10.21608/avmj.2025.336860.1474 Assiut University web-site: www.aun.edu.eg

IMMUNOHISTOCHEMICAL ASSESSMENT OF VARIOUS CONCENTRATIONS OF TOPICAL VITAMIN C SOLUTION ON CUTANEOUS WOUND HEALING IN RABBITS

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Received: 9 December 2024; **Accepted:** 19 March 2025

ABSTRACT

Vitamin C, a water-soluble antioxidant, is important in almost every phase of wound healing. However, oral routes provide some obstacles to delivering vitamin C to the skin, such as absorption limits and issues in achieving adequate bioavailability. Consequently, the current study aimed to evaluate the effectiveness of various dosages of topically applied vitamin C solution on intentional skin wound healing. 18 male albino rabbits were divided into three groups according to the time of euthanasia. Each rabbit had four full-thickness excisional skin wounds on its dorsum; one was left untreated, the second had ultrasound gel applied to it, the third had 5% topical vitamin C, and the fourth had 10% vitamin C (both covered by ultrasound gel). The animals were euthanized after 24 hours, 3 days and 7 days and MMP-9 was used for immunohistochemical analysis. The group that received 10% vitamin C had significant increases in MMP-9 expression on the first and fifth days, while the control group and the ultrasonic gel group both displayed a moderate increase. According to the study, using vitamin C topically accelerates the healing of skin wounds; higher concentrations (10%) have more beneficial effects.

Keywords: Vitamin C, ascorbic acid, skin wound healing, Immunohistochemistry, MMP-9.

INTRODUCTION

The skin, the largest organ in the body, accounts for approximately 16 % of the total body weight. It is essential for maintaining homeostasis and for the defense system it creates against external stimuli (Diaz-Garcia *et al.*, 2021). The epidermis provides structure, immunity, and nutrition to the integumentary system. Sweat, sebaceous, and hair follicles are in the dermis, which hascategories micro element and macro higher concentrations of vascular, mechano-receptor, and extracellular matrix (ECM). Whereas growth hormones and energy are obtained from subcutaneous adipose tissue (Leclere, 2017).

A wound is known as a break in the skin's or mucosa's epithelial lining brought on by physical or thermal trauma, which may result in either temporary or permanent malfunction (Qi *et al.*, 2022). The process of cutaneous wound healing is a highly structured and dynamic one that consists of several linked and overlapping stages (Baron *et al.*, 2020). In order to achieve wound closure and maintain one's quality of life, skin healing from wounds is crucial. Numerous cell types

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and mediators are included, and their functions are extremely complex (Sorg *et al.*, 2017).

In skin wound healing therapies and the management of severe and chronic wounds, there remain unmet needs, despite much research undertaken in these areas. The principal causes of these problems are inaccuracies in wound assessment and inefficient care management. Therefore, the discovery of improved and innovative methods for treating skin wounds has significant global medical significance (Armstrong *et al.,* 2020).

Vitamin C or ascorbic acid (AA), among the vitamin family members, is most familiar in the population, and functional foods and supplements are easily accessible. Water-soluble vitamin C is a powerful antioxidant and a reducing agent essential to many biological functions, and found to have anti-scurvy properties (Gandhi *et al.*, 2023, Ellulu *et al.,* 2015). When vitamin C is taken orally, insufficient amounts reach the skin, preventing proper bioavailability. As a result, taking ascorbic acid orally is not recommended for delivering the required amount to skin and other peripheral structures. Application, either local or topical, is a crucial way to supply AA to the skin (Padayatty *et al.,* 2010). Vitamin C has a major effect on the healing process at every stage. It facilitates the synthesis and maturation of collagen during the proliferative phase, and the removal of neutrophils during the inflammatory phase (Mandi *et al.,* 2019).

Many biological processes, including tissue regeneration and wound healing, are significantly impacted by matrix metalloproteinases like MMP-9, which are zinc-dependent endopeptidases (Van den Steen *et al.,* 2002). Protease hydrolysis can be used to alter the structure of extracellular matrix (ECM) by cleaving and degrading specific ECM proteins and non-ECM molecules (Reinhard *et al.,* 2015). Therefore, the current study aimed to evaluate the effectiveness of topically administered vitamin C solutions in several dosages on intentional skin wound healing, and to determine the precise dosage of vitamin C that has the greatest healing impact on the wound.

MATERIALS AND METHODS

The study was approved by our institution's research ethics committee under approval number (UoM. Dent. 24/1016). It involved 18 male albino rabbits of 6 months old, weighed between 0.5 and 1.5 kg, and divided into three groups of six rabbits each according to time of euthanasia 24 hours, the 3rd day and after the 7th day. Every rabbit was housed in identical environmental and dietary settings and kept in individual cages with free access to water and food (grain, vegetables, fruit) and a temperature of (15-28°C). The cages were cleaned regularly, and rats' health was monitored by a veterinarian (Shawky and Hassouna, 2021).

Dose preparation

The 500 mg/5 ml vitamin C container (Redox-c® 500mg/5ml (Bayer) made in Turkey) was used to prepare the 10% vitamin C solution (1) ml^3 was withdrawn from the ampule and diluted with (9) ml^3 of sterile distilled water. The 5% vitamin was prepared by withdrawing (0.5) ml^3 of the ampule and diluted with (9.5) ml^3 of sterile distilled water (Pathy, 2018).

Surgical Procedure

Under sterile circumstances and general anesthesia, the study was carried out. Subsequent intraperitoneal injections of xylazine® (5 mg/kg), a sedative and analgesic solution, and ketamine® (ketamine hydrochloride) 50 mg/kg, a general anesthetic drug, were used to sedate the animals (Naser, 2021).

Each rabbit would have (4) wounds on its dorsum, each of them would be square 1cm in width and length and would be full thickness (till we reach the fascia), the

wounds would be 1 cm apart from each other (horizontally and vertically) the four wounds would be treated as follows:

1. The first wound was controlled without any interfering, labeled Control.
2. An ultrasound gel only covered the second wound, labeled G0.
3. The third wound was treated with a 5% vitamin C solution, and then covered by an ultrasound gel, labeled G1.
4. The fourth wound was treated with 10% vitamin C solution, and then covered by an ultrasound gel, labeled G2.

Every (24) hours, the gel is washed off carefully with normal saline, then the vitamin C solution is reapplied. The use of ultrasound gel is important to keep the vitamin C solution on the wound, and a normal saline solution was used to remove the gel. Following the euthanasia of rabbits, autopsies were carried out right away, and the entire incision site was included in the tissue sample, which was prepared for immunohistochemical analysis. After being fixed in formalin, the specimens were prepared for paraffin sections. Blocks of tissue were sectioned at a thickness of 5 µm on charged glass. Before performing immunohistochemistry, each section was deparaffinized and hydrated using a series of xylene and ethanol concentrations, reducing it to deionized water (Ni *et al.*, 2021). Immunostaining for MMP-9: All sections were rinsed with deionized water after antigen retrieval. Hydrogen peroxide 3% for 10 minutes to quench endogenous peroxidase. To reduce the quantity of nonspecific background staining, Slides were exposed to 3% normal goat serum (DAKO) for 20 minutes, and avidin and biotin blocking solutions (biotin from DAKO and streptavidin from Jackson Immuno Research,

After being cleaned with TBST, sections were biotinylated for 30 minutes at room temperature using goat anti-mouse IgG. Following Streptavidin-HRP (DAKO) incubation for 30 minutes at room temperature, the sections were counter-stained with hematoxylin and chromogen-stained with diaminobenzidine (EnVision FLEX DAB+ Chromogen (DM827, DAKO) (Singh *et al.,* 2019). MMP9 protein kit obtained from (My BioSource company, San Diego, California, USA).

To avoid bias, three histopathologists who are only given the codes for each group evaluated blindly all slides. The evaluation of staining intensity was performed by a light microscope with visual analysis (McCarty *et al.*, 1986). The slides are inspected at x400 magnification using a light microscope linked to a digital camera.

Immunohistochemical scoring criteria

The cytoplasm was shown to have brown granules upon positive immunostaining. On a semi-quantitative assessment scale, the intensity and proportion of positively stained cells were used to rate the MMP 9 immunostaining. The following was used to score the MMP 9 cytoplasmic staining intensity: (Liu *et al.*, 2019).

Table 1: criteria for immunohistochemistry

Score	Dye intensity
Score 0	none
Score 1	Weak
Score 2	Moderate
Score 3	Strong

Data analysis

Data was analyzed by IBM SPSS Statistics version 25 and Microsoft Excel 2016 as there was a non-normal distribution of the study data (Shapiro-Wilk test, $p<0.05$), and the median, as well as the interquartile range (IQR), were used. For comparisons, non-parametric tests were also used.

Mann-Whitney U Test was used to compare between two groups, and the Kruskal-Wallis Test was employed to compare among groups on the same day. It has been determined that a P-value of 0.05 or less is statistically significant.

RESULTS

All rabbits in this study remained through the experimental process without any problems, and none experienced any post-operative consequences, such as wound infection or bleeding. After 24 hours, both control and G0 showed weak positive reactions for MMP9, with a median and interquartile range (IQR) of 1 (0.25). In the G1 group, there was moderate positive reaction expression for MMP9 with a median and interquartile range (IQR) of 2 (0.25), and in the G2 group, the sections revealed a highly positive reaction with a median and interquartile range (IQR) of 2.5 (1), as seen in Figure (1 A-D). The median and interquartile range (IQR) for the control and G0 on day three were 1 (1) and 1.5 (1), respectively, indicating a mild positive reaction for MMP9. The sections of G2 showed a highly positive reaction with a median and interquartile range (IQR) of 3 (1), as shown in Figure (2 A-D), whereas the G1 group showed moderately positive expression for MMP9 with a median and IQR of 2.5 (1).

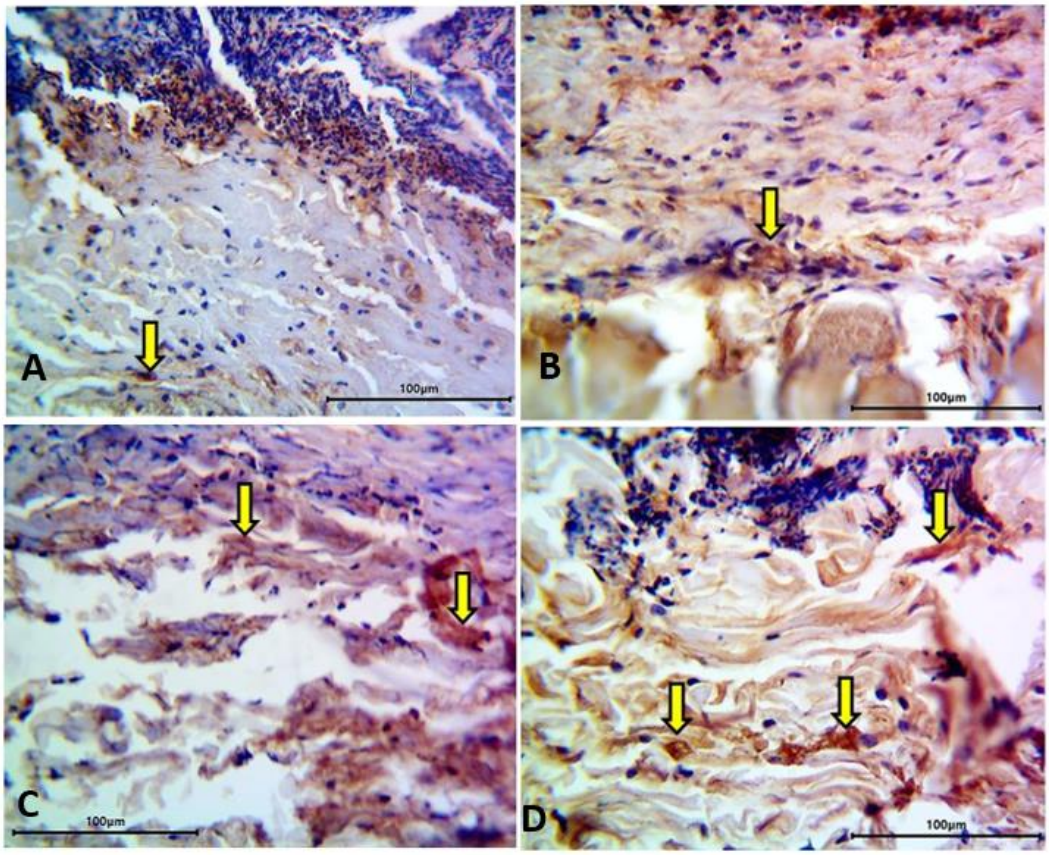

Figure (1): Immunohistochemical expression of the MMP9 in the skin of rabbit from
(A) The control group at Day 1 revealed weak positive reaction (arrow) (score 1+).
(B) The G0 group at Day 1 revealed weak positive reaction (arrow) (score 1+).
(C) The G1 group at Day 1 revealed moderate positive reaction (arrow) (score 2+).
(D) The G2 group at Day 1 revealed highly positive reaction (arrow) (score 3+).

On day seven, the control group showed a moderate positive reaction for MMP9, with a median and interquartile range (IQR) of 2 (1). Furthermore, the specimens in the G0 group showed a moderate positive reaction, with a median and interquartile range (IQR) of 2 (1). MMP9 expression was strong in both G1 and G2 groups, as shown in Figure (3 A-D), with a median and interquartile range (IQR) of 3 (0). Immunohistochemical findings, which highlight variations among the groups, are briefly presented in Table (2).

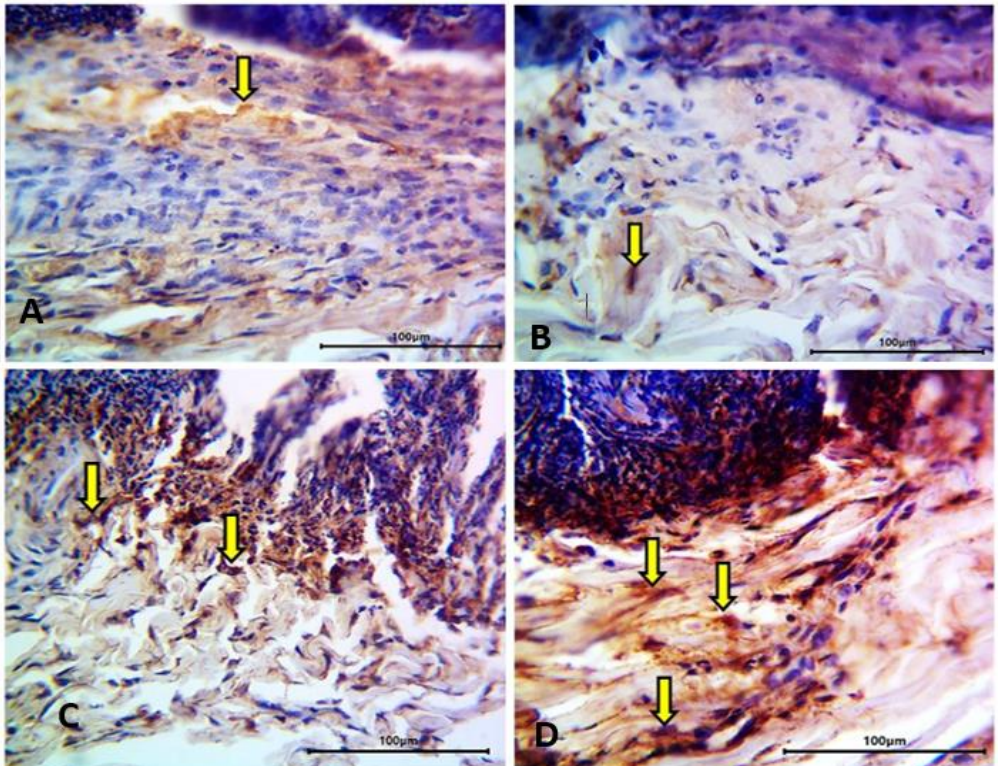

Figure (2): Immunohistochemical expression of the MMP9 in the skin of rabbit from
(A) The control group at Day 3 revealed weak positive reaction (arrow) (score 1+).
(B) The G0 group at Day 3 revealed weak positive reaction (arrow) (score 1+).
(C) The G1 group at Day 3 revealed moderate positive reaction (arrow) (score 2+).
(D) The G2 group at Day 3 revealed highly positive reaction (arrow) (score 3+).

Table 2: The Scores of the MMP9 at study periods.

Periods	Median and interquartile range (IQR) for control Group.	Median and interquartile range (IQR) for G0.	Median and interquartile range (IQR) G1.	Median and interquartile range (IQR) G2.
Day 1	1(0.25)	1(0.25)	2(0.25)	2.5(1)
DAY 3	1(1)	1.5(1)	2.5(1)	3(1)
DAY 7	2(1)	2(1)	3(0)	3(0)

All scores appear as Median and interquartile range (IQR)

MMP-9 Using Kruskal-Wallis Test:
Comparing MMP-9 (Matrix Metallo-proteinase-9) between the groups showed that on day one, day three (P-value of 0.003), and day seven (P-value of 0.009), there was a statistically significant difference in MMP-9. According to these findings, the treatments (G1 and G2) had a positive impact on raising MMP-9 levels, compared to the control group, which may have improved the healing process and decreased tissue degeneration throughout the course of three days. All scores appear in Table (3).

Table 3: Comparison of MMP9 for skin among groups within same day.

Group	DAY 1 MMP9	DAY 3 MMP9	DAY 7 MMP9
Control	0.003*	0.003*	0.009*
G0			
G1			
G2			

Kruskal-Wallis test t was used to compare among groups. (*)Significant difference at p≤0.05

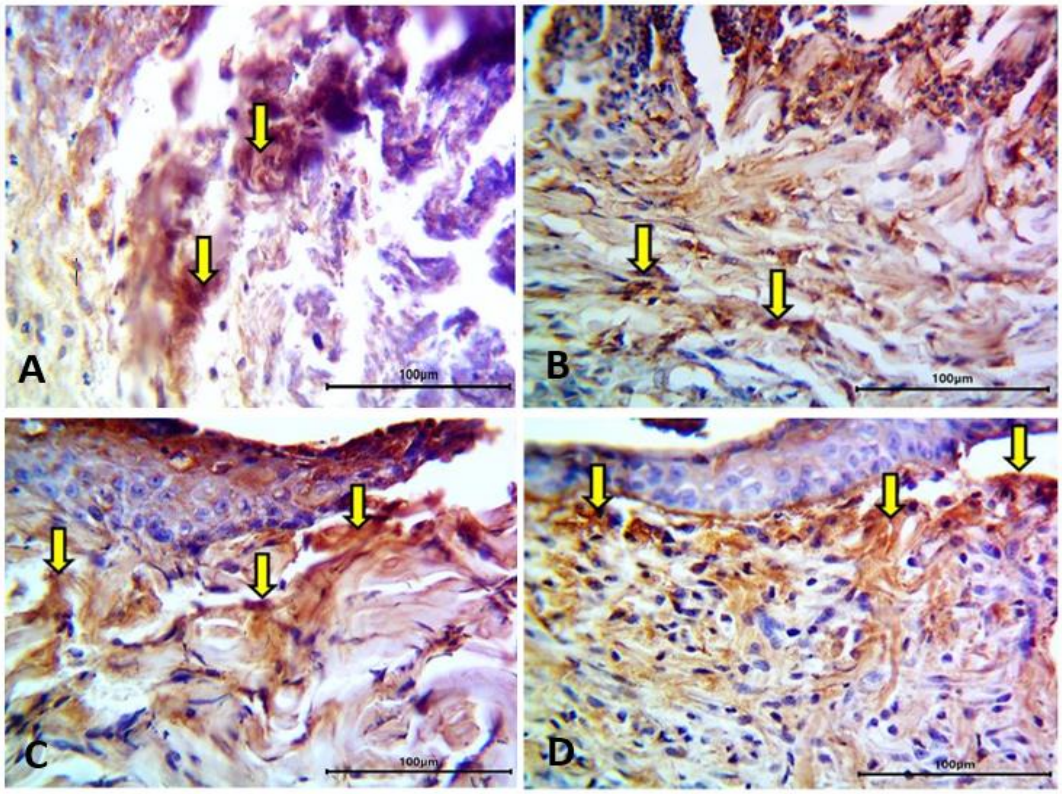

Figure (3): Immunohistochemical expression of the MMP9 in the skin of rabbit from
(A) The control group at Day 7 revealed moderate positive reaction (arrow) (score 2+).
(B) The G0 group at Day 7 revealed moderate positive reaction (arrow) (score 2+).
(C) The G1 group at Day 7 revealed highly positive reaction (arrow) (score 3+).
(D) The G2 group at Day 7 revealed highly positive reaction (arrow) (score 3+).

MMP-9 Using Mann-Whitney U Test:

The MMP-9 comparison using the Mann-Whitney U Test revealed no statistical differences between the control and G0 across all study periods. On days 1 and 3, there were statistical differences between the control and G1, and on day 7, there were no differences. G0 displayed significantly different results, compared to G1 or G2, on Day 1 (P-value = 0.041, 0.004) and Day 3 (P-value = 0.026, 0.015).

In all research periods, there were no statistically significant changes between G1 and G2. According to these findings, the treatments, in particular the gel containing 10% vitamin C (G2), were successful in raising MMP-9 levels, compared to the control group on days one and three. However, on Day 7, the differences were not statistically significant. All scores are seen in Table (4).

Table 4: Comparison of MMP9 (Mann-Whitney U Test: Comparison of Group Vs Group (same day).

Group	DAY 1 MMP9	DAY 3 MMP9	DAY 7 MMP9
Control vs G0	1.000	0.690	1.000
Control vs G1	0.041*	0.015*	0.065
Control vs G2	0.004*	0.009*	0.065
G0 vs G1	0.041*	0.026*	0.065
G0 vs G2	0.004*	0.015*	0.065
G1 vs G2	0.240	0.699	1.000

Mann-Whitney U test was used to compare between groups. (*) Significant difference at $p \leq 0.05$.

DISCUSSION

Studies have shown that applying vitamin C topically enhances the healing of surgical wounds and encourages tissue reconstruction (Ravetti *et al.,* 2019, Gref *et al.,* 2020). MMP-9 is a significant protease necessary for several biological functions, including wound healing (Zariffard *et al.,* 2015).

We found that on day one there was a statistically significant difference between groups, with a higher median score in the G2 group. Most of the G2 group had strong staining, while weak staining was seen in the control group. This may be because of the ability of vitamin C to hasten the healing process by increasing the inflammatory infiltrate. When tissue injury occurs, MMP-9 represents the early inflammatory response (Gill *et al.,* 2008). Our results go with a previous study, which determined that this signaling protease plays a crucial role in wound healing and can control the inflammatory response (Nagy *et al.*, 2015).

Our findings are consistent with a study that found that MMP-9 is an important mediator in the healing process, which was more active during the first 24 hours. MMP-9 is stored by neutrophils and can be rapidly released (Triebel *et al.,* 1995). Following injury, immune cells like monocytes, lymphocytes, macrophages, and dendritic cells, as well as epithelial and endothelial cells, exhibit both local and systemic increases in MMP-9 expression (Vandooren *et al.,* 2013).

In the early inflammatory phase of wound healing, MMP-9 aids in breaking down the extracellular matrix (ECM) components, collagen and elastin, and the removal of injured tissue. As a result, immune cells can get to the wound and initiate the healing process (Larouche *et al.,* 2018).

Since early MMP-9 activity triggers the pro-inflammatory cytokines IL-1β and IL-8, which in turn causes magnified leukocyte inflow and increased inflammation, it would be advantageous during the early inflammatory phase of healing (Van den Steen *et al.,* 2002).

Day three groups revealed a statistically significant difference in MMP-9 gene expression between G2 and control groups, with the G2 group exhibiting a strong stain and most of the control groups exhibiting a weak stain. This is due to MMP-9's role in extracellular matrix breakdown and remolding, which promotes cellular migration. MMP-9 possesses anti-inflammatory properties and regulates inflammatory processes. In experimental animals, their absence resulted in a severe inflammatory reaction, and animals deficient in MMP9 show delayed healing and re-epithelialization (Zhang *et al.,* 2020, kyriakides *et al.,* 2009). Our study agrees with the previous study, which stated that the treatment group showed favorable regulation of fibroblasts, including increasing cell migration and collagen formation coupled with an elevated expression of MMP-9, compared to the control group (Yi *et al.,* 2022). Another study revealed that MMP-9 produced from macrophages enhanced post-MI remodeling via both direct and indirect effects on the healing of heart wounds (Meschiari *et al.,* 2018). Also, the effects of vitamin C on extracellular matrix remodeling in mouse corneal epithelial stem/progenitor cells were examined by Chen and colleagues in their study on the beneficial effect of vitamin C on mouse corneal epithelial wound healing in vivo (Chen *et al.,* 2017). They found that ascorbic acid accelerated corneal epithelial wound healing in vivo. The function of MMP-9 in wound healing is intricate. Its capacity to proteolytically cleave matrix components, growth factors, and cytokines is consistent with its functional complexity (kyriakides *et al.,* 2009).

On day seven, groups didn't demonstrate a statistically significant change in the staining intensity. All the slides in both G2 and G1 groups showed intense staining, while most of the G0 control showed moderate staining. Higher MMP-9 indicates continuous

remolding. Keratinocytes must travel through the extracellular matrix to wounds to heal successfully. The activation of metallo-proteases like MMP-9's gelatinolytic activity facilitates this process. However, these enzymes also drive cell proliferation, downstream cytokines, and growth factor-stimulated signaling pathways (Balaji *et al.*, 2015). MMP-2 and -9 stimulate the migration and proliferation of keratinocytes, fibroblasts, and endothelial cells to promote the formation of new granulation tissue in the final stages of wound healing (Dekoninck *et al.*, 2019). MMP-9 helps in the transporting of keratinocytes and the movement of endothelial progenitor cells. The endothelial mesenchymal transition is initiated by basal keratinocytes at the wound edge, which breaks their hemidesmosome attachment to the basal membrane and their desmosome connection to each other, enabling the cells to start migrating into the temporary matrix to fill the void. Concurrently, the keratinocytes which are still below the edge start to multiply (Rousselle *et al.*, 2019). The overall high staining observed in all groups could be attributed to the normal physiological high matrix metalloproteinases that present at this point of the healing process. Using human tracheal xenografts, the existence of MMP 7 and MMP 9 has been confirmed in naked mice when their expression and activity peaked in well-differentiated regenerated airway epithelium during the final stages of wound healing. Inhibition of these respective MMPs leads to disruption in epithelial cell differentiation. All this data indicates that MMPs are required for wound healing and appropriate epithelial cell differentiation (Coraux *et al.*, 2005).

CONCLUSION

Vitamin C can fasten wound healing, and this property depends on its concentration, as increasing the concentration leading to increased healing viability of the tissue and decreasing the healing time.

ACKNOWLEDGMENT: All authors thank the affiliates of the College of Dentistry at Mosul University.

FUNDING: This research does not have any source of funding.

DATA AVAILABILITY: All the raw data are available when asked.

DECLARATIONS: All Authors declare no conflict of interest

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التقييم المناعي الهستوكيميائي لتراكيز مختلفة من محلول فيتامين ج الموضعي في شفاء الجروح الجلدية في الأرانب

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فيتامين ج، أحد مضادات الأكسدة القابلة للذوبان في الماء، مهم في كل مرحلة من مراحل التئام الجروح تقريبًا. ويواجه تناول الطعام عن طريق الفم بعض التحديات في توصيل فيتامين ج إلى الجلد المتمثل في حد الامتصاص وصعوبة الوصول إلى التوافر البيولوجي الكافي. الهدف: تقييم فعالية محلول فيتامين C المطبق موضعياً بجرعات مختلفة على التئام الجروح الجلدية المتعمدة. المواد والطرق: تم تقسيم ١٨ أرنبًا ألبينو ذكرًا إلى ٣ مجموعات، وكان لكل أرنب ٤ جروح جلدية استئصالية كاملة السُمك على الظهر، واحدة تركت بدون أي إضافة، والثانية تمت تغطيتها بجل الموجات فوق الصوتية، والثالثة تمت اضافة ٥٪ فيتامين ج موضعي والرابع ١٠٪ فيتامين ج (كلاهما مغطى بهلام الموجات فوق الصوتية)، تم الموت الرحيم للحيوانات بعد ٢٤ ساعة للمجموعة الاولى و٣ أيام للمجموعة الثانية و٧ أيام للمجموعة الثالثة، وتم استخدام MMP-9 في التقييم المناعي الهستوكيميائي للانسجة. النتائج: في اليوم الأول والخامس، كانت هناك زيادة كبيرة في تعبير MMP-9 في المجموعة التي تلقت ١٠٪ من فيتامين C افضل من تركيز ٥٪، في حين أظهرت كل من المجموعة الضابطة ومجموعة الهلام بالموجات فوق الصوتية زيادة معتدلة. الخلاصة: أثبتت الدراسة أن فيتامين ج يساعد على شفاء جروح الجلد بشكل أسرع عند استخدامه موضعياً، وتظهر النتائج الأفضل عند استخدام التركيز الأعلى (١٠٪).

الكلمات المفتاحية: فيتامين ج، حمض الأسكوربيك، التئام جروح الجلد، الكيمياء المناعية، MMP-9.