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Enhanced Wound Healing with Engineered Dermal Tissues: Collagen-Based Scaffolds Enriched with Chitosan, Multi-walled Carbon Nanotubes, and Angiotensin II

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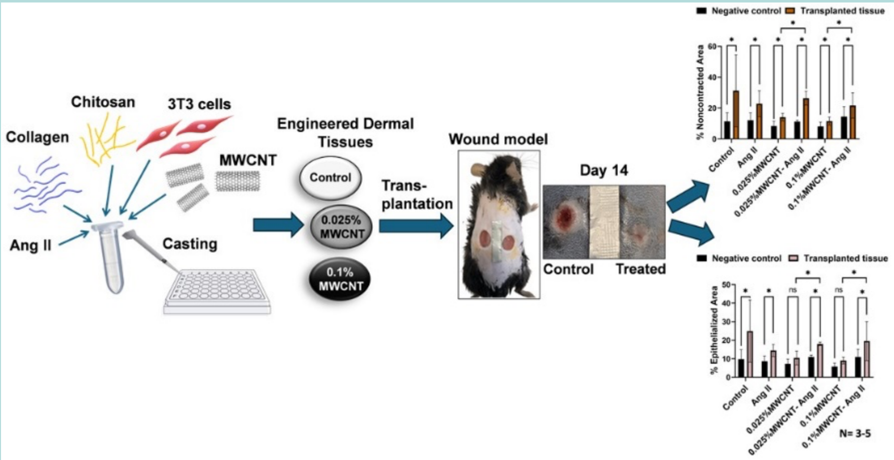
Abstract: Chronic wounds represent significant health challenges due to the complex, multistep nature of wound healing, which involves numerous key factors. Effective wound healing is not all about wound closure but also about restoring the skin's normal texture, appearance, and function. Engineered skin substitutes have emerged as a potential treatment. This project developed novel engineered dermis tissues (EDTs) using collagen-based scaffolds enriched with chitosan, multi-walled carbon nanotubes (MWCNTs), and angiotensin II (Ang II) to enhance wound healing. These EDTs were tested in a mouse full-thickness wound model and evaluated macroscopically and histologically after 14 days. Results showed that all transplanted EDTs significantly reduced wound contraction and increased epithelialization, especially those containing Ang II. The transplanted EDTs did not affect wound closure or the thickness of the new epidermis or dermis. Overall, our EDTs improved wound healing quality by promoting epithelialization and reducing contraction.



Keywords: Engineered tissues, regenerative medicine, wound healing, chronic wounds, multi-walled carbon nanotubes, nanomedicine

Introduction

Millions of patients worldwide suffer chronic non-healing wounds, such as diabetic foot, venous ulcers, pressure ulcers, and ischemic ulcers (1-3). These wounds bring forth physical and emotional suffering, diminishing quality of life and elevating the risk of death, in addition to a vast economic burden (4, 5). A main underlying cause for the failure of wound closure is a defect in the healing capacity of the dermis layer of the skin (6-9), which is a fibrous connective tissue that constitutes the thickest layer of the skin and is responsible for the physicomechanical and properties and functions of the skin. It is made up mainly of fibroblasts that are scattered through an extracellular matrix (ECM), which is mainly composed of a scaffold of organized collagen fibers that are cross-linked with other biomaterials such as glycoproteins, proteoglycans, and glycosaminoglycans that contribute to the homeostasis and mechanical properties of the dermis (10, 11).

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The limited available therapeutic options, including plastic surgery, are frequently associated with several complications and a high rate of unsatisfactory outcomes (12, 13). Engineered dermis tissues (EDT) have recently emerged as one of the potential alternatives to surgical options, which may spare patient suffering and probably provide better therapeutic outcomes (14-16). The composition of the EDTs should ideally mimic that of a natural dermis; therefore, collagen should constitute the highest proportion. Collagen is the main determinant of the mechanical properties of the tissue. It also facilitates cell attachment, migration, growth, and differentiation. In addition, it may provide a substrate that guides the host cells at the wound margin to infiltrate the wound bed and grow there into the desired functional tissue (17-19). However, the mechanical properties of the synthetic collagen matrix are not optimal, therefore it can be modulated by enriching the collagen matrix with other polymers like chitosan (20), which is a natural polysaccharide polymer that is non-poisonous, biocompatible, and biodegradable (18, 19, 21). Its presence could be favorable for the scaffold of EDTs as it has been reported to boost the production of collagen and work well in conjunction with fibroblast growth factor in accelerating wound healing (18, 22). Additionally, it has significant antimicrobial properties and may improve wound re-epithelialization (23-25), making it an effective option for wound care. In addition, chitosan is a glycosaminoglycan-like biodegradable polymer, which when combined with collagen, may improve its biomechanical stability, and prevent excessive contraction of collagen by the inhabitant fibroblasts (26).

The biomechanical properties of the EDTs could be further enhanced by incorporating multi-walled carbon nanotubes (MWCNTs), which are made up of several layers of rolled graphene sheets into cylinders with diameters in the nanometer scale and lengths in the micrometer scale and have distinct mechanical properties like great tensile strength (27-29). MWCNTs are hydrophobic in nature and thus tend to precipitate as aggregates in aqueous media (27, 30, 31). However, by adding polar functional groups or coating them with polar molecules like chitosan, their dispersibility in aqueous media can be significantly improved (32-34). It has been shown that the incorporation of chitosan complexed with MWCNTs within the scaffold of an engineered connective tissue significantly improved its biomechanical properties as demonstrated by enhanced tissue stiffness, a reduction in tissue contraction, and an increase in tissue elasticity, extensibility toughness, and resilience (35). Moreover, our group showed in a previous project that topical application of a gel composed of chitosan-MWCNT complex demonstrated a pro-healing effect on a mouse wound model (36).

In addition, recent evidence has emerged on the role of some hormones like angiotensin II (Ang II) in the organization of wound healing via several mechanisms like enhancing fibroblast and keratinocyte migration and tissue remodeling (37). Angiotensin type-1 receptors (AT1R) were found to be expressed in rat skin tissue during the wound healing process, and it was found that wound healing was hindered in rats treated with an AT1R blocker (38).

In this project, we developed novel EDTs based on collagen and chitosan, supplemented with MWCNTs and Ang II. Then, we investigated the effect of transplanting these tissues on the wound healing process using an in vivo wound model.

Materials and Methods

Chemicals and reagents

Calcium-free Dulbecco's phosphate-buffered saline (DPBS) (02-023-1A), Dulbecco's Modified Eagle Medium (DMEM) (01-055-1A), and penicillin/streptomycin solution (03-031-1B) were procured from Biological Industries, Jerusalem. Other essential materials included Trypsin-EDTA solution 1X (59417C), Fetal bovine Serum (FBS) (C8065), DMEM powder (56436C-10L), L-glutamine solution (03-020-1B), Bovine skin collagen solution (C4243-20ML), Angiotensin II human (SLCC4027), chitosan powder (CAS912764), and Xylazine (X1126-1G), all sourced from Sigma-Aldrich, USA. Ketamine (as HCL) 50 mg/ml (CA-LA SH110617) was obtained from Pfizer, USA. MWCNTs were obtained from Nanostructured and Amorphous Materials, USA. Isoflurane 99.9% solution for inhalation (Lot # 6041795) was gifted from Abbott.

Preparation of chitosan-MWCNTs complex

The complex was formulated using the procedure described by Kittana et al., 2021(20), with certain modifications. Briefly, a 1.5% w/v chitosan solution was prepared in 1% acetic acid. Subsequently, MWCNTs powder was introduced into the chitosan solution to form a 4% (w/v) suspension. The resulting suspension underwent a 2-hour sonication process, followed by autoclaving.

Culturing and maintenance of 3T3 cell line

The 3T3 cells were cultured in DMEM-based growth medium containing 10% FBS, 1% glutamate and and 1% penicillin/streptomycin. The cells were maintained under standard cell culture conditions (37 °C, 5% CO2, and a humidity of 99%).

Generation of EDTs

The method was adopted with modifications from Kittana et. al. and Assali et. al. (20, 39). All tissue components were kept ice-chilled throughout the experiment. The master formula required to prepare a single EDT of each of the investigational tissue types is presented in Table 1. First, the 3T3 cells were collected by using 0.05% trypsin, and the cell concentration was adjusted to 12 x 106 cell/ml and was kept chilled in ice bath. Then, collagen, chitosan and 2X DMEM solutions (prepared from DMEM powder) were added sequentially in a pre-cooled Eppendorf tube. The 4% MWCNTs suspension was added at this step were appropriate to obtain a final diluted concentration of either 0.1% or 0.025%. The pH of the acidic mixture was neutralized with 0.1% NaOH, then the cell suspension was added to the mixture. The mixture was immediately poured into a gelatin-coated 48-well plate (300 µl/well). Each single EDT contained about 400,000 cells. The culture plates were kept at room temperature under the sterile laminar flow for about 15 min to allow collagen polymerization in the presence of an intact gelatin coat to prevent the adhesion of the polymerizing tissue with the surface of the plates. Then, the plates were transferred to the cell culture incubator for 50 minutes. During this time collagen polymerization accelerated while the gelatin coat melted due to the warm temperature there, rendering the forming EDTs floating in the medium. At this point, about 2 ml of the growth medium was added per well, and the plates were kept in a cell culture incubator, with the medium being replaced every other day. For the experiments that investigate the effects of Ang II on the wound healing capacity of the tissue, 0.2 µl of a stock Ang-II solution (1 mM) was added to the master mix just after the addition of the cell suspension, so that the final concentration of Ang II in the master mix was about 660 nM. Also, the culture medium of these tissues contained 1 µM Ang II.

Table 1: Master formula for a single EDT

Tissue components	Control tissue	0.1% MWCNT tissue	0.025% MWCNT tissue
Chitosan	50 µl	50 µl	50 µl
Collagen	50 µl	50 µl	50 µl
2X DMEM	100 µl	100 µl	100 µl
4% MWCNTs	-	8 µl	2 µl
0.1% NaOH	90 µl	90 µl	90 µl
Cell Suspension (12 x 10^6 cells/ml)	33.33 µl	33.33 µl	33.33 µl
1 mM Ang II	+/- 0.2 µl	+/- 0.2 µl	+/- 0.2 µl
Total	~323 µl	~330 µl	~325 µl

In vivo Experiments

Experimental animals

The study employed C57BL/6 mice aged between 4 to 6 weeks, with an average weight of 23.9±6 g. The mice were individually housed in cages with standard lighting (12 hr light/12 hr dark) and maintained at a temperature of 24°C, ensuring optimal conditions. They were provided with unrestricted access to both food and water. All animal handling and treatment procedures adhered to the ethical guidelines set forth by the Ethics Committee of the International Association for the Study of Pain. Additionally, the study protocol received approval from the institutional review board (IRB) at An-Najah National University.

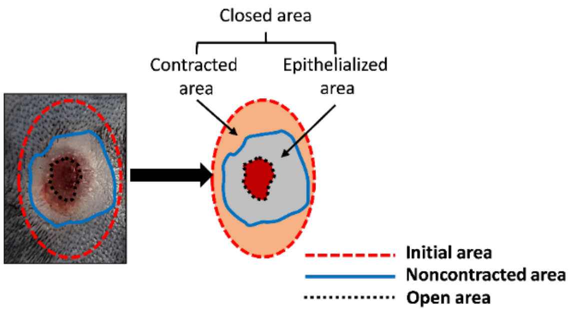
EDT implantation in a mouse wound model

The mice were anesthetized following the instructions and guidelines of the Vertebrate Animal Research guidelines of the University of Iowa, USA (40). The Ketamine cocktail was prepared by combining 800 µl of ketamine solution (5% w/v), 50 µl xylazine (10% w/v), and 1150 µl normal saline. The mice were anesthetized by a combination of intraperitoneally administered Ketamine cocktail (2.5 µl/g bodyweight) and inhaled isoflurane.

After that, the dorsal surface of the mice was eliminated with an electric clipper and a commercial depilatory cream. Then the skin was sterilized with povidone-iodine and 70% ethanol. After that, a fold of the dorsal skin from the middle region was stretched on the table and punctured with a biopsy puncture (8 mm diameter) to create two identical circular full-thickness excisional wounds. Then one side received a test EDT, while the other side was left untreated to serve as an internal negative control. After that, the wounds were covered with a Tegaderm® Transparent Film dressing. Then, the mouse was transferred to a separate cage with free access to water and food. After 14 days, the mice were euthanized by over anesthesia, and the tissue biopsies were collected from the wound sites and fixed in 10% formalin buffer.

Morphometrical studies

For the macroscopic evaluation of the wound-healing process, the wounds were imaged on the day of wound induction and the day of euthanasia (the 14th day). The evaluation of the wound healing was performed according to Cifuentes et al. 2020 with modifications as shown in Figure 1 (41). ImageJ (NIH, Bethesda, MD, USA) was used to measure the initial wound area, the contracted area, the non-contracted area, the epithelized area, the open area, and the closed area on the day of euthanasia. These measurements were used to calculate four parameters; the relative change in the wound area which was calculated by dividing the non-contracted area by the initial area. The percentage of wound contraction was calculated as the percentage of the contracted area to the initial area. The re-epithelialization was calculated as the percentage of the epithelized area to the initial area. Lastly, wound closure was calculated as the percentage of the closed area to the initial wound area.



$$\% \, Noncontracted \, area = \frac{Noncontratced \, area}{Initial \, area} \, x \, 100$$

$$\% \, Epithelialization = \frac{Epithelialized \, area}{Initial \, area} \, x \, 100$$

$$\% \, Wound \, Closure = \frac{Closed \, area}{Initial \, area} \, x \, 100$$

Figure 1: Illustration of the parameters used for the macroscopic evaluation of wound healing. The scheme was reproduced from Cifuentes et al 2020 with modifications (41) The initial area is the area of the wound measured immediately after wound induction. The contracted area is the area of the healthy skin that is pulled towards the center of the wound. The epithelized area is the healed wound area that has reached the stage of neoepidermis formation. The noncontracted area represents the epithelized area plus the open area. The open area is the remaining wound area that is still not healed.

Histological analysis

The collected tissue biopsies were processed and stained with Masson-Trichrome stain according to the routine methodology employed for similar clinical samples at the Histopathology Department at An-Najah National University Hospital. The stained tissue sections were imaged using a digital microscope (Leica ICC50 HD camera from Leica Camera AG, located in Wetzlar, Germany). ImageJ software was used to make the measurements necessary to calculate the ratio of the average thickness of the new epidermis at the wound bed to the average thickness of the healthy epidermis around the wound bed for the same sample (internal control). Similar measures were used to calculate the ratio of the average thickness of the new dermis at the wound bed to the average thickness of the healthy dermis around the wound bed for the same sample (internal control).

Statistical analysis

The collected data were statistically analyzed by GraphPad Prism version 9.5.1 for Windows. The paired Student t-test and the two-way ANOVA statistical test were used as appropriate to compare the means. The results were reported as the mean ± standard error mean. The difference between means was considered significant when the p-value was ≤ 0.05.

Results and Discussion

Result and discussion

Although advancements have been made in treating chronic wounds, they persist as a major challenge for the healthcare system. Consequently, the development of novel and enhanced treatment modalities remains of top priority. In this study, our objective was to generate an innovative therapeutic approach for chronic wound healing. We accomplished this by formulating a hydrogel based on collagen and chitosan, supplemented with varied concentrations of chitosan-coated MWCNTs, along with or without the inclusion of Ang II (Figure 2).

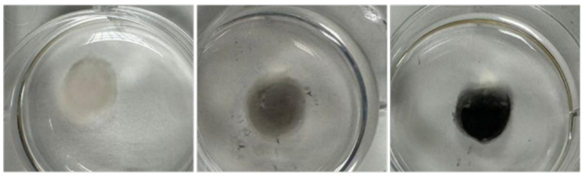


Figure 2: Macroscopic image of the EDTs with various concentrations of MWCNTs. Left, Control tissue, composed of collagen and chitosan. Middle, composed of collagen, chitosan, and 0.025% MWCNTs. Right, composed of collagen, chitosan, and 0.1% MWCNTs.

Effect of EDT transplantation on mice body weight

The follow-up of mice bodyweight throughout the course of the experiment could be utilized as a general indicator of overall health status. As shown in Figure 3, the mean body weight for the mice in all treatment groups did not significantly change over the 14-day study period. In addition, the mice in all treatment groups were active and behaved normally, suggesting that the procedure had no negative impact on the overall health of the mice.

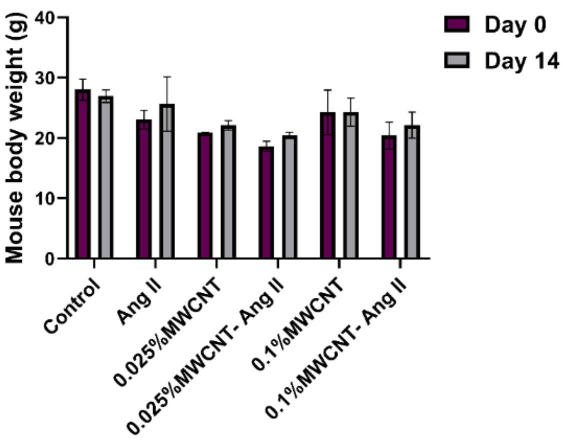


Figure 3: Effect of the transplantation procedure on mice body weight. no significant change in the weight of the mice 14 days after the procedure. Data is presented as mean ± standard error mean. N= 3-5 for each group. The level of significance was set at p-value ≤ 0.05.

Macroscopic evaluation of wound healing

The macroscopic evaluation of wound healing (Figure 4) was carried out to investigate the effect of the transplanted tissues on wound contraction, re-epithelialization, and closure. The extent of wound contraction is an important parameter in evaluating the efficiency and quality of wound healing, as it helps reduce the size of the wound by bringing the edges of the wound closer. However, excessive wound contraction can lead to scarring and permanent disfiguring of the skin, which may have a negative impact on the appearance and quality of the wound and possibly restrict the underneath joint movement (42).

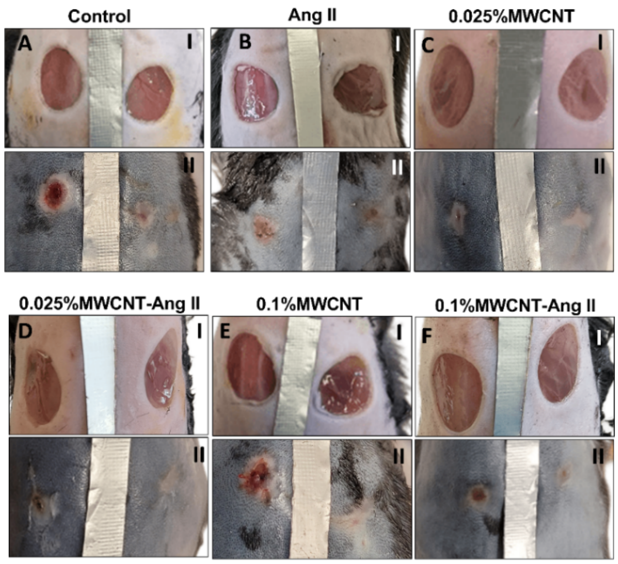


Figure 4: Macroscopic images of the induced standard wounds at day 0 (I) and at day 14 (II). On the right side is the internal negative control wound (without any treatment). On the left side is the wound that received the test tissues. The standard constituents of the ECM in all tissues were collagen and chitosan. (A) Control (B) Ang II (C) 0.025% MWCNT; (D) Ang II and 0.025% MWCNT; (E) 0.1% MWCNT; and (F) Ang II and 0.1% MWCNT.

The effect of the transplanted tissues on wound contraction was evaluated indirectly by calculating the percentage of noncontracted areas after 14 days of wound induction and treatment. As shown in Figure 5A, all groups that received transplanted tissues had a significantly greater percentage of the noncontracted area than the corresponding negative control wounds. Likewise, one can notice in Figure 4 that the skin around the healed wounds that received transplanted tissues appeared more relaxed as compared to the corresponding untreated wounds on the same mouse. However, there was no reliable method to quantify this observation.

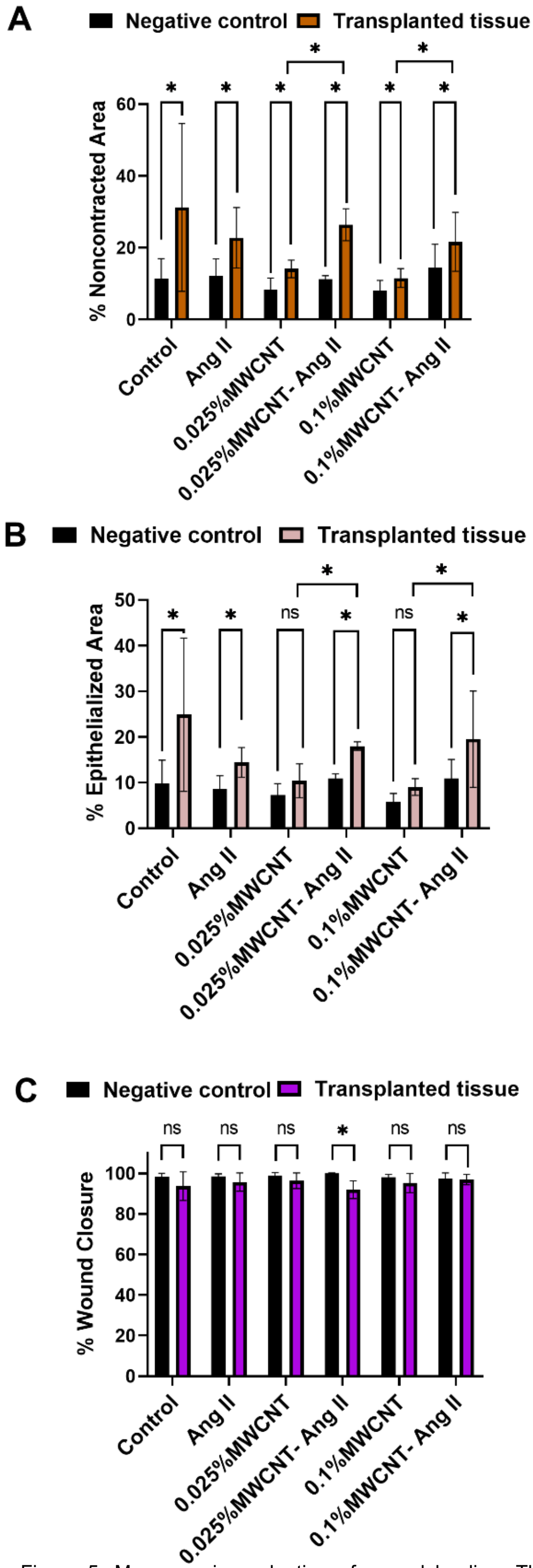


Figure 5: Macroscopic evaluation of wound healing. The graphs show the comparison of different wound healing parameters between the wounds treated with transplanted test tissues and their corresponding negative control wounds. (A) The percentage of noncontracted areas. (B) The percentage of epithelialized area (C) percentage of wound closure. The data is presented as mean ± standard error mean. A paired student t-test was used to compare the means. The difference was considered significant when p-value ≤ 0.05. N= 3-5 for each group.

At the same time, we investigated the effect of the transplanted tissues on the process of epithelialization by calculating the percentage of the epithelialized area in the wounds that received the transplanted tissues compared to the corresponding negative control wound as shown in the graphs in Figure 5B. It was found that in all wounds that received tissues without MWCNTs, there was a significant increase in the percentage of the epithelialized area as compared to their corresponding negative control wounds, which was not the case with the wounds that received tissue transplants containing MWCNT unless Ang II was incorporated in the tissues. In principle, we think that these findings are reasonable, because when the wound contraction is reduced this means that the wound needs to regenerate a wider skin area to fill in the gap, however, the transplanted ECTs may at least partially substitute for the lost dermis layer of the skin and may allow the growth of new epidermal cells on their surface towards the center of the wound, resulting in wider epithelialized areas as compared to the negative control wound, which appears to have contracted to a larger extent, so the remaining area for the regeneration of the epidermis was smaller. However, the data suggest that the presence of MWCNTs might suppress the epithelialization process to some extent, however this effect could be reversed by Ang II, which highlights a potential role for Ang II in promoting the quality of the healing process, and this is in line with previous results by other research groups who demonstrated in their work that the expression of the Ang II receptor subtypes (AT1R and AT2R) in cells plays a major role in the function of both keratinocytes and fibroblasts during wound healing. It was demonstrated that AT1R are expressed in both keratinocytes and fibroblasts, while AT2R are expressed mainly in dermal fibroblasts. Also, they showed that AT1R signaling enhances the growth and migration of myofibroblasts during wound healing, while this is suppressed by AT2R signaling. Myofibroblasts are differentiated contractile form of fibroblasts that are active in ECM deposition during wound healing and are key players in fibrosis and scar formation, therefore, it was suggested that maintaining balanced signaling between AT1R and AT2R might be crucial for proper wound healing (38, 43). Additionally, it was found by other researchers that Ang II boosted the movement of keratinocytes and fibroblasts in a dose-dependent manner by activating AT1R which was associated with the release of heparin-binding epidermal growth factor, which in turn stimulated the epidermal growth factor (EGF) receptor, that is essential for the growth and differentiation of cells (37). In the same context, other studies investigated the changes in the expression of AT1R and AT2R during the wound healing process in mice. They found that Ang II levels increased in the first seven days and then declined afterward, at the same time, the expression of AT1R and AT2R followed a similar pattern. Interestingly, they also found that AT2R expression increased again after the wound epithelialization phase (44).

Moreover, in our project, we measured the percentage of wound closure, which represents the percentage of the closed area (sum of the contracted area and the epithelialized area) at day 14 to the initial area. The data did not generally demonstrate a statistical or clinically significant difference in the percentage of wound closure between the wounds treated with transplanted tissue and the corresponding negative control wounds (Figure 5C), which is consistent with the other findings as the increase in the epithelialized area in the wounds that received the tissue transplants was at the expense of reduced wound contractions, which implies that the presence of transplanted tissue does not greatly impact the rate at which the wound closes during the two-week study period but rather the quality of the healing process. However, this should be further investigated in future projects on wound models with dysfunctional healing mechanisms.

Taken together, the data demonstrated that the transplanted tissues may improve wound healing by reducing wound contraction and enhancing the epithelialization process, however, they do not speed up overall wound closure. Moreover, Ang II may reverse the inhibitory effect of MWCNTs on wound epithelialization, highlighting its potential role in healing.

Microscopic evaluation of wound healing

Skin tissue samples excised from the wound area were processed, sectioned, and stained with a Masson-trichrome stain (Figure 6A). The thickness of the dermis and epidermis at the wound bed and outside the wound was measured using ImageJ software. For the same tissue section, we calculated the ratio of the new epidermis to the thickness of the epidermis outside the wound (control) (Figure 6B), and the ratio of the new dermis thickness to the thickness of the dermis outside the wound (control) (Figure 6C). These ratios were calculated for the wounds that received tissue transplants and for the negative control wounds.

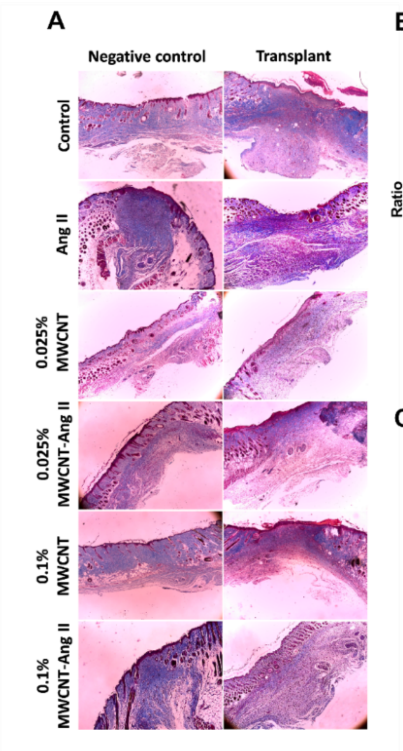


Figure 6: Histological evaluation of the healing wounds. A: tissue sample sections stained by the Masson-trichrome stain. B: the ratio of the new epidermis to the thickness of the control epidermis outside the wound. C: the ratio of the new dermis thickness to the thickness of the control dermis outside the wound. These ratios were calculated for the wounds that received tissue transplants and for the negative control wounds that healed without intervention. The data is presented as mean ± standard error mean. A paired student t-test was used to compare the means. The difference was considered significant when p-value ≤ 0.05. N= 3-5 for each group.

As shown in Figure 6B, there were no noticeable distinctions between the wounds treated with any of the transplanted tissues and the negative control wounds in terms of the thickness of the new epidermis that was generated during the healing process. Taken together with the data presented in Figure 5B, we propose that the EDT transplantation may increase the epithelization area, without affecting the thickness of the new epidermis, which indicates that the transplanted tissues are not likely to cause abnormalities in the epithelization process, but rather it improves it. At the same time, the data in Figure 6C suggest that the thickness of the newly formed dermis in the wound beds that received EDT transplants tended to be higher than in those of the negative control wounds, although the difference was not high enough to reach the threshold of the statistical significance. We propose that this could be the case because the thickness of the EDTs was close to that of the natural dermis and the biodegradation of collagen of the EDTs in the wound bed during the process of wound remodeling, which might have contributed to the observed enhanced re-epithelialization process (45). This can be perceived as an advantage that the transplanted tissues may not render anomalies in skin thickness

Conclusion

This study demonstrated potential therapeutic benefits for EDTs composed of collagen, chitosan, MWCNTs, and Ang II in enhancing the quality of wound healing. Treatment of the excisional wounds with the EDTs, particularly those containing Ang II, promoted the process of epithelialization, which is crucial for the formation of a protective barrier over the wound site. Additionally, the transplanted tissue significantly reduced wound contraction, which is particularly important for wounds that are prone to a high risk of scarring. These findings hold promising implications for the development of potential treatments targeting slow-healing wounds or those at risk of excessive scarring, providing hope for improved therapeutic interventions in the future.

Disclosure Statements

Ethics approval and consent to participate

All animal handling and treatment procedures adhered to the ethical guidelines set forth by the Ethics Committee of the International Association for the Study of Pain. Additionally, the study protocol received approval from the institutional review board (IRB) at An-Najah National University

Consent for publication

Not applicable

Availability of data and materials

The raw data required to reproduce these findings are available in the body and illustrations of this manuscript.

Author's contribution

The manuscript was written through the contributions of all authors. All authors have approved the final version of the manuscript. Amal Alqato: experimentation and data analysis, Naim Kittana: project design, supervision, auditing and manuscript writing, Mohyeddin Assali: Project design and manuscript writing, Hanood Abu-Rass: histology studies.

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Conflicts of interest

The authors declare that there is no conflict of interest regarding the publication of this article

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