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Topical folinic acid enhances wound healing in rat model

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ABSTRACT

Purpose: Folic acid is an essential vitamin participating in DNA synthesis and repair. Recently folic acid has been shown to stimulate DNA-repair capacity in dermal fibroblasts in response to injury. Thus, the present study aimed to investigate the effects of topical folinic acid, a 5-formyl derivative of tetrahydrofolic acid, on wound healing using rat wound model.

Materials and methods: A rat wound model was established, and the wound healing was evaluated by macroscopic and histological analyses among vehicle control, 2.5% folinic acid, 1% folinic acid, and dexpanthenol treatment groups. While an image-analysis program was used to evaluate macroscopic wound closure, connective tissue properties, mast cell numbers, and the expressions of matrix metalloproteinase 1 (MMP-1) and 9 (MMP-9) were evaluated by microscopy.

Results: The 2.5% folinic acid-treated group exhibited enhanced wound healing by increased reepithelialization, neo-vessel formation, inflammatory cell migration, collagen deposition and progressive mast cell increase. Furthermore, 2.5% folinic acid induced higher expressions of MMP-1 and MMP-9.

Conclusions: Folinic acid enhances both macroscopic and microscopic wound healing in rat wound model.

1. Introduction

Folic acid is a water-soluble vitamin B mainly involved in DNA synthesis and repair and is essential for normal cell metabolism [1]. Insufficient folic acid intake has been associated with impaired normal growth and development, neural tube defects, DNA instability, cardiovascular diseases, and various cancers [2]. The knowledge about the role of folates on the skin is limited, however, there are ongoing promising studies on folate status and human skin diseases [3].

In mammalian cells and tissues including skin, major transport system of folic acid is folate-carrier [1]. It has been shown that skin fibroblasts, which are essential components of connective tissue metabolism and predominant cell type in the course of wound repair in the skin, increase folate-carrier 1 expression, and their intracellular folic acid uptake in response to UV radiation [3]. Also, it has been shown that treatment of cultured dermal fibroblasts with folic acid stimulates DNA repair capacity of dermal fibroblasts suggesting a promising treatment option for photoaged skin [3,4]. In-vitro and in-vivo efficacies of topical folic acid-and creatine-containing formulation on epidermal skin regeneration were previously reported, indicating a role of folic acid in the treatment of photoaged skin [4]. In addition, treatment of cultured dermal fibroblasts with folic acid and creatine increased collagen gene expression and procollagen levels, and improved collagen fiber density [1].

Given all these study findings, we suggested that topical folic acid might play a role in promoting skin wound repair.

Folinic acid is a 5-formyl derivative of tetrahydrofolic acid. In contrast to folic acid (a synthetic form of folate), folinic acid is one of the forms of folate found naturally in foods. In the body, folinic acid can be converted into the other active forms of folate and has the full vitamin activity of folic acid. And, in contrast to folic acid, its function is unaffected by the inhibition of dihydrofolate reductase [5]. Thus, in this

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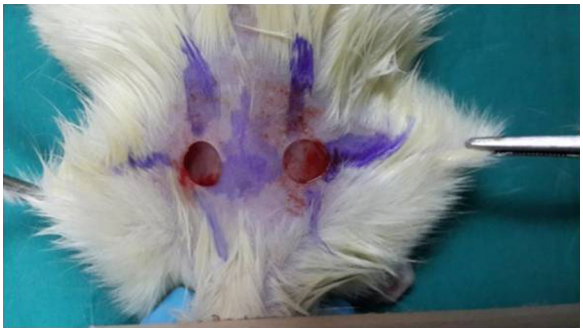

Fig. 1. Wound model used in the study.

study, we aimed to investigate the effects of topical folinic acid on wound repair using in vivo rat wound model.

2. Material and methods

2.1. Animals and wound repair model

Forty adult male Sprague-Dawley rats, with average weight 200–250 g were included in the study. The study protocol was in compliance with current guidelines for the care of the laboratory animals and was approved by the Afyon Kocatepe University Animal Experimentation Ethics Committee (approval number: AKUHAD-YEK-414-15). The animals were maintained in the standard laboratory conditions (temperature: 22 ± 2 °C, humidity: 55 ± 5%, 12/12 reversed light cycle). Each rat was housed in a separate cage and provided with standard diet and water ad libitum.

All rats were anesthetized with an intramuscular injection of ketamine/xylazine (87 mg/kg/13 mg/kg). Dorsal skin hair of the rats was shaved bilaterally and 2 circular full-thickness skin wounds were obtained using a 6-mm punch biopsy tool (Fig. 1). The wounds were not sutured and left open. The rats were then randomly divided into four groups based on treatment applied on wounds: pure vaseline (negative control, G1), 1% folinic acid cream (G2), 2.5% folinic acid cream (G3), and dexpanthenol (positive control, G4). Ten animals were included in each group, and starting from the day of biopsy, topical creams were applied two times daily in sufficient amounts to cover the entire wound until the epithelisation was complete. Animals were observed closely for any signs of infection and excluded from the study in case of infection.

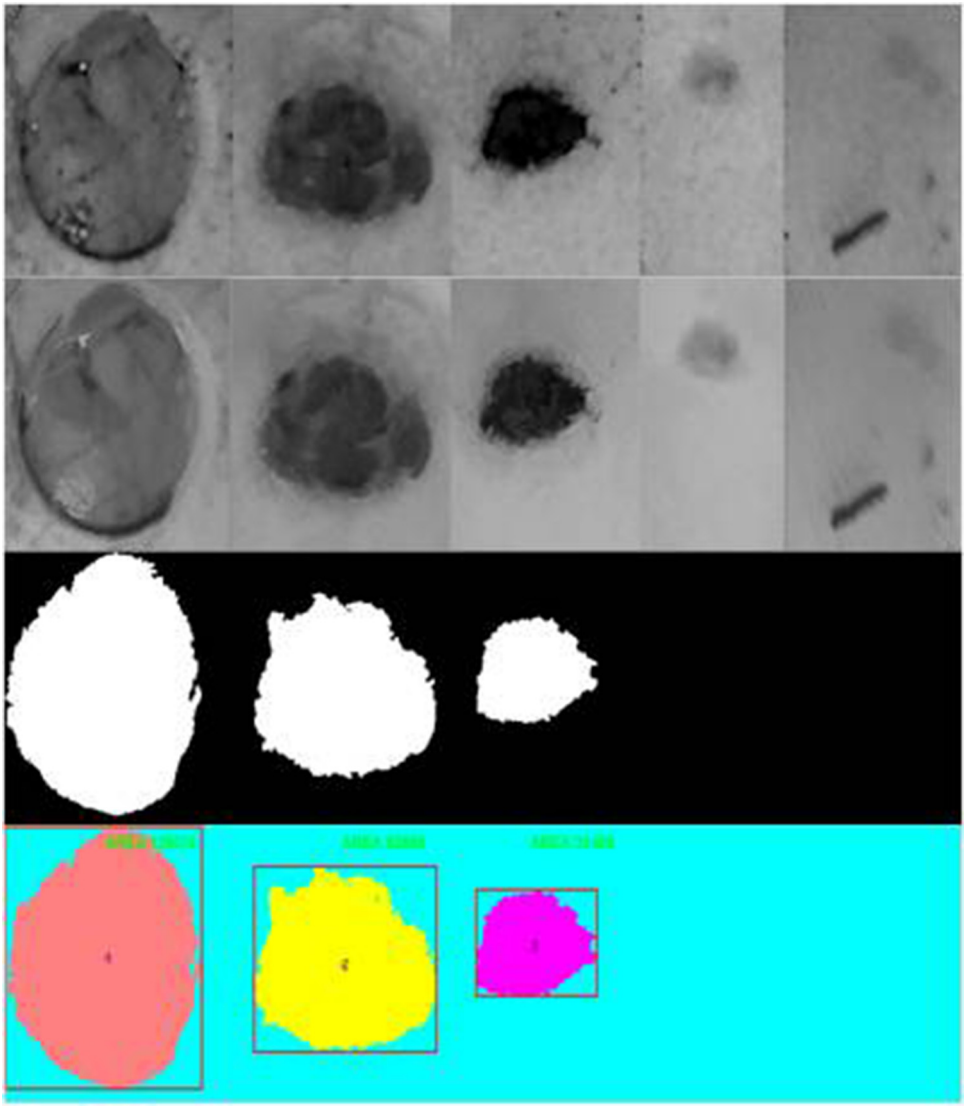

Fig. 2. Implementation of image processing on the images.

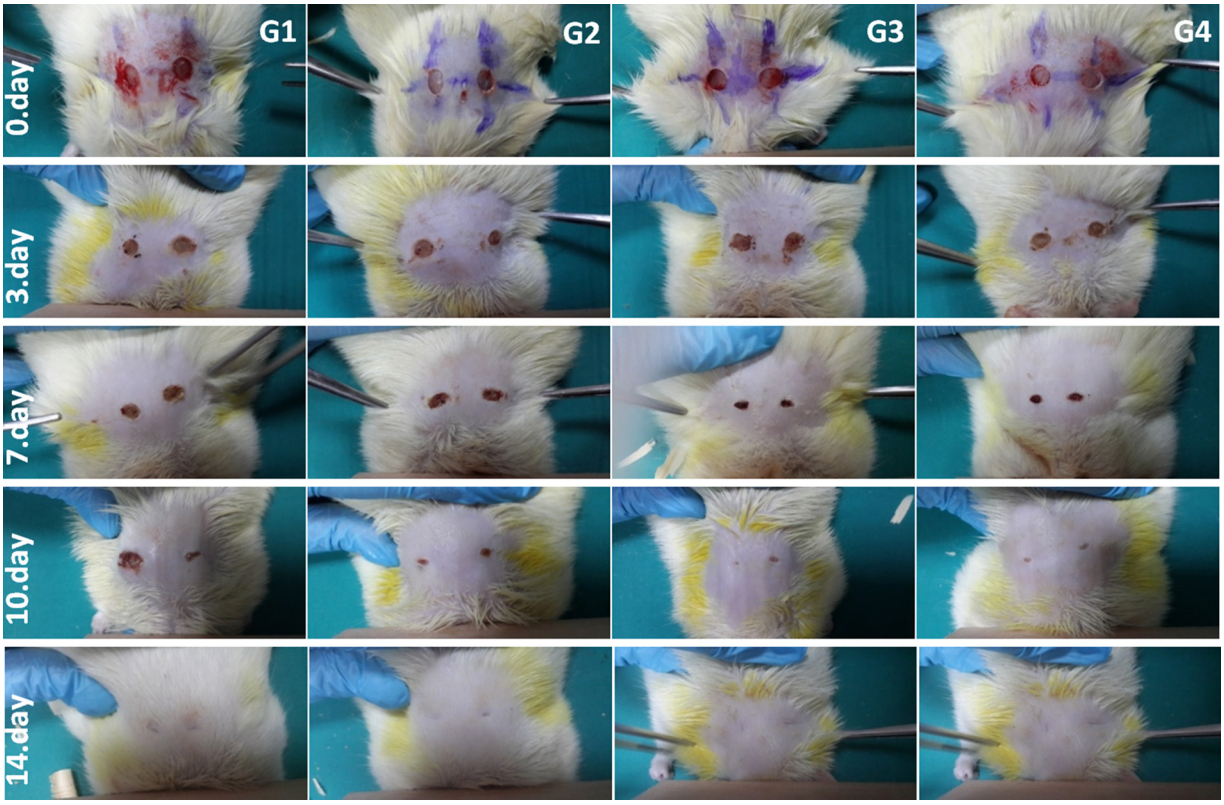

Fig. 3. Comparison of macroscopic wound healing between 4 groups (G1: negative control; G2:1% folinic acid; G3: 2.5% folinic acid; G4: positive control).

2.2. Preparation of topical 1% and 2.5% folinic acid creams

For 1% and 2.5% folinic acid creams, pure folinic acid calcium salt hydrate powder (250 mg; > = 99.0% (HPLC); 47612; Sigma Aldrich Chemie GmbH, Steinheim, Germany) was dissolved in warm, ultra pure water, added to melted vaseline and cooled by stirring.

2.3. Macroscopic wound analysis

For macroscopic analysis, digital photographs of the wound were taken on days 0, 3, 7, 10, and 14. The image analysis was done using Matlab Image Processing Toolbox on the images of the healing process of the wounds on the rat. The first-day image of the wound was processed and obtained as pixels. The same process was done on the other days. Fig. 2 shows examples of processed images from day 0 to day 14. Sharpening filter was used for sharpening images, Watershed transformation was used for enhancing and refining, and image re-construction filter was used for reconstructing images. In this way wound area and the border of the area were determined. Using these algorithms shortened image processing.

Additionally, the percentage of wound closure was calculated as follows: (area of initial wound - area of the actual wound) / area of initial wound $\times 100$.

2.4. Microscopic analysis of wounds

For microscopic evaluation, wound and its surrounding skin tissues were taken from 3 animals in each group on day 3, 3 animals in each group on day 7, and from the remaining 4 animals in each group on day 14.

All tissues were fixed in 10% neutral formalin, processed with classical histological techniques and embedded in paraffin. Then, sections of 5 μ thickness were taken from the blocks and mounted on slides. For histological evaluation, we used Hematoxylin & Eosin (H&E) staining to determine general tissue properties and Masson Trichrome staining to determine connective tissue properties. We evaluated epithelial healing, inflammatory cell migration, neovascularization, edema, collagen structure regularity and matrix formation, and each parameter was scored as follows: 1 - none, 2 - silent, 3 - middle, 4 - marked, and 5 – total. The total sum of scores on day 3, 7, and 14 were compared between the groups.

For staining mast cells, we used 0.1% Toluidin blue. The mast cells were counted under a light microscope in 6 different areas with Image Analysis Software (Nikon DS4 Image Analysis Software), and the numbers were compared between the groups.

For immunohistochemical analysis, new 5 μm thickness tissue sections were cut from the formalin fixed, paraffin-embedded H&E-stained tissue blocks and sections were stained using antibodies against matrix metalloproteinase 1 (MMP-1, TA323955; Acris) and matrix metalloproteinase 9 (MMP-9, SC-6840; Santa Cruz).

Statistical analyses were performed using SPSS v.18.0 for Windows (SPSS Inc., Chicago, IL, USA). Continuous variables were presented as a mean ± standard deviation and categorical variables as frequencies and percentages. Differences between the groups were determined using one-way analysis of variance test. The level of statistical significance was set at $p < 0.05$.

3. Results

3.1. Macroscopic findings

There were no significant differences between healing rates of the 4 groups on day 3 ($F(3,43) = 0.41$, $p = 0.748$)) however on day 7 and 10, 2.5% folinic acid-treated group and dexpanthenol group had significantly higher healing rates compared to other groups ($F(3,24) = 8.03$, $p = 0.001$, and $F(3,18) = 5.03$, $p = 0.01$, respectively) (Fig. 3). The wounds were healed or almost healed in each group on day 14 (mean wound % closure on day 14 was 95.16 ± 6.78,

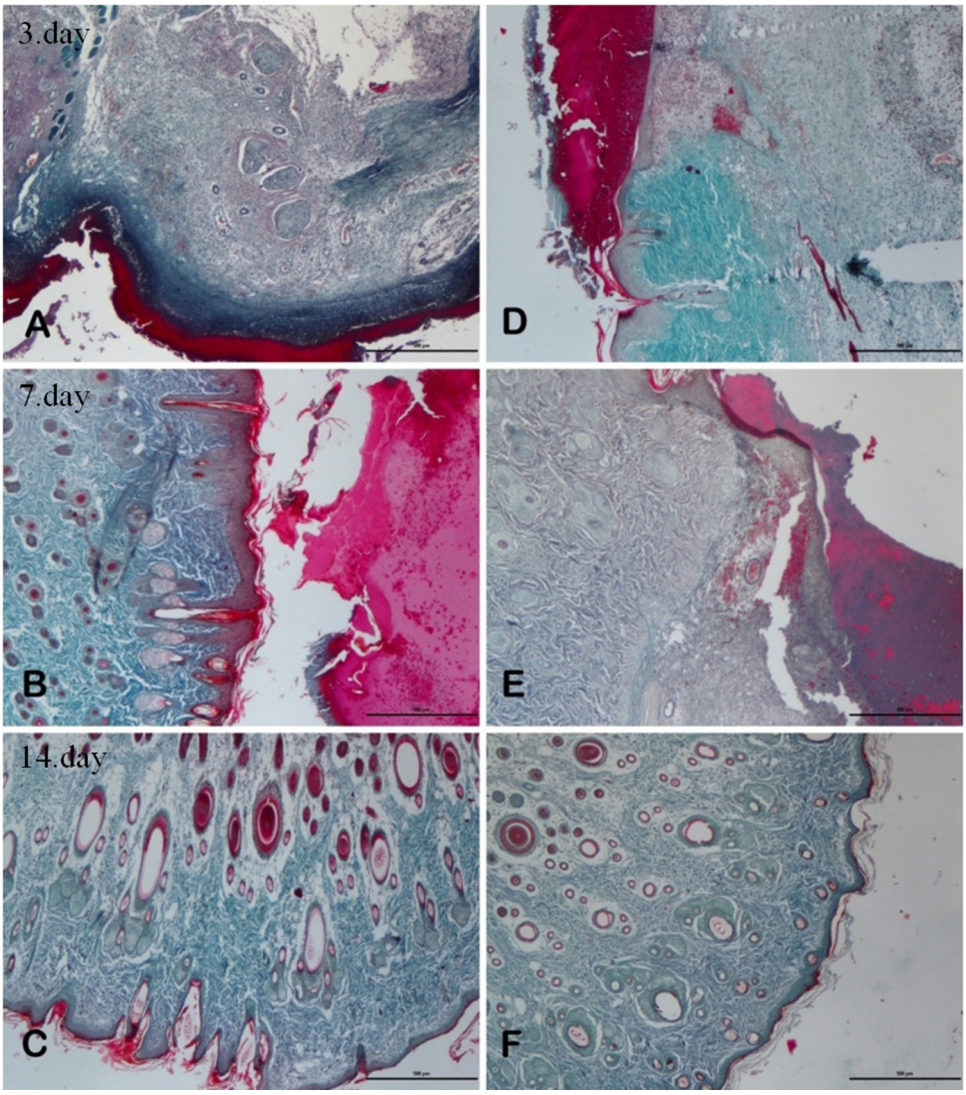

Fig. 4. Comparison of wound healing phases between Group 3 (A, B, C) and 4 (D, E, F) (Masson Trichrome × 10, G3: 2.5% folinic acid, G4: positive control).

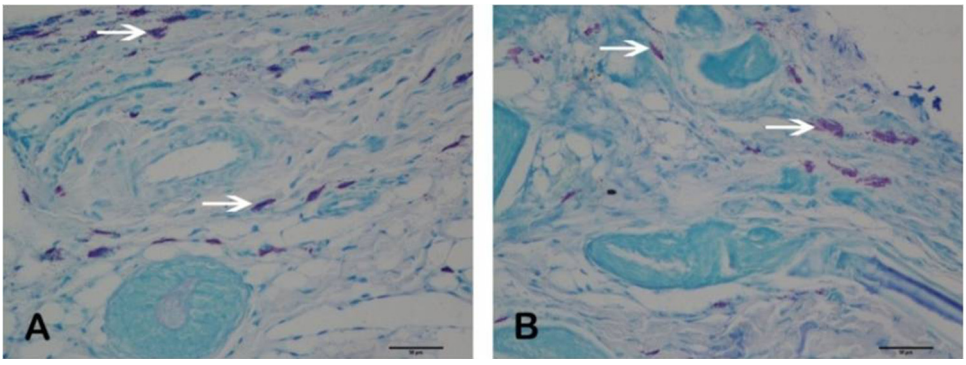

Fig. 5. Dermal mast cells in Group 3 (A) and Group 4 (B) on day 7 (Toluidin blue × 40, G3: 2.5% folinic acid, G4: positive control).

97.99 ± 2.45, 100, and 100 in negative control, folinic acid 1%, folinic acid 2.5%, and positive control group, respectively).

3.2. Microscopic findings

Histological evaluation on day 3 did not differ significantly between the groups, whereas on day 7 2.5% folinic acid-treated and dexpanthenol-treated groups had significantly accelerated tissue regeneration by increased reepithelialization, neo-vessel formation, inflammatory cell migration and collagen deposition compared to negative control and 1% folinic acid-treated groups. On day 14, reepithelialization was completed or almost completed in all groups, however, collagen fiber density was higher and fiber distribution and scar formation were more regular in 2.5% folinic acid group as compared to the remaining groups (Fig. 4).

Mast cell evaluation in all groups showed similar numbers and

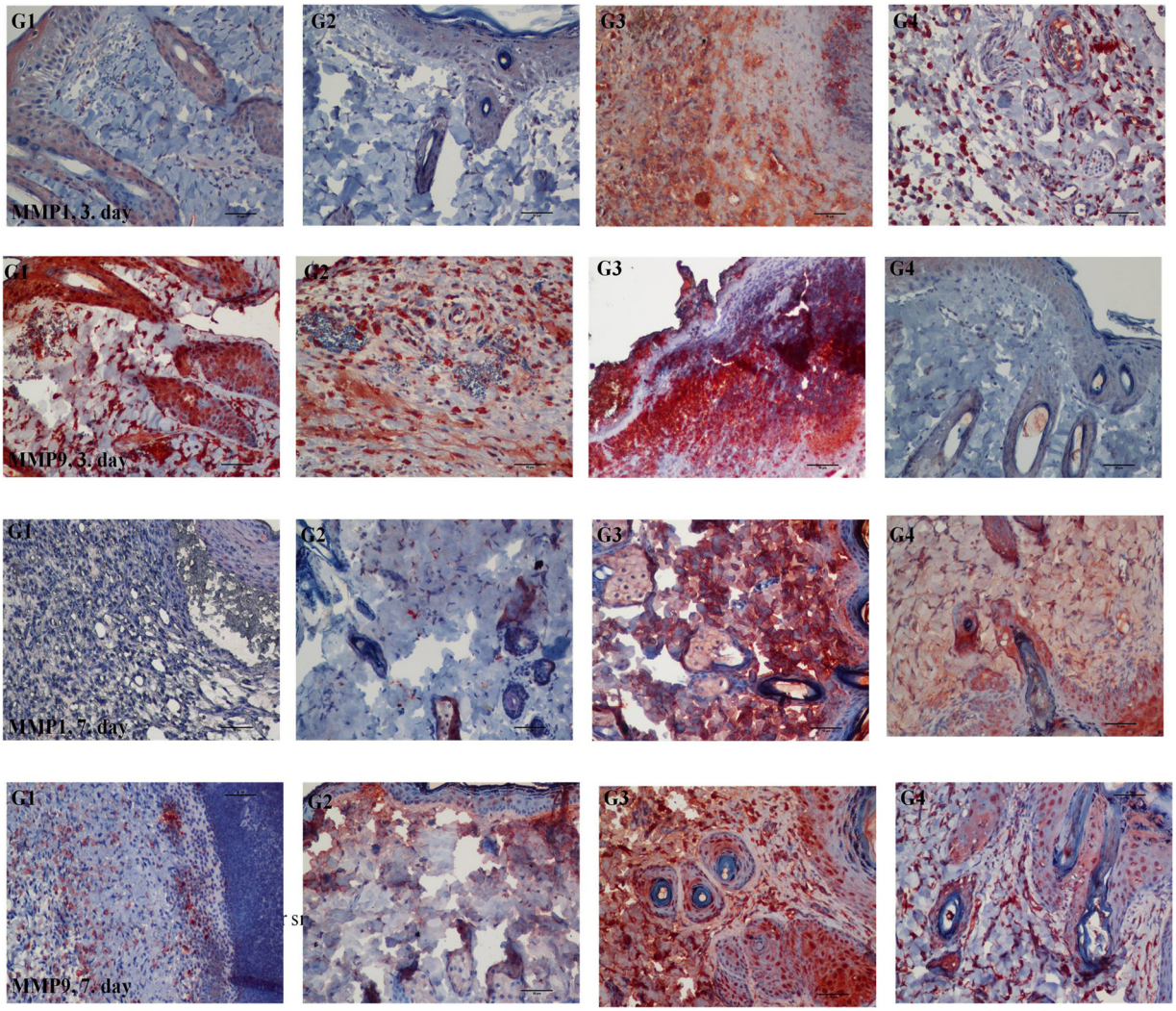

Fig. 6. MMP-1 and MMP-9 expressions in all groups on day 3 and 7 (G1: negative control; G2: 1% folinic acid; G3: 2.5% folinic acid; G4: positive control).

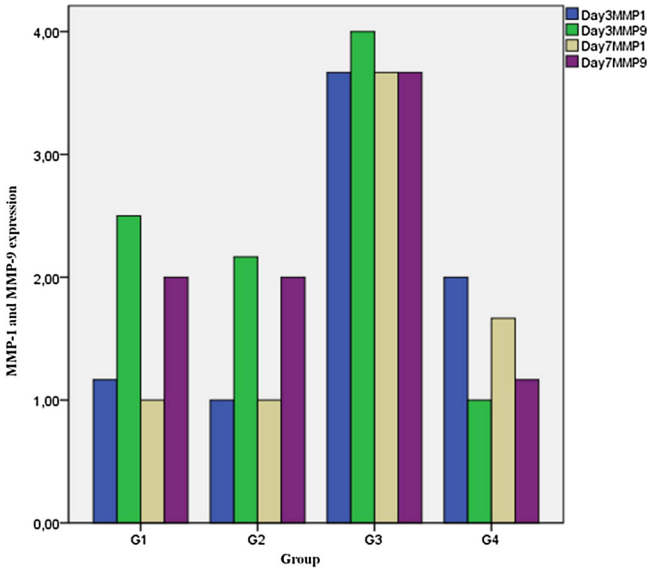

Fig. 7. Comparison of MMP-1 and MMP-9 expressions between study groups on day 3 and 7 (G1: negative control; G2:1% folinic acid; G3: 2.5% folinic acid; G4: positive control).

distribution in all groups on day 3, however, on day 7 and 14, folinic acid-treated groups had significantly progressive mast cell increase compared to negative and positive control groups (Fig. 5). Despite progressive mast cell increase in both folinic acid groups, 2.5% folinic acid group had higher mast cell numbers and better tissue regeneration as compared to 1% folinic acid group

In addition, MMP-1 and MMP-9 expressions on day 3 and 7 were significantly higher in 2.5% folinic acid group compared to other groups (Figs. 6 and 7).

4. Discussion

Wound healing is a dynamic and complex process consisting of four overlapping phases: coagulation, inflammation, cellular proliferation, and matrix formation and remodeling, occurring spontaneously in response to injury [6,7].

Skin fibroblasts and the dermal collagen network which form the extracellular matrix play a major role in the skin wound repair process. Beneficial actions of folic acid on the skin fibroblasts and collagen neosynthesis have been previously reported [2]. Folic acid stimulates DNA-repair capacity of dermal fibroblasts and intracellular folic acid uptake increases in both dermal fibroblasts and epidermal cells after UV radiation to protect the skin cells from UV-induced damage [2,3].

In their study, Fischer et al. [1], evaluated the effects of topical folic acid- and creatine-containing formulation on skin firmness, collagen

gene expression, procollagen synthesis, collagen fibril organization and collagen density using cutometric analysis, multiphoton laser scanning microscopy and cultured fibroblasts. The authors found that the folic acid- and creatine-containing formulation significantly improved skin firmness in vivo. In addition, treatment of fibroblasts with folic acid and creatine improved the collagen metabolism by increasing collagen gene expression, procollagen levels, and collagen fiber density. In the present study, we showed that topical 2.5% folinic acid enhanced wound healing process. The 2.5% folinic acid-treated wounds had significantly accelerated tissue regeneration by increased reepithelialization, neovessel formation, inflammatory cell migration and collagen deposition. In addition, collagen fiber density was higher and fiber distribution and scar formation were more regular in 2.5% folinic acid group as compared to the remaining groups. This finding was in consistence with previous studies reporting improvement of collagen metabolism by folic acid.

To elucidate the potential mechanisms of better wound healing in the folinic acid-treated group, we also analyzed mast cells and MMP-1 and MMP-9 expressions in wounds. Our present study findings reveal that 2.5% folinic acid-treated group show progressive increase in mast cells, enhanced collagen deposition and scar formation, MMP-1 and MMP-9 expressions as compared to other groups. Enhanced collagen deposition by folinic acid may be explained by direct effects of folic acid on fibroblasts as previously mentioned in the introduction section. However, the progressive increase in mast cells in folinic acid-treated groups may also be a contributing factor to accelerated wound healing. In recent years, the role of mast cells in wound healing gained attention, and literature data shows that activated mast cells enhance acute inflammation, stimulate keratinocytes aiding in reepithelialization, activate endothelial cells causing neoangiogenesis and also support the cellular proliferation and migration in the skin by producing cytokines and growth factors. Moreover, several studies have shown that mast cells promote scar formation by stimulating fibroblasts and affecting collagen maturation and remodeling [7,8]. It has been reported that wound healing in mast cell-deficient mice is partially impaired, whereas high number of mast cells have been associated with hypertrophic scars, keloids and fibrotic skin and internal organ diseases [9,10]. Direct mast cell-fibroblast interactions or several pro-fibrotic mediators released from activated mast cells have been suggested to be possible mechanisms by which mast cells stimulate fibroblasts [7,11,12].

It's clearly known that MMPs and their inhibitors play an important role in regulating extracellular matrix degradation and deposition that is essential for wound healing, both in acute and chronic wounds [13]. MMPs regulate cell-cell and cell-matrix signaling through the release of several cytokines and growth factors and can control inflammation in the wound area [13]. Especially MMP-1, MMP-3, and MMP-9 are the major cytokine regulators during wound healing [13]. In the present study, we showed higher expressions of MMP-1 and MMP-9 in the 2.5% folinic acid group, which were in correlation with better macroscopic and microscopic wound healing in that group [13].

5. Conclusions

In conclusion, 2.5% topical folinic acid enhances wound healing in rats. Improved collagen synthesis, increased MMP-1 and MMP-9 expressions and progressive mast cell increase due to folinic acid all seem to play a role in this process.

Conflict of interests

The authors declare no conflict of interests

Financial disclosure

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Author contributions

Study Design: Nilay Duman, Reşat Duman, Oğuzhan Alagöz.
Data Collection: Nilay Duman, Reşat Duman, Murat Tosun, Murat Akıcı, Engin Göksel.
Statistical Analysis: Nilay Duman, Murat Tosun, Barış Gökçe.
Data Interpretation: Nilay Duman, Reşat Duman, Murat Tosun, Murat Akıcı, Engin Göksel, Barış Gökçe.
Manuscript Preparation: Nilay Duman, Reşat Duman.
Literature Search: Nilay Duman.
Funds Collection: Nilay Duman.

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