The Impacts of Hybrid and Hyaluronic Acid Fillers on Skin Regeneration Post-Operatively

Original Article

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ABSTRACT

Introduction: Cells, growth factors, and cytokines interact uniquely during skin wound healing. Long-term comfort and reducing hospital costs and scars are being examined in wound treatments.

Aim of the Work: To compare the efficacy of Hybrid filler (hyaluronic acid +calcium hydroxylapatite) and hyaluronic acid filler on skin healing after induced incisions and evaluate the expression of cluster of differentiation 31 (CD31), matrix metalloproteinase-9 (MMP-9), wound healing biomarkers, using immunohistochemistry.

Materials and Methods: Three sets of three male Albino rats were randomly allocated to the 3rd, 7th, and 14th healing periods. Rats have three circular dorsal incisions. The top wound got hybrid filler (NEAUVIA STIMULATE®), the bottom right wound received hyaluronic acid filler (TEOSYAL RHA® 3), and the lower left wound was uninjected as a control. Biopsies were taken from each rat on the 3rd, 7th, and 14th days to determine the expression of CD31 and MMP-9 using immunohistochemistry.

Results: The immunohistochemical analysis of MMP-9 demonstrated that both treatment groups showed high expression of MMP-9 throughout the whole period in contrast to the control group which revealed weak expression. Regarding CD31, the highest scores were for the hybrid filler group more than HA and control groups on all days. However, median scores of CD31 were higher in the HA group compared to the control group on the third and seventh days. Re-epithelialization on the fourteenth day was highest in the hybrid filler group followed by the HA group, and the control group was the lowest.

Conclusion: Rat skin healed faster with hybrid and HA fillers, they may enhance wound healing.

Received: 31 July 2023, **Accepted:** 13 September 2023

Key Words: Hybrid fillers, hyaluronic acid fillers, skin healing.

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ISSN: 1110-0559, Vol. 47, No. 3

INTRODUCTION

Tissue healing after injury is the result of a coordinated set of cellular and molecular actions. When tissues are harmed, a series of reactions are triggered, starting with inflammation and continuing with the production and remodeling of new tissues. Platelets and damaged blood vessels create chemicals and growth factors that go to work immediately to start the healing process. Once a blood clot has formed, inflammatory cells are able to enter the damaged area and defend it from further infection. During the proliferative phase, dermal fibroblasts multiply all around the wound site after keratinocytes have migrated and multiplied along the wound margin. Extracellular matrices are then rapidly produced by the fibroblasts. While the wound is still in the proliferative phase, granulation tissue is created, earning its name from the granular appearance caused by the presence of newly developed capillaries. Scar tissue is considered mature once collagen has been repeatedly generated and destroyed. Scar tissue is weak due to its absence of epidermal appendages[1,2].

Non-branched hyaluronic acid (HA) is a biopolysaccharide with a high molecular weight. This natural linear dipolysaccharide has β-(1,4)-linked D-glucuronic acid and β-(1,3) N-acetyl-D-glucosamine units. It's a polyanionic polymer with unique physicochemical and biological properties. HA was chosen as a polymer because it is biological, endogenous, and natural. HA is used in ophthalmic surgery, arthritis treatment, polymeric scaffolds for wound healing, tissue engineering, cartilage regeneration, drug delivery, and more recently medication delivery[3].

Animal research used the HA because it accelerates healing by promoting re-epithelization, which creates elastic tissue and increases microvascular density in full-thickness surgical wound models[4]. Hyaluronic acid aids tissue regeneration, inflammatory response, and angiogenesis during wound healing[5].

Dermal fillers may reduce facial lines and wrinkles. Most wrinkle fillers are transitory since the body absorbs

DOI: 10.21608/ejh.2023.226217.1929

them. Hyaluronic acid injectable fillers are the most popular because they are safe, effective, may be undone, and provide long-lasting, natural-looking results with low recovery time. Cross-linking HA chains with 1,4-butanediol diglycidyl ether creates these fillers[6].

Artificial calcium hydroxylapatite microspheres in an aqueous carrier gel make up the biodegradable filler. At the injection site, the soluble carrier gel uniformly distributes and spaces the microspheres. After gel absorption, microspheres induce local collagen synthesis[7].

Hybrid filler reinforcement mixes two or more fillers in a composite for surprising outcomes. 'Hybrid' means a multifaceted thing. This category's fillers differ chemically and physically. Many clinicians inject both HA and CaHA fillers at the same time to take advantage of their synergistic benefits[8].

MATERIALS AND METHODS

Experimental Model

The research used nine 4-6-month-old albino rats weighing 250-300 grams[9]. Three rats each group were randomly assigned to three healing periods (3rd, 7th, 14th[10]. The animals were fed water and commercial feed (grain, fruit, and vegetables) and lived at the animal house in special cages at temperatures (18-22 °C). All animals' experiments followed up the animal care protocol to prohibit any health problems and to enhance efficiency.

Surgical Procedure

Anesthetic Method

Ketamine® 50mg/kg and xylazine® 5mg/kg intraperitoneal injections sedated the animal[11].

Surgical Method

1. The rat was anesthetized and shaved using scissors and an electric hair clipper. After that, the surgical site was disinfected with 10% povidone-iodine.

2. Each rat's dorsum was marked with three circular sections. Wounds were 1.5 cm wide and 4 cm apart. One wound (upper wound) was selected for injection with hybrid filler (NEAUVIA STIMULATE®), another one (lower right) with HA filler (TEOSYAL RHA® 3), and the control site (lower left) was left uninjected.

3. Wound creation: Blade no.15 was used to make 1.5cm circular incisions on the designated skin, maintaining the underlying muscle layer.

4. The upper wound was immediately injected with 0.1ml of NEAUVIA STIMULATE dermal filler (HA+ CaHA) in the periphery of the circle (intradermal)[12]. The lower right wound was injected with 0.1ml of TEOSYAL RHA® 3 hyaluronic acid dermal filler. The control wounds were left uninjected.

5. After skin removal, the surgical site was compressed with a compression bandage and cleansed with normal saline and wrapped to avoid infection. To avoid wound contamination, the animals were housed in separate cages (Figure 1).

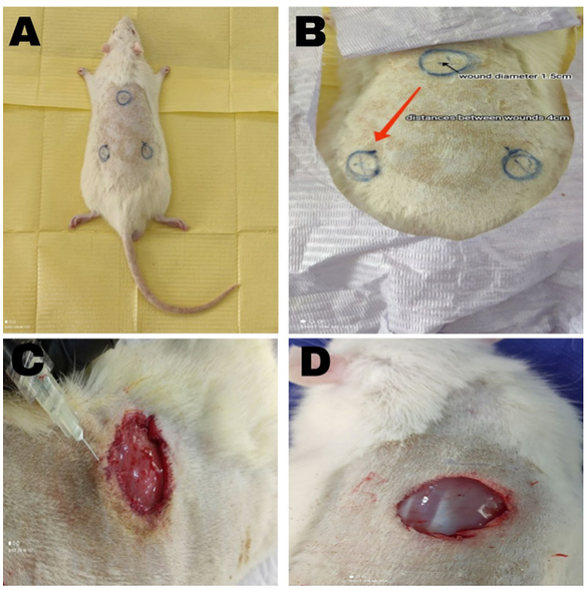

Fig. 1: Surgical procedure. A, B: shaving the area and markings the circles, C, D: wound creation and filler injection

Biopsy collection

On the 3rd, 7th, and 14th days of investigation, three rats in each group had excisional biopsies. The rat wounds were biopsied.

Skin samples were stored in sterile containers with 10% formalin solution and transmitted for immunohistochemistry at given intervals.

RESULTS

All slides were blindly examined by three qualified histopathologists who received only the codes for each group to prevent any bias.

In our study`s sections, group (A) refers to hybrid filler (HA+CaHA), group (B) for hyaluronic acid (HA) filler, and group (C) refers to the control group.

Immunohistochemical Expression of CD31

Third Day

The hybrid filler group showed moderate positive expression (++) of CD31 at the wound site. While the HA group revealed weak positive expression (+) of CD31 to immunohistochemistry. On the other hand, the control group viewed negative expression to the test (-) at this period, (Figures 1,2, Table 2).

Seventh Day

The hybrid filler slides revealed intense positive expression of CD31 (+++), while HA filler group showed

moderate positive expression (++) at wound site. Control group during this period illustrated weak positive expression (+) of CD31 to immunohistochemistry, (Figures 2,3, Table 2).

Fourteenth Day

In the hybrid filler group, there was intense positive expression (+++) of CD31 at wound site. While for HA and control groups, there were moderate positive expressions (++) of CD31 during the same period, (Figures 3,4, Table 2).

Immunohistochemical Expression of MMP9

Third Day

The hybrid and HA filler groups demonstrated moderate positive expression (++) of the MMP9 to immunohistochemistry. On the contrary, there was weak expression of the MMP9 (+) in the control group, (Figures 4,5, Table 2).

Seventh Day

Both the hybrid filler and HA groups showed intense expression of MMP9 (+++) at wound site. The control group also showed weak expression of MMP9 (+) to immunohistochemistry, (Figures 5,6, Table 2).

Fourteenth Day

The hybrid filler group demonstrated intense expression (+++) of the MMP9 to immunohistochemistry. The HA group also illustrated strong positive expression of the MMP9 (+++). On contrast, there was weak expression of the MMP9 (+) in the control group, (Figures 6,7, Table 2).

Skin Histology Findings of Fourteenth Day

Inflammatory Cells Infiltration

The hybrid filler and HA filler slides revealed no inflammatory cells infiltration. The control group demonstrated scanty inflammatory cells infiltration, (Figures 7,8, Table 1).

Granulation Tissue Formation

The amount of granulation tissue formation in the wound is scanty in hybrid filler and HA filler groups during two weeks with newly formed blood vessels (angiogenesis). The control group demonstrated well developed granulation tissue, (Figures 7,8, Table 1).

Re-epithelialization

In hybrid filler group, re-epithelialization covered the entire wound with regular thickness. HA specimens showed well developed re-epithelialization covered the whole wound with irregular thickness. The control group revealed re-epithelialization covering more than half of the wound, (Figures 7,8, Table 1).

Statistical Analysis of Histological Findings

In this study, we used Mann-Whitney U test for comparison between two groups within the same day, (Tables 3,4,5).

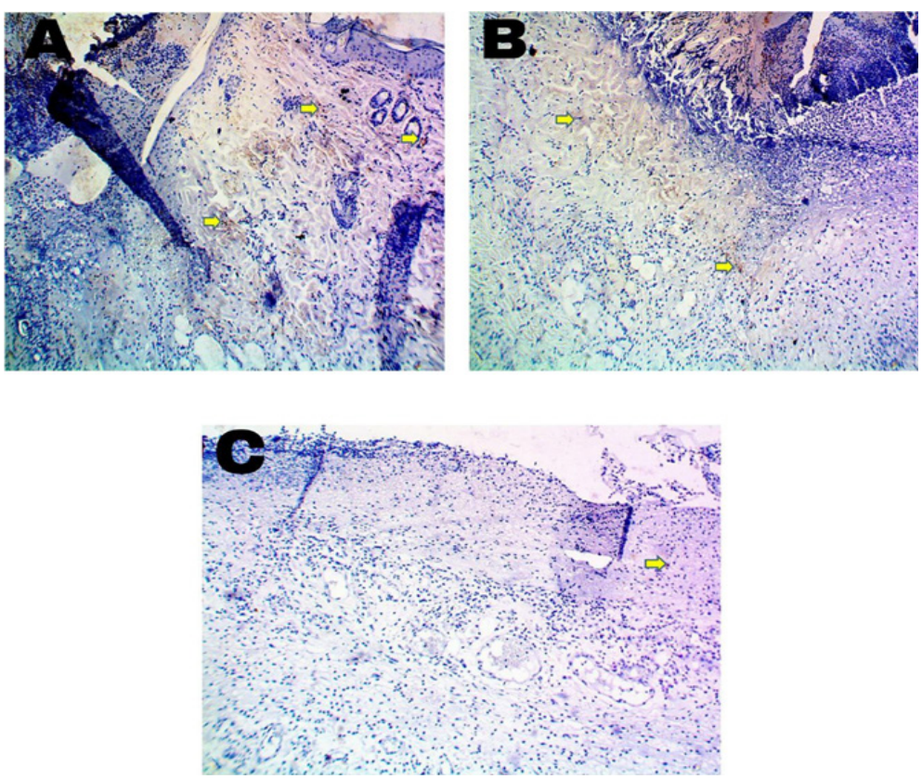

Fig. 2: Immunohistochemistry expression of the CD31 (3 days). Hematoxylin stain, 100X. A (hybrid filler) showed moderate expression, B (hyaluronic acid filler) showed mild expression, and C (control) showed weak expression.

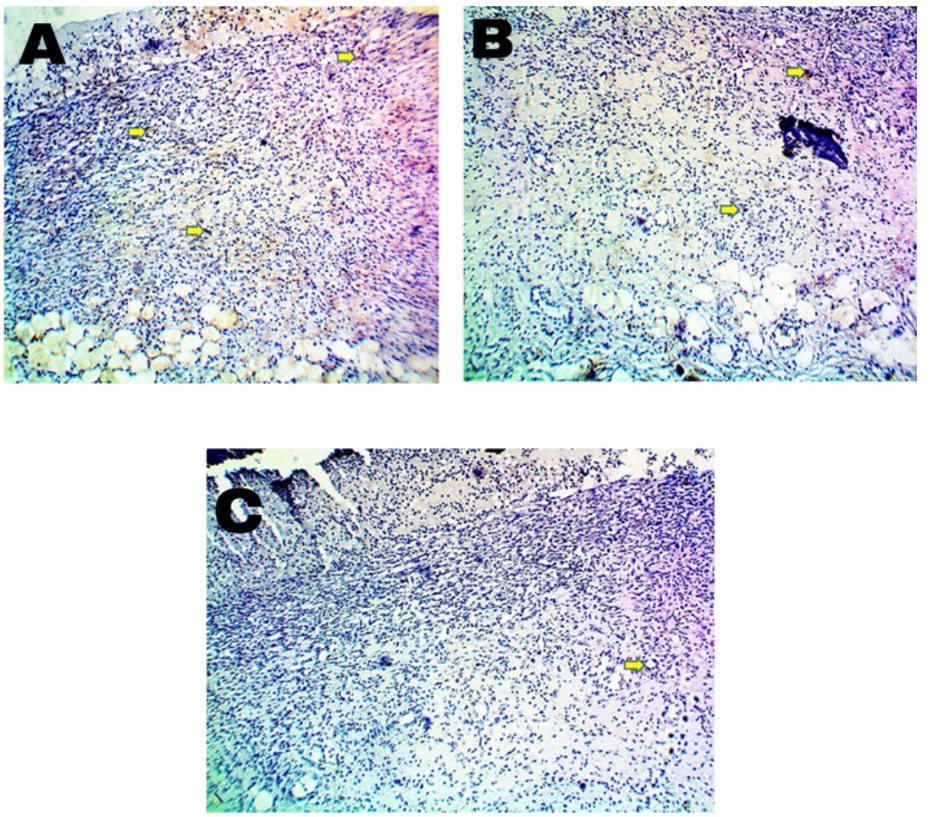

Fig. 3: Immunohistochemistry expression of the CD31 (7 days). Hematoxylin stain, 100X. A (hybrid filler) showed intense expression, B (hyaluronic acid filler) showed moderate expression, and C (control) showed mild expression.

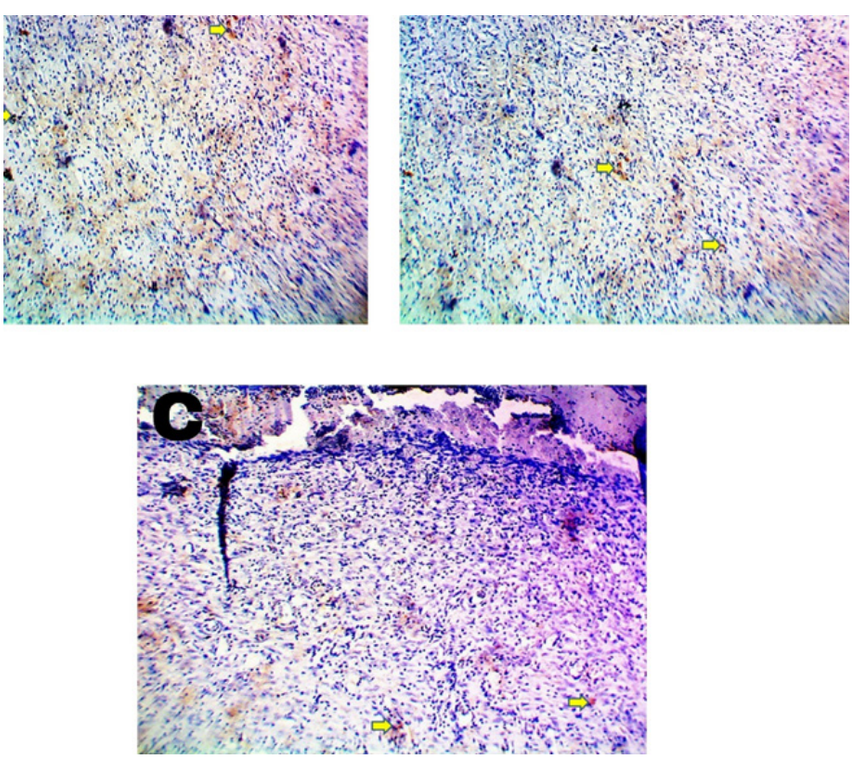

Fig. 4: Immunohistochemistry expression of the CD31 (14 days). Hematoxylin stain, 100X. A (hybrid filler) showed strong expression, B (hyaluronic acid filler) showed moderate expression, and C (control) showed moderate expression.

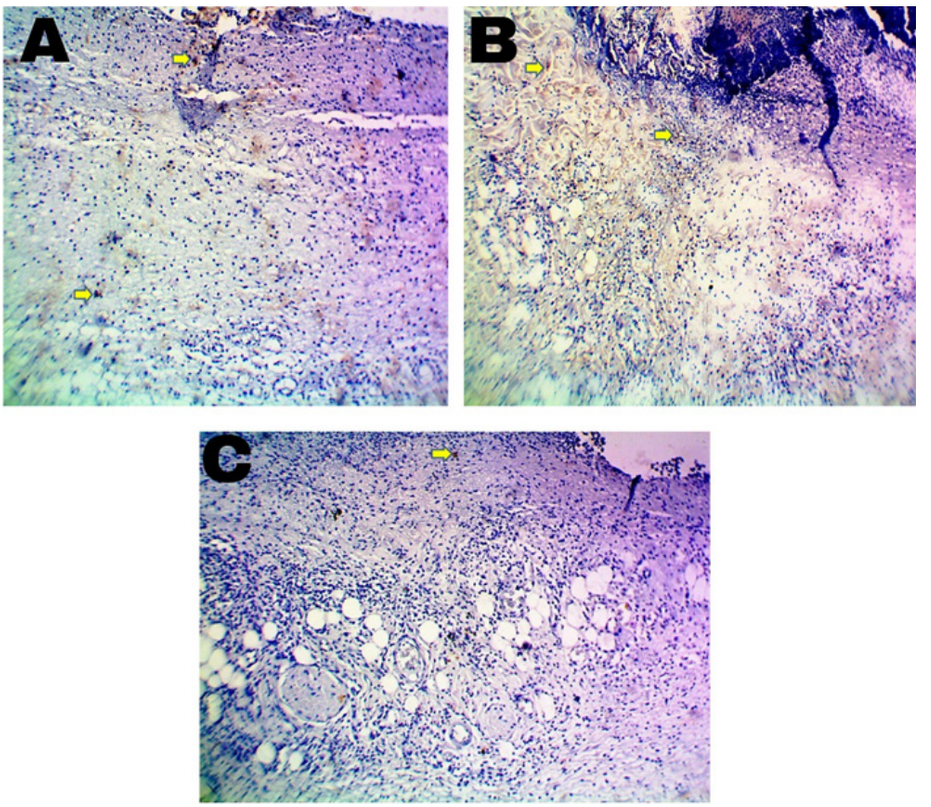

Fig. 5: Immunohistochemistry expression of the MMP-9 (3 days). Hematoxylin stain, 100X. A (hybrid filler) showed moderate expression, B (hyaluronic acid filler) showed moderate expression, and C (control) showed mild expression.

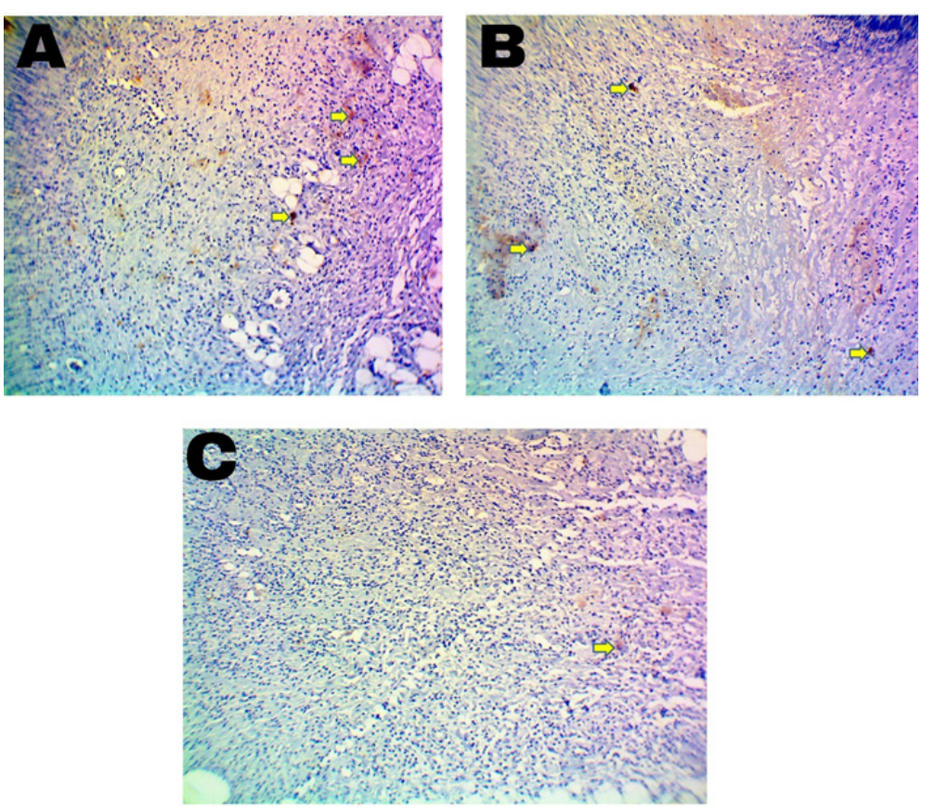

Fig. 6: Immunohistochemistry expression of the MMP-9 (7 days). Hematoxylin stain, 100X. A (hybrid filler) showed intense expression, B (hyaluronic acid filler) showed strong expression, and C (control) showed mild expression.

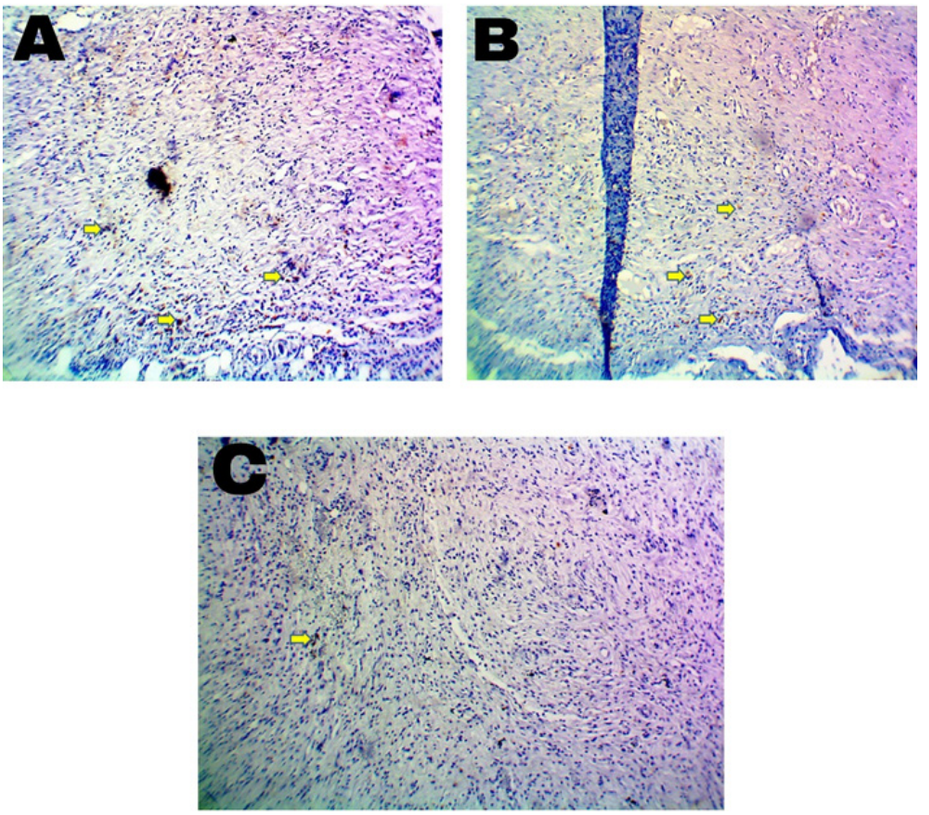

Fig. 7: Immunohistochemistry expression of the MMP-9 (14 days). Hematoxylin stain, 100X. A (hybrid filler) showed intense expression, B (hyaluronic acid filler) showed strong expression, and C (control) showed mild expression.

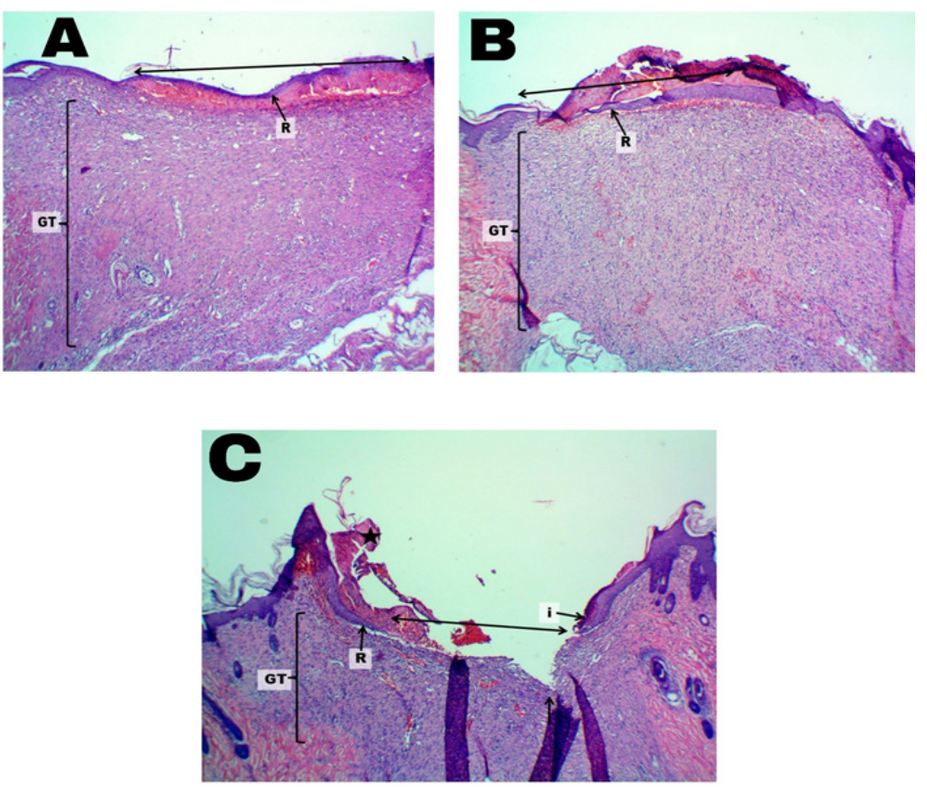

Fig. 8: Histological section of rat skin (after 14 days) showing wound site (↔), containing inflammatory cells infiltration (i), re-epithelialization (R), granulation tissue (GT). H&E stain, 40X. A (hybrid filler), B (hyaluronic acid filler), and C (control).

Table 1: The histopathological Scores of the inflammatory cells infiltration (ICI), granulation tissue formation (GTF), and re-epithelialization (RE) of the treatment groups (A: hybrid filler, B: hyaluronic acid filler) and the control group (C) as Median at 14th days of the study period.

Time period	Group	Median of ICI	Median of GTF	Median of RE
14th Day	A	1	2	4
	B	1	2	3
	C	2	4	2

Table 2: CD31 and MMP-9 expression intensity in the skin specimens. The intensity means scores of CD31 and MMP-9 immunohistochemistry expression from treatment groups (A: hybrid filler, B: hyaluronic acid filler) and the control group (C). Each group had 3 specimens measured at time intervals (3rd (G1),7th (G2) and 14th (G3) days). The scores represent: 0 (- negative expression), 1 (+ mild positive expression), 2 (++ moderate positive expression), and 3 (+++ strong positive expression).

Time period	Group	Median of CD31	Median of MMP-9
3rd Day	G1A	2	2
	G1B	1	2
	G1C	0	1
7th Day	G2A	3	3
	G2B	2	3
	G2C	1	1
14th Day	G3A	3	3
	G3B	2	3
	G3C	2	1

Table 3: Comparisons of the intensity scores of MMP-9 and CD31 immunohistochemistry expression between the control group and the treatment A (hybrid filler) group on the same day.

Time period	CD31 (*P-value*)	MMP9 (*P-value*)
3rd Day	0.034*	0.043*
7th Day	0.025*	0.025*
14th Day	0.043*	0.025*

Mann-Whitney U test was used for the comparisons between groups at $p \leq 0.05$. (*): Significant difference.

Table 4: Comparisons of the intensity scores of MMP-9 and CD31 immunohistochemistry expression between the control group and the treatment B (hyaluronic acid filler) group at the same day.

Time period	CD31 (*P-value*)	MMP9 (*P-value*)
3rd Day	0.034*	0.043*
7th Day	0.043*	0.025*
14th Day	1.000	0.034*

Mann-Whitney U test was used for the comparisons between groups at $p \leq 0.05$. (*): Significant difference.

Table 5: Comparisons of the intensity scores of MMP-9 and CD31 immunohistochemistry expression between both treatment (A) and (B) groups at the same day.

Time period	CD31 (*P-value*)	MMP9 (*P-value*)
3rd Day	0.025*	1.000
7th Day	0.034*	0.700
14th Day	0.043*	0.317

Mann-Whitney U test was used for the comparisons between groups at $p \leq 0.05$. (*): Significant difference.

DISCUSSION

One of the most common wound healing models, excisional wounds, requires second intention healing without sutures like acute clinical wounds. Hemorrhage, inflammation, granulation tissue, re-epithelialization, angiogenesis, and remodeling may be examined in this model[13].

The hybrid filler and HA filler groups had greater median MMP-9 expression than the control group, increasing from moderate expression at three days to intense expression at seven and fourteen days. MMP-9 expression was low in the control group, hence our research groups improved wound healing.

Our results agrees with Kyriakides *et al*. 2009 who found that animals lacking matrix metalloproteinase-9 had delayed wound healing, delayed re-epithelialization, and disrupted collagen fibrillogenesis[14]. Dysregulation causes chronic inflammation and delayed wound healing[15]. HA substantially elevated MMP-9 in keratinocytes, indicating wound healing advantages[16]. Isnard *et al*. 2001 found that fibroblasts and keratocytes activated latent MMPs and increased MMP-9 expression in the presence of hyaluronan (HA)[17]. Manuel and Gawronska-Kozak 2006 hypothesized that MMP-9 expression in the remodeling phase of wound healing in nude mice may be a crucial factor in their capacity to scar-free healing[18].

In the hybrid filler group, CD31 expression increased from moderate on the third day to strong on the fourteenth day. The HA filler group was lower than the hybrid filler group through all days. The HA filler group was greater than the control group at third and seventh days. On the third day, the control group had a negative CD31 score, but on the fourteenth day, it matched the HA group.

According to Chen *et al*. 2022, HA filler injection boosted CD31 expression and cutaneous blood vessel growth compared to the control group[19]. Angiogenesis was enhanced by CaHA, which could indicate better blood flow and nutrient supply to the skin[20]. Hu *et al*. 2023 reported that CD31 staining results after calcium hydroxylapatite application showed the newly formed blood vessels in the gap between the CaHA particles[21].

On the fourteenth day, unlike the control group, the hybrid filler and HA filler groups had full remission of the

inflammatory process. After employing the hybrid filler, Urdiales-Gálvez *et al*. 2023 found no irritation[22]. Neither treatment groups had much granulation tissue on this day. The control group, however, had a lot of granulation tissue. On day 14, macrophages produce TNF-α from HA. TNF-α decreases granulation tissue, fibroblastic collagen production, and scarring[23]. Hybrid filler covering the whole wound with normal thickness had the greatest median ratings for re-epithelialization. HA fillers had a lower median score than hybrid fillers with uneven thickness. The control group scored lowest for wound re-epithelialization encompassing more than 50%.

CONCLUSION

Our research shows that both drugs significantly improved secondary intention wounds and had positive effects on healing acceleration in terms of epithelial healing after injection.

We propose that you give some thought to use hybrid filler or hyaluronic acid filler as an alternative approach to treat wounds.

CONFLICT OF INTERESTS

There are no conflicts of interest.

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الملخص العربى

آثار الحشو الهجيني وحشو حمض الهيالورونيك على تجديد الجلد بعد الجراحة

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المقدمة: تتفاعل الخلايا وعوامل النمو والسيتوكينات بشكل فريد أثناء التئام الجروح الجلدية. يتم فحص الراحة طويلة الأمد وتقليل تكاليف المستشفى والندوب في علاجات الجروح.

الأهداف: لمقارنة تأثيرات الحشو الهجيني (حمض الهيالورونيك + هيدروكسيلاباتيت الكالسيوم) وحشو حمض الهيالورونيك على التئام الجلد بعد الجروح المستحثة باستخدام المناعة النسيجية (مجموعة التمايز ٣١ وميتالوبروتيناز المصفوفة ٩).

المواد وطرائق العمل: تم وضع ثلاث مجموعات من ثلاثة ذكور جرذان ألبينو بشكل عشوائي في أوقات الإصلاح الثالث والسابع والرابع عشر. الفئران لديها ثلاث جروح دائرية على ظهورهم. حصل الجرح العلوي على الحشو الهجيني، الجرح الأيمن السفلي حصل على حشو حمض الهيالورونيك، ولم يتم ملء الجرح الأيسر السفلي كاختبار. في الأيام الثالث والسابع والرابع عشر، تم أخذ الخزعات من كل فأر لتحديد التعبير عن مجموعة التمايز ٣١ وميتالوبروتيناز المصفوفة ٩ باستخدام المناعة الكيميائية.

النتائج: اظهر التحليل المناعي الكيميائي ل ميتالوبروتيناز المصفوفة ٩ ان كلتا مجموعتي العلاج اظهرتا تعبيرا عاليا عن ميتالوبروتيناز المصفوفة ٩ طوال الفترة بأكملها على عكس مجموعة التحكم التي اظهرت تعبيرا ضعيفا. فيما يتعلق ب مجموعة التمايز ٣١، كانت اعلى الدرجات لمجموعة الحشو الهجيني أكثر من حشو الهيالورونيك ومجموعات التحكم في جميع الايام. ومع ذلك، كانت الدرجات المتوسطة ل مجموعة التمايز ٣١ اعلى في مجموعة حشو الهيالورونيك مقارنة بمجموعة التحكم في اليومين الثالث والسابع. كانت اعادة الغشاء الظهاري في اليوم الرابع عشر اعلى في مجموعة الحشو الهجيني تليها مجموعة حشو حمض الهيالورونيك، وكانت مجموعة التحكم هي الأدنى.