, considering that systemic administration of L-arginine may disturb body homeostasis, we studied the effects of local administration of L-arginine on the healing of cutaneous excisional wound.

Materials and Methods

Adult male Sprague-Dawley (Pasteur institute, Tehran, Iran) rats weighting 200-250 gram were used in this study. Animals were acclimatized to standard laboratory condition for 7 days before use. The room temperature ($22 \pm 2\ ^{o}C$) and lighting (7 am-7 pm light) were controlled. The rats were maintained on a commercial rat chow with water *ad libitum*. L-arginine was purchased from Merck (Germany). Wounds were generated on the back of 30 rats according to the method described previously (21). Briefly, on the day of surgery, rats were anesthetized by an intraperitoneal injection of thiopental sodium (Nesdonal; 40 mg/kg body wt) and given an intramuscular injection of a pain killer (Xylazine; 6 mg/kg body wt.). The dorsum of animals was then shaved, and the skin was prepped with povidone-iodine and sterile saline. Four full thickness excisional wounds (2 cm apart, paravertebral) were generated on the back of each animal using sterile 6 mm-diameter skin punchers. The wounds were then randomly divided to four groups. The test wounds were treated with two intralesional injections of 200 µm or 400 µm of L-arginine solution during postinflammatory phases; the first injection was performed on day 3 and the second one on day 5 post-wounding. Normal saline was injected into the sham wounds which were otherwise treated as the test

wounds. The volume (20 µl) of the injected solution was kept constant for all treatments. To assure a uniform delivery, the center of wounds was chosen as the site of injection. Control wounds were remained unmanipulated throughout the experiment. Animals with infected wounds and/or edge deformity wounds were excluded from the study regardless of the phase of healing. Seventeen to twenty two wounds were tested in each group. On day 3 post-wounding the area of all wounds were measured immediately before the treatments, and used for normalization of the wound area when the rate of wound closure was to be determined on day 5 post-wounding. Using a transparent sheet, wound size was monitored daily by imaging. The images were then scanned, imported to a PC, digitalized and analyzed by an image-processing software, ScnImage. Using 8 mm-diameter skin punchers, the wound and normal adjacent tissue were harvested on day 12 post-wounding. Harvested tissues were fixed in 4% paraformaldehyde solution, processed, embedded in paraffin blocks and serial sections (4 µm) were prepared. Sections were then stained with hematoxylin and eosin using standard procedure. Re-epithelialization and morphometric analysis of epidermal layers were assessed by microscopic examination of the serial sections.

Results

To assess the effects of L-arginine on the rate of wound closure, the reduction in wound surface area of different wound groups were measured and compared on the indicated days. All wounds showed a similar reduction in wound area on day 3 post-wounding, prior to the first injection (data not shown). As compared to size reduction in control wounds (19.6%±2), the wounds treated with 200 µm or 400 µm of L-arginine showed a significant increase (P<0.05) in the reduction of wound size (28.4%±2.9 and 29.6%±2.9, respectively) 2 days after the first injection (Figure 1). A similar result was found when the area of test wounds was compared with that of sham wounds, 28.4%±2.9 and 29.6%±2.9 vs 20.2%±2, (P<0.05). Most often the presence of scab on the surface of wounds (Figure 2) limits accurate evaluation of wound closure; therefore, on day 12 post-wounding, the number of open wounds were determined by microscopic examination of serial sections prepared from harvested wounds. Microscopic examination of tissue sections revealed that only 15% of the wounds treated with 200 µm of L-arginine (n= 20) were open and 85% were close or fully re-epithelialized, whereas the open or partially re-epithelialized wounds in sham (n= 17) and control (n= 22) groups were 47% and 40%, respectively (Figure 3). Similar results (n=15) were observed when 400 µm of L-arginine was tested (Figure 3). L-arginine had profound effects on the keratinocytes in the epidermal layer of neoepidermis. Basal keratinocytes in the wounds treated with L-arginine showed a marked alteration in morphology. The columnar basal keratinocytes in these wounds assumed cuboidal morphology and became more flat in shape (Figure 4). The thickness of epidermis was also affected by L-arginine. The epidermal layer in the L-arginine-treated wounds were markedly thinner than that in control or sham wounds, suggesting that cell proliferation, migration and/or differentiation were also affected by L-arginine (Figure 5).

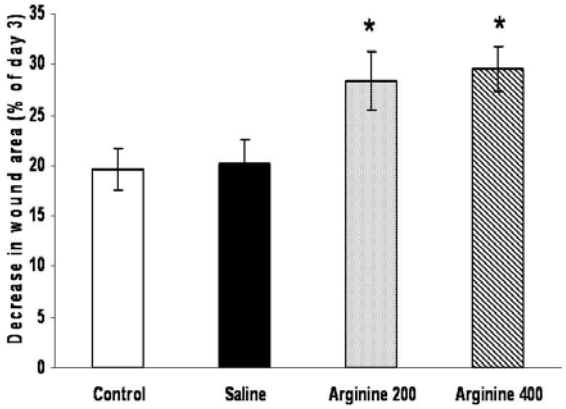

Figure 1. The effect of L-arginine on the wound size decrement. The surface area of wounds was measured two days after the treatments. Each bar shows the mean±standard error of the mean of wound surface area reduction of control wounds (open bar), wounds treated with saline (solid bar) and wounds treated with 200 µm of L-arginine (doted bar) or 400 µm of L-arginine (hatched bar).
* P< 0.05

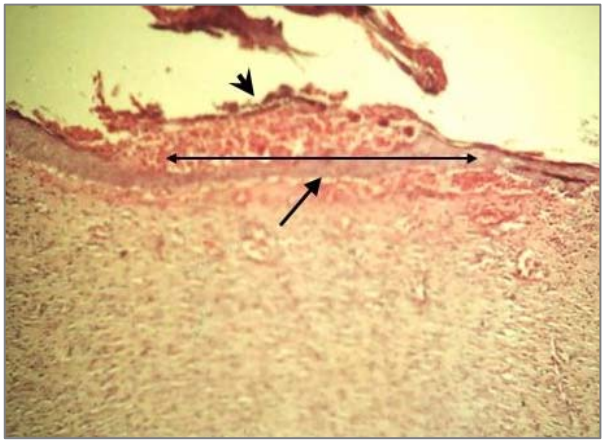

Figure 2. The presence of scab limits an accurate evaluation of wound closure by macroscopic methods. A tissue section obtained from the central portion of a wound harvested on day 12 post-wounding and stained with hematoxyline and eosine. The photomicrograph shows a remarkable part of neoepidermis (arrow) covered by the overlaying scab (arrow head).

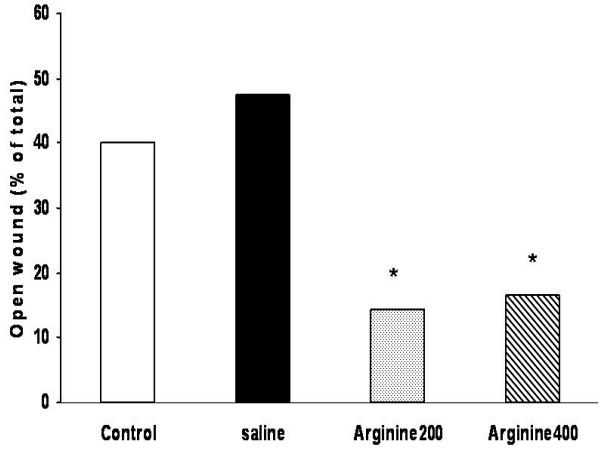

Figure 3. The effect of L-arginine on the rate of wound closure. Wound closure was evaluated by microscopic examination. Wounds with complete epithelial layer were considered close. A significantly larger number of control wounds (open bar) and saline-treated (solid bar) wounds were still open on day 12 post-wounding as compared to the wounds treated with 200 μm (dotted bar) or 400 μm of L-arginine (hatched bar). At least 15 wounds were examined for each treatment. * $P< 0.05$

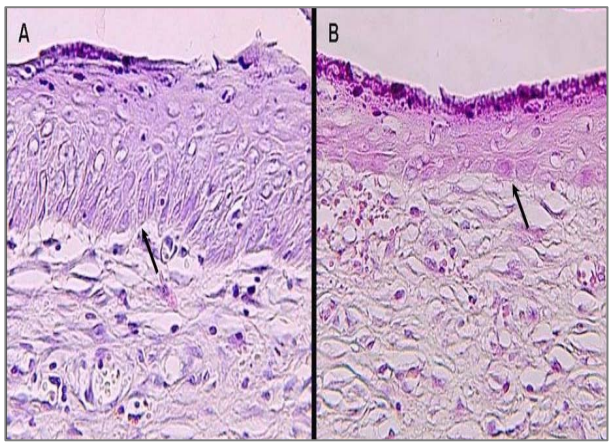

Figure 4. Intralesional injection of L-arginine alters the morphology of keratinocytes in the neoepidermis. Representative hematoxylin and eosin- stained tissue section of a close wound. The epidermal keratinocytes in wound treated with 400 μM of L-arginine (B) have assumed cuboidal morphology and become flat (arrow) whereas they have kept their columnar shape (arrow) in wound treated with saline (A).

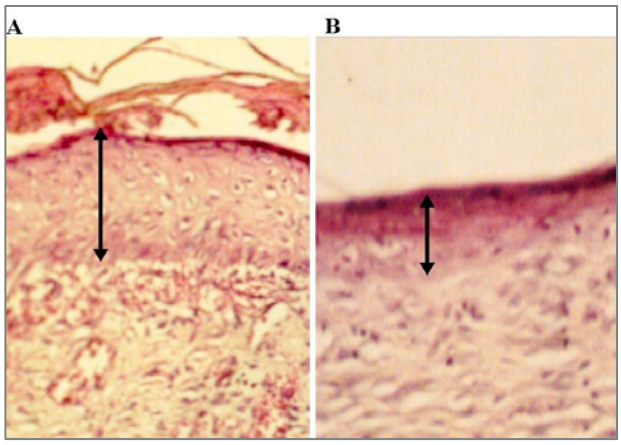

Figure 5. Intralesional injection of L-arginine profoundly affects the thickness of neoepidermis. Representative hematoxylin and eosin- stained tissue section of closed wound. Neoepidermis (two head arrows) of the wound treated with 400 μm of L-arginine (B) is much thinner than that of wound treated with slain (A).

Discussion

These data clearly show that local administration of L-arginine accelerates wound closure, and profoundly modulates the keratinocytes activities in regenerating skin. Although the positive effect of systemic administration of L-arginine has been shown previously, the merit of our study is that L-arginine effects are confined to the skin wound site. Since the high concentration of arginase released by wound macrophages in the wound microenvironment leads to substrate limitation and impairs production of L-arginine metabolites, NO and proline (15), multiple local administration of L-arginine can be more effective than its systemic administration. It is now accepted that the positive effect of L-arginine on the healing process is mediated mostly by its two major metabolites. In contrast to local administration, systemic administration of L-arginine exposes all tissues to the metabolites that may lead to disturbance of normal tissue function.

Likewise, L-arginine-induced growth hormone may exert its biological effects globally leading to disturbance of body metabolism.

A significant increase in the rate of wound closure, marked morphological changes of basal keratinocytes and reduced epidermis thickness in the wounds treated with L-arginine indicate that this amino acid may play an important role in epithelial cell biology, at least, during regeneration and tissue repair process. Perhaps, increased re-epithelialization rate is induced by stimulation of epithelial cell migration as the basal keratinocytes assumed cuboidal morphology and became more flat in shape covering a larger surface of the underlying neodermis. Although we did not investigate the involved mechanism, these effects seem to be mediated, at least in part, by NO. Accumulating data support this idea (12-15). Using the same animal model, Amadeu and Costa showed that inhibition of nitric oxide synthesis delay wound closure (22). Moreover, Noiri *et al* have shown that NO is essential for a switch from stationary to migratory phenotype in epithelial cells (19). In accordance to these data, the migratory phenotype of keratinocytes in L-arginine-treated wounds could also be induced by NO. Gosselink *et al* reported a positive effect of local administration of NO donors or L-arginine on mucosal wound healing (23). They have also found that the positive effects of exogenous NO donors but not L-arginine, as an endogenous NO donor, are associated with headache, indicating that nature of NO provider plays roles in development of the NO- induced side effects.

Interestingly, we found that the thickness of epidermal layer is profoundly affected by the L-arginine. This indicates that the proliferation and/or differentiation of keratinocytes are affected by the L-arginine treatments. A high concentration of NO synthesized and released by keratinocytes is considered a potent inhibitor of proliferation and inducer of differentiation for these cells (22). Therefore, increased production of NO due to the high concentration of local L-arginine may cause a reduction in the epidermis thickness. The NO-induced alteration in epidermis thickness is previously reported by other investigators (24, 25). Intriguingly, data reported by Bruch-Gerharz and colleagues show that arginase-1 is persistently overexpressed in psoriasis lesion. This can limit inducible nitric oxide (iNOS) activity and, therefore, suppress the delivery of anti-proliferative NO concentration (24). Further studies are needed to better understand the precise mechanism involved in the L-arginine--induced alterations in epidermis. However, from clinical point of view these findings are quite important. Epidermis thickening is a common characteristic of several skin disorders. The effects of L-arginine on the thickness of epidermis make this amino acid as a target molecule which can be potentially used in clinics.

Conclusion

Our data show that local administration of L-arginine accelerates cutaneous wound healing, and has profound effects on keratinocytes activities leading to alteration in the thickness of neoepiderm. Therefore, it can be potentially used for improving impaired wound healing and treatment of skin disorders; in particular those charachterized by hyperkeratosis.

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