An-Najah National University

Faculty of Graduate Studies

GENERATION OF ENGINEERED DERMAL TISSUES ENRICHED WITH CHITOSAN-MULTIWALL CARBON NANOTUBE COMPLEX FOR UTILIZATION IN WOUND HEALING

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Dedication

This dissertation is a heartfelt expression of gratitude to Allah, who has been my constant source of inspiration, wisdom, knowledge, and strength.

My parents, siblings, and friends have played an invaluable role in my academic journey, and I dedicate this work to them. Their unwavering encouragement and support have been my driving force and I am deeply grateful for their love and motivation.

Finally, I would like to extend my gratitude to all those who have assisted me along the way. Whether it was offering a helping hand or simply being there for me when I needed it, their contributions have been greatly appreciated. I dedicate this thesis to anyone who has supported me, especially in my efforts to tackle tasks with enthusiasm and persistence.

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Lastly, my heart is overflowing with gratitude to my family. Their love, encouragement, and support have been my anchor and I am proud to have made them proud. Their unwavering love and support have been a constant source of motivation throughout my years of study and research.

Declaration

I, the undersigned, declare that I submitted the thesis entitled:

GENERATION OF ENGINEERED DERMAL TISSUES ENRICHED WITH CHITOSAN-MULTIWALL CARBON NANOTUBE COMPLEX FOR UTILIZATION IN WOUND HEALING

I declare that the work provided in this thesis, unless otherwise referenced, is the researcher's own work, and has not been submitted elsewhere for any other degree or qualification.

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Date: 29/5/2023

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GENERATION OF ENGINEERED DERMAL TISSUES ENRICHED WITH CHITOSAN-MULTIWALL CARBON NANOTUBE COMPLEX FOR UTILIZATION IN WOUND HEALING

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Abstract

Introduction and background: The process of wound healing is complicated and involves four precise stages: hemostasis, inflammation, proliferation, and remodeling. When interrupted, this process can result in chronic wounds, constituting significant health and economic burden. Engineered skin tissues (EST) with different constituents have been proposed as a potential treatment.

Aim of the project: To generate engineered dermis tissues (EDTs) as a substitute for the dermis layer to enhance wound healing.

Material and Methods: The scaffolds of the generated EDTs were based on collagen, which is similar to the natural dermis, and it was enriched with chitosan, a natural biocompatible and biodegradable polymer that possesses wound-healing properties, and different concentrations of multiwall carbon nanotubes (MWCNTs) that can enhance the mechanical properties of the EDTs. The effect of incorporating angiotensin-II (Ang II) in the tissues on angiogenesis was also investigated. All tissues were populated by 3T3 cells. The EDTs were transplanted in a mouse wound model, and the wound sites were analyzed macroscopically and histologically by masson-trichrome stain after 14 days of transplantation to evaluate the quality of wound healing.

Results: Overall, our study found that transplanted tissue had no negative impact on animal health. It reduced contraction and facilitated epithelization, but did not affect the percentage of wound closure. EDTs transplantation did not affect the thickness of the

new epidermis, but it increased the thickness of the dermis. The incorporation of Ang II in the matrix of the EDT did not affect the degree of angiogenesis.

Conclusions and Recommendations: The transplanted tissue enhanced the quality of wound healing by promoting epithelialization and reducing contraction. This finding is significant for the development of potential treatments for slow-healing or high-risk scarring wounds.

Keywords: chitosan; multiwall carbon nanotubes; angiotensin II; engineered dermic tissue; wound healing.

Chapter one

Introduction

1.1 Chronic wounds as a major health burden

In the vast realm of the United States, a staggering 3 to 6 million souls are plagued by non-healing wounds. The majority of these cases burden individuals over the age of 65. The financial toll surpasses $3 billion annuall [1, 2]. These wounds bring forth physical and emotional suffering, diminishing quality of life and elevating the risk of death [3]. Shockingly, a study revealed that 28% of chronic wound patients met their demise within two years due to related complications [4]. Consequently, urgent action is imperative to develop more effective treatments for this pressing health issue.

Chronic wounds are injuries that do not heal in a typical way and may persist for over four weeks [5]. These types of wounds may become stuck in the inflammation stage of healing [6, 7]. There are various types of chronic wounds, including those caused by poor circulation, local pressure, or certain diseases. Despite variations in the underlying molecular causes, these wounds share certain characteristics such as high levels of inflammation, persistent infection, lack of functional stem cells, formation of microbial biofilms that are resistant to drugs, and incapability of skin cells to repair. These factors prevent the wound from healing. However, chronic wounds can have a wide variety of underlying reasons [6, 8].

Factors impeding wound healing can be grouped into several categories; factors that directly impact the wound in its specific location such as oxygen levels and infection. Other factors are related to the person's overall health, such as age, gender, sex hormones, stress, ischemia, alcoholism smoking, and nutrition. Moreover, some diseases like obesity, diabetes, keloids, fibrosis, hereditary healing disorders, immunocompromised conditions, jaundice, and uremia can underly chronic wounds. In addition, some medications can delay wound healing like glucocorticoids, and chemotherapy [7, 8].

1.2 Tissue repair and wound healing process

Damage or injury to the skin, whether from skin wounds or other causes, can greatly impair the skin's functions and in severe cases even lead to death [9]. When our tissues endure injury or undergo deterioration, a fascinating phenomenon called "repair" takes center stage. During this extraordinary process, our body's innate healing mechanisms spring into action, working diligently to restore the damaged tissues to their optimal health and functionality. This process is divided into two parts: regeneration and replacement (fibrosis). Both regeneration and fibrosis share a similar sequence of events that occur in response to tissue injury. Despite this similarity, they are distinct processes with different outcomes.

Regeneration, a remarkable phenomenon, entails the replacement of damaged cells with brand-new ones of the same kind, leading to the complete restoration of tissues. However, in the realm of humans, such profound regeneration is a rare occurrence, transpiring only under specific conditions. Two pivotal factors must align: firstly, the cells dwelling within the afflicted tissue must possess the remarkable capacity to divide and replicate, and secondly, the structural framework of the tissue must remain unscathed, untouched by harm's relentless hand. Conversely, let us explore the realm of fibrosis, a distinctive phase of the healing process. During this stage, the body undertakes a different strategy to mend and overcome injury. Rather than replacing damaged or wounded tissue with like-for-like counterparts, a transformation takes place. Connective tissue emerges, gradually encasing the affected area, as enduring scar tissue is meticulously fashioned. Fibrosis only partially restores the original tissue structure and may lead to structural abnormalities that impact the function of the organ. There are several causes for fibrosis, including repeated injuries, chronic inflammation, or normal repair processes that have gone wrong. Moreover, when fibrosis occurs, an excessive buildup of elements found in the extracellular matrix, such as collagen, happens, creating long-lasting scar tissue [10].

In most cases, tissue repair involves a combination of both regeneration and replacement.The nature of the tissue in which an injury occurs significantly influences the subsequent healing process. It is fascinating to witness how different types of tissue respond uniquely to injury, shaping their recovery. Some tissues are continuously dividing by nature (also known as labile tissues), such as epithelial and hematopoietic

tissues. Such tissues have a higher ability to regenerate due to their high cell proliferation rate. Let us delve into the captivating world of quiescent tissues, also known as stable tissues. These remarkable components of our biological fabric are composed of cells that generally maintain a state of rest, refraining from the bustling activity of cell division. However, when faced with specific cues, such as cellular damage or injury, these cells awaken from their slumber and embark on a transformative journey of replication and multiplication. Within the intricate tapestry of our anatomy, examples of such wondrous quiescent tissues include the parenchyma of select organs and the mesenchymal cells. In contrast, nondividing tissues (or permanent tissues), such as cardiac and skeletal muscle, are permanently unable to regenerate and will always leave evidence of injury in the form of a scar [10].

The process of healing a wound is a complex journey, encompassing four distinct stages that unfold with remarkable intricacy. Let me guide you through this captivating expedition of restoration. The first stage, known as hemostasis, is all about bringing the bleeding under control and initiating clot formation. It is commonly referred to as the vascular response. Moving forward, we enter the realm of inflammation, where the body's cellular response takes the spotlight, orchestrating a harmonious interplay of immune cells to combat pathogens and clear away debris. As we progress further, we encounter the stage of proliferation, a phase marked by the astonishing proliferation of cells and the formation of new blood vessels, paving the way for tissue regeneration. Finally, we reach the stage of remodeling, where the newly formed tissue undergoes refinement and reorganization, restoring its optimal structure and functionality. For a wound to heal properly, all four stages must occur in the correct order and at the appropriate time [8, 11].

In humans, optimal wound healing includes quickly stopping bleeding, appropriate inflammation, differentiation, proliferation, and migration of mesenchymal cells to the wound site, the formation of new blood vessels, the rapid re-growth of epithelial tissue over the wound (promote re-epithelization), and the proper formation, cross-linking, and alignment of collagen to give strength to the healed tissue [1, 12]. In the realm of wound healing, various mediators come into play, including platelets, inflammatory cells, cytokines, growth factors, and enzymes like matrix metalloproteinases and their

inhibitors. They work together to regulate and facilitate the intricate processes involved in the healing of wounds [13].

The process of stopping bleeding and initiating the healing process after a wound occurs is known as hemostasis. Blood clots and vasoconstriction are the first steps in the process of hemostasis. Inflammatory cytokines and growth factors including transforming growth factor beta (TGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), and epidermal growth factor (EGF) are produced as a result. The use of chemicals helped stem the flow of blood and set in motion the inflammatory phase of the healing process. Lymphocytes, macrophages, and neutrophils are present throughout this time [12, 14, 15]. Toxic chemicals such as proteases and reactive oxygen species (ROS) are produced by neutrophils during their crucial role in clearing the body of harmful microorganisms and debris [8].

The function of macrophages in wound healing is crucial. When an injury occurs, the first cells to react are called macrophages. These cells secrete chemical signals that activate and attract other inflammatory cells. They also dispose of dead cells, such as neutrophils, which aid in regulating inflammation. As the macrophages eliminate these cells, they shift into growth- and repair-promoting mode. New blood vessel formation, keratinocyte proliferation, and fibroblast proliferation are all part of this process [16, 17]. In this way, macrophages are instrumental in transitioning the healing process from the initial inflammatory phase to the next stage of growth and repair [8].

The involvement of T-lymphocytes, which arrive at the area of injury later on, is not well understood. Lack of T-lymphocytes has been demonstrated to slow wound healing, and different types of T-lymphocytes may have opposite effects on the restoration process [18-20]. Other types of immune cells may also have a role [21, 22].

Next, the proliferative phase of the healing process occurs either simultaneously with or soon after the inflammatory phase. This is the stage where new epithelial cells form and migrate across the wound site (a process known as re-epithelialization). In this phase, fibroblasts and blood vessel cells are prominent in the wound and support the formation of fresh blood vessels, collagen, and the development of granulation tissue. The wound-healing process eventually reaches its final remodeling stage, which can take several years to complete. In this stage, the wound's extracellular matrix is remodeled to

resemble normal tissue, and many of the new blood vessels regress. Additionally, the healing process also involves the gradual shrinking of the wound, thought to be caused by cells known as contractile fibroblasts or myofibroblasts. This happens throughout the entire healing process [12, 14].

Recent research has also focused on the function of stem cells in the repair and rejuvenation of damaged tissue during the healing process. Epidermal stem cells and bone marrow-derived cells are two examples of multipotent cells that aid in recovery by acquiring the features of specialized cell types. An example of how stem cells contribute to wound healing is by producing the cells that move to the wound site and cover it again, these cells called keratinocytes are derived from epidermal stem cells, and bone marrow-derived cells help support blood vessel growth and tissue regeneration [23-26].

When the healing process is disrupted, delayed, or prolonged, it can result in setbacks in wound healing or non-healing chronic wounds, which is a significant issue for healthcare systems as discussed above [8, 27].

1.3 The role of Ang II in wound healing

Angiotensin is a hormone that is an essential component of the renin-angiotensin-aldosterone system. This system is responsible for regulating the volume and blood pressure. The hormone is formed from angiotensinogen, a peptide prohormone that is produced mainly in the liver. Renal cells produce renin in response to a drop in blood pressure or other signals; this enzyme cleaves two amino acids from angiotensinogen to produce angiotensin I; this is then converted to angiotensin II (Ang II) by the angiotensin-converting enzyme (ACE), an enzyme primarily found in the pulmonary endothelium but also present in other organs like the heart [28]. It is well-known and extensively studied that Ang II regulates both systemic blood pressure and volume balance. Evidence is mounting, however, that Ang II may also play a role in the healing of skin wounds [29].

As mentioned earlier, Several processes, including epithelialization, dermal repair, and angiogenesis, are involved in the skin's wound healing process. Keratinocytes are responsible for epithelialization, while fibroblasts produce a matrix to facilitate dermal healing [29].

Takeda H. and his team conducted a study aimed to understand the role of the tissue angiotensin system in skin wound healing. Heart and kidney tissue repair has traditionally been attributed to the tissue angiotensin system, which is assumed to operate independently of the renin-angiotensin system. Its function in skin wound healing, however, was unknown until recently. Researchers found that wound healing was hindered in rats when they were given an oral drug that suppresses the angiotensin type-1 receptor (AT1). Laboratory tests confirmed the presence of both AT1 and angiotensin type-2 receptor (AT2) in skin cells during the wound healing process. Experiments showed that activating the AT1 receptor has been shown to speed up the skin's repair and regeneration processes, while activating the AT2 receptor has the opposite effect. Overall, their findings suggest that the balance of signals between AT1 and AT2 plays a role in regulating skin wound healing [30].

Wound healing was severely slowed in mice lacking the Ang II type 1 receptor (AT1R), according to a fascinating study conducted by Yahata Y. and colleagues in 2006. Then, the impact of Ang II on the migration of keratinocytes and fibroblasts, two crucial cells in the wound-healing process, was examined. A dose-dependent increase in cell migration was seen when Ang II was added to the culture medium. Heparin-binding EGF-like growth factor (HB-EGF) has been proposed as a mediator of Ang II-induced migration. AT1R activation results in the production of HB-EGF. The epidermal growth factor (EGF) receptor is activated by this growth factor to promote cell development and differentiation. Data showing that inhibiting AT1R, HB-EGF, or EGFR inhibited Ang II-induced keratinocyte and fibroblast migration provided further evidence for the critical role played by Ang II in stimulating the migration of these cells during skin wound healing via the release of HB-EGF. This is the first direct proof that Ang II is involved. This has significant significance for the future development of treatments for skin wounds [29] since it sheds light on hitherto unknown systems involved in the healing process [29].

Another study conducted by Steckelings et al.'s sought to determine whether or not angiotensin II receptor expression changes in humans when skin wounds heal. The researchers used three models to analyse the expression of angiotensin II receptors in human skin as it healed from wounds. First, by scraping primary human keratinocytes, the scientists were able to alter the mRNA expression of the angiotensin receptor.

Second, they used punch biopsies of human skin to examine the first stages of wound healing. Finally, the team looked at scars on human skin to see how they heal in vivo. The study found that both AT1 and AT2 receptor expression increased across the board, with AT2 expression being highest in the damaged area. There was an increase in receptor expression as soon as 24 hours post-injury, and it lasted for at least three months. Angiotensin II may play a role in cutaneous wound healing, according to the study's findings, which show that angiotensin AT1 and AT2 receptors are upregulated throughout the wound-healing process [31].

Heng-Jun Wu and co-authors conducted a study to better understand the function of Ang II in the healing process. Their goal was to look at Ang II and its receptors (AT1 and AT2) as they fluctuate throughout wound healing to see what kind of effect that would have. Full-thickness wounds were made on the backs of mice for the experiment. ELISA, bromodeoxyuridine (Brdu), immunostaining, terminal deoxynucleotidyl transferase, and reverse transcription polymerase chain reaction were only some of the methods used to examine tissue samples acquired at various times during the wound healing process. The levels of Ang II were found to be highest within the first week following wound creation, before levelling off. The expression of AT1 and AT2 receptors was similarly observed to be elevated during the first seven days before gradually declining. After the wound had been epithelized, there was also a subsequent upsurge in AT2 expression. This study adds to the growing body of evidence suggesting angiotensin II (Ang II) is involved in wound healing and tissue remodelling, particularly in the last phases of the process. In the context of wound healing, AT1 receptors have been linked to cell proliferation, while AT2 receptors have been linked to cell death and tissue remodelling [32].

1.4 Current options for the treatment of chronic wounds

Historically, surgically-required wounds like burns and chronic wounds have had just one viable alternative for covering: split-thickness skin grafts. It's a term for a transplant that consists of both the epidermis and the dermis. However, if a significant quantity of healthy skin is not available for use as a donor site, then this process may be difficult to carry out. This is especially the case if the donor area is not easily accessible. This operation is not without its dangers, including the possibility of pain and scarring as

well as an unsatisfactory outcome. Donor-site problems are another area of concern [7, 33].

1.5 Engineered skin tissues as a treatment for chronic wounds

Tissue engineering is a rapidly growing branch of biomedical study that incorporates concepts and methods from cell biology, materials science, engineering, and medicine. When a person's ability to heal on their own isn't enough, tissue engineers work to create artificial organs and tissues in a laboratory setting. We may then employ these synthetic organs and tissues as replacements. In situations when there is a severe shortage of organ donors, this might be of paramount relevance. Tissue engineering is a crucial field of study because it seeks to solve the problem of organ shortages by developing functional substitutes for the missing organs [34].

Recently, artificial tissue products have been developed thanks to research into the biology of persistent wounds. These products focus on the phase of the wound-healing process known as the proliferation stage, during which cells proliferate [35-37].

1.5.1 General requirements of engineered skin tissue

Engineered skin tissue (EST) must meet certain requirements to be effective. These include: (a) Having a layer of keratinocytes (the cells that make up the outer layer of skin) that can be replenished, and that is firmly attached to the underlying dermis (the layer below the epidermis). (b) The inclusion of the dermis layer in artificial skin replacement is crucial for successful wound healing. Just having the top layer of skin, the epidermis, is usually not enough to bring back the skin's original structure and functions and can lead to issues such as fragile grafts, wound contraction, and scar formation. (c) Being well-vascularized (having a good blood supply). (d) Provide elastic structural support for the skin. (e) Being biocompatible (not causing an adverse reaction when implanted in the body). (f) Having a suitable microstructure, such as a pore size with a mean range of 100-200 micrometers and a porosity greater than 90%. (g) Being able to be controlled in terms of its biodegradability. (h) Having appropriate mechanical properties. (i) Having a surface that encourages cells to attach, grow, and develop in a specific way through its chemical makeup and small and large structures [9, 37, 38].

Finally, An important aspect of skin tissue engineering is creating a supportive structure, referred to as a scaffold, that serves as a guide for the organization and growth of cells and the integration of the engineered skin into the host tissue [36].

1.5.2 Currently available engineered skin tissues

There are a variety of engineered skin tissue products that are available for commercial use, with many more being developed. These products have been used to treat over 200,000 patients. These products can be broadly categorized into two types: acellular and cellular. Acellular products consist only of a matrix created from natural or artificial materials that have the appropriate physical and chemical makeup to allow the body's cells to access the wound during the healing process. Cellular products, on the other hand, contain cells, either in combination with a matrix or not. Examples of cellular products include epidermal cell sheets, epidermal constructs, dermal replacements, and bilayered skin equivalents [9].

Although these methods exist, they are not often employed because of drawbacks like as antigenicity and a lack of donor locations [37]. Wound contraction, scarring, and poor integration with the surrounding tissue are common problems with many of the currently available skin replacements for wound healing [9]. The development of pharmacological preparations that hasten the healing process has been a primary focus of researchers and pharmaceutical businesses in response to these difficulties [39].

1.6 Scaffolds for skin tissue engineering

Cells are able to connect, migrate, develop, and differentiate with the aid of the scaffold because it acts as a replacement for or stimulant of the extracellular matrix. Scaffolds are three-dimensionally designed substrates that physically support host cells as they infiltrate and develop into the intended functional tissue or organ [35-37]. To promote cellular adhesion, proliferation, and differentiation, a tissue scaffold must have the appropriate physical and mechanical qualities and surface chemistry, including nano and microstructures [36].

1.6.1 Collagen as a scaffold for engineered tissues

The extracellular matrix contains significant amounts of collagen, a fibrous protein. Tensile strength, regulation of cell adhesion, help in chemotaxis and migration, and guidance of tissue formation are just some of the many functions that this fundamental structural component of the extracellular matrix performs [7, 40]. There are 28 different kinds of collagen, although types I (80-85%), II, III (8-11%), V, and XI predominate in connective tissue. Most collagen is of the type I kind [7, 41]. . While collagen may be dissolved in an acidic environment, it sets up as a gel in a basic one [42]. Since it poses no harm to the body and can be broken down quickly, it is a useful substance for tissue engineering. However, untreated collagen scaffolds have other issues, such as rapid deterioration and weakness. A collagen-based scaffold's strength and breakdown rate may be modified by cross-linking. This approach may be used to address the aforementioned problems [37].

The effectiveness of collagen scaffolds can only be improved by cross-linking. The most frequent types of cross-linking are chemical and physical. Collagen materials may be kept biocompatible by the application of physical cross-linking techniques such as photooxidation, dehydrothermal treatments, and UV irradiation [43]. However, there are times when the cross-linking accomplished by these physical processes is insufficient, and chemical methods must be used instead. The chemicals used in chemical cross-linking include glutaraldehyde, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide, polyglycidyl ether, and polyepoxide resins [37]. Since glutaraldehyde may join amino groups on two distinct polypeptide chains, it is often used as a bifunctional cross-linking reagent. Because of its low price, great efficiency in cross-linking, and high water solubility, it is widely used in skin tissue engineering [44].

1.6.1.1 Collagen-based skin substituent

Bell and his team found that by using human fibroblasts to compress a collagen lattice in vitro, they could create a structure with properties comparable to those of tissue. This realisation came about in the 1970s. They discovered that fibroblasts may force water out of a collagen structure by interacting with the fibres in a manner that makes the structure more compact. The pace and magnitude of contraction might be modulated by modifying the protein concentration and cell density of the lattice, as well as by using inhibitors like Colcemid. The result is a fabric-like tissue with potential as a treatment

for burns and other skin ailments. The lattice protein collagen is well suited for use as a transplant since it is not readily rejected by the immune system. In addition, they found that resting fibroblasts are just as effective as growing ones. This is likely due to the fact that as cells leave the cell cycle they undergo physical and structural changes that might be helpful in the healing process [45].

In 1981, Bell and his colleagues used fibroblasts cast in collagen lattices to construct live skin tissue in the lab, which they subsequently seeded with epidermal cells. As a result, a skin-like tissue was produced that had the same texture and thickness as real skin. After being effectively grafted onto the donor, these tissue-engineered skin replacements displayed properties such as vascularization, immune response suppression, wound contraction prevention, wound space filling, and wound retention. The success of this technique laid the groundwork for the creation of several more tissue-engineered skin products [46].

A variety of collagen-based dressings are commercially available in the form of gel, sheet, lattice, or sponge, such as Promogram, Johnson & Johnson, Puraply, and Royce Medical. These products have been used in various clinical settings for temporary wound coverage, such as ulcers and burns, with positive outcomes [47]. Additionally, Researchers have also created other types of skin substitutes for wound healing, including those made with acellular tissue, a combination of cellular layers, and bi-layered equivalents that use collagen-based materials [36].

Donor skin may be used as either a short-term bandage for a wound or a permanent supply of allodermis when it comes to dermal restoration. Integra, developed by Integra LifeSciences, is an alternative to utilising donor skin that provides a vascularized dermis for a split-thickness skin transplant. Lifecell also offers Alloderm, a product created from freeze-dried human donor dermis. Advanced Biohealing's Dermagraft and Transcyte are synthetic materials modified with donor fibroblasts. The silicone membrane of Transcyte is there to protect the skin temporarily. Last but not least, a pig skin substitute called Permacol was created by Tissue Science Laboratories and may be used as a short-term wound dressing. Donor skin may be used as either a short-term bandage for a wound or a permanent supply of allodermis when it comes to dermal restoration. Integra, developed by Integra LifeSciences, is an alternative to utilising donor skin that provides a vascularized dermis for a split-thickness skin transplant.

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Several products have been developed for epidermal and dermal replacement, such as Apligraf, Orcel, and Permaderm, which are all appropriate for use in long-term injuries or sores. These products are made by combining allogeneic keratinocytes, fibroblasts, and bovine collagen to provide a temporary or permanent skin replacement material[36, 48]. It is important to note, however, that there are restrictions on the use of collagen-based materials as scaffolding or dressing. These components often don't last long and aren't particularly sturdy. It may also cause the wound to constrict and shrink too much when used for healing [36].

An artificial dermis layer was created by Seo YK and a coworker in 2008. The scaffold is made of reinforced collagen. A collagen sponge was woven with a mesh formed from numerous layers of collagen thread to produce this scaffold. The reinforced scaffold demonstrated enhanced cell adherence and penetration, as well as dramatically increased tensile strength compared to a conventional collagen sponge [49].

Although advancements have been made with collagen-based wound healing materials, issues such as wound shrinking and scarring persist. Collagen is underutilised because of its expensive cost and inconsistent quality depending on harvest site and processing techniques [50]. Some researchers are investigating natural and synthetic polymers as potential solutions to these problems [51, 52].

Collagen fibres of varying sizes and shapes make up the dermal extracellular matrix. The skin's structure and mechanical strength are supported by this matrix. Scientists have attempted to develop synthetic ECM analogues composed of nanoscale fibres to imitate these qualities and promote wound healing [53, 54]. These fibres may be fabricated by a number of different techniques, including drawing, template synthesis, temperature-induced phase separation, self-assembly, and electrospinning [55]. Among these techniques, electrospinning has grown in favour as a fast, cheap, and versatile means to create polymeric nanofibers. This method involves using an electrostatic force

to pull fibers from a polymer solution or melt, using a metal electrode as a collector. Additionally, the large surface area to volume ratio of the generated scaffolds is also beneficial because it encourages cell-matrix interactions, allows for good oxygen flow, and can store fluids, all of which are essential for the wound healing process [56-58].

In 2002, Bowlin GL and colleagues were among the first to demonstrate the feasibility of using electrospinning to produce scaffolds composed of collagen nanofibers. They used calf skin type I collagen dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFP) and found that the technique allowed for tailoring the mechanical properties of the matrix through control of fiber orientation. The electrospinning process also allows for the creation of complex, three-dimensional shapes [59]. Studies have revealed that dermal fibroblasts and keratinocytes respond favourably to electrospun collagen, and that it promotes cellular proliferation and matrix penetration [60, 61]. However, some concerns have been raised about the high cost of collagen material and the potential for denaturation with commonly used solvents like HFP or TFE, which could affect its biological activity and structural integrity. Additional research is required to determine the entire effect of these variables [36, 62].

1.6.2 Chitosan as a scaffold for engineered tissues

De-acetylation of the naturally occurring polysaccharide chitin yields synthetic polymer chitosan. Chitin is one of the most abundant natural polysaccharides [63]. Chitin is a naturally occurring polysaccharide that is quite plentiful. It may be utilized as a medication delivery vehicle, a surgical thread, and a wound-healing substance, among many other biological purposes. Some of its numerous benefits include not being poisonous, being biocompatible and biodegradable, and having hemostatic properties [36, 37, 64]. Due to its electrostatic properties, Chitosan can boost the production of collagen and work well in conjunction with a growth factor called fibroblast growth factor, resulting in accelerated wound healing [36, 65]. Additionally, it has strong antimicrobial properties, making it an effective option for wound care [66].

Chitosan-based treatments speed up the infiltration of polymorphonuclear cells, a type of white blood cell that aids in the fight against infection in the wound, and improve the process of re-epithelialization when tested on an animal model of open skin wounds [67, 68]. Furthermore, Clinical Research has shown that using chitosan in wound

treatment can enhance structural organization and blood vessel formation in the dermal layer [69]. The presence of a high quantity of amino groups in chitosan is significant in its ability to connect and bind to other types of polymers, such as collagen, gelatin, and GAGs [37, 70]. However, despite the promising results seen in these studies, chitosan has some drawbacks, such as poor mechanical properties, meaning that it is not very strong or durable, excessive shrinkage, and warping after drying which has limited its use in tissue engineering [71]. Scientists have tried to get around this problem by joining chitosan to even bigger molecules, such carbon nanotubes. The goal was to make the chitosan more effective in adherence to the affected area and improve the way it interacts with cells in the wound [64].

1.6.2.1 Collagen-chitosan scaffold

In the year 2003, a group of researchers under M. Gingras's direction created a lab-grown skin substitute for treating severe burns. Collagen and chitosan were combined, then human cells were seeded and cultured for a month to create the replacement. The purpose of the research was to determine whether or not this replacement promoted nerve regeneration in burn victims. The skin replacement was put into mice, and the researchers then used techniques including confocal microscopy and immunohistochemistry labelling to track nerve development over time. The data revealed that nerve development started to occur at day 60 post-transplant and increased significantly at 90 and 120. The three-dimensional architecture of the collagen-chitosan tissue-engineered skin also aided in the development of nerves, the research showed. This study expands our knowledge of how to enhance neuron regeneration in laboratory-grown skin by pre-treating the sponge with neurotrophic substances [72, 73].

The original extracellular matrix of the skin was the inspiration for the scaffold developed by Sarkar and colleagues (2013) for use in skin tissue creation. The scaffold consisted of nano/microfibrous layers of chitosan and collagen that had been electrospun, freeze-dried, and cross-linked. The scaffold was evaluated, and its tensile strength, swelling behaviour, and biodegradability were all determined to be satisfactory. In vitro research showed that the scaffold is conducive to cell growth, with both fibroblasts and keratinocytes responding positively. The scaffold showed promise in stimulating keratinocyte migration and wound re-epithelization, both of which are critical for wound healing and tissue regeneration, in an ex vivo human skin comparable

wound model. When it comes to tissue engineering for the skin, the chitosan/collagen scaffold shows a lot of promise [74].

1.6.2.2 Collagen-chitosan-based three-dimensional scaffold

Current wound healing treatments, such as scaffolds and artificial skin replacements, have limits, according to research published by Zhang and colleagues in 2010. These drawbacks include shrinking, sluggish blood vessel creation, high cost, and scarring. They proposed employing ECM-like three-dimensional structures to guide the attachment, proliferation, and differentiation of cells into functional and structural skin tissue as a potential solution. These scaffolds are used in skin tissue engineering as a wound dressing to prevent infection and as a physical framework for dermal fibroblast and keratinocyte proliferation and differentiation [36].

While collagen shows promise for use in skin engineering, Ma L. and colleagues (2003) noted that its quick breakdown rate and limited mechanical strength might be drawbacks. To address this, they attempted to improve the properties of collagen by cross-linking it with glutaraldehyde (GA). Due to its water solubility, excellent cross-linking effectiveness, and cheap cost, GA is a common bifunctional cross-linking reagent; nevertheless, it may also be cytotoxic. To mitigate this, the team added chitosan to the collagen scaffold, as it is natural, biodegradable, and biocompatible, and this polymer can function as a linking agent to boost the ability of GA to create cross-links. Using fibroblasts isolated from the human dermis, they created a chitosan-collagen scaffold by freeze-drying the mixture and cross-linking with different concentrations of GA. The scaffold was then tested for biodegradation in vitro and in vivo using a rabbit model. The group observed that the GA treatment affected the scaffold's shape and swelling characteristics, but that there were no discernible variations across scaffolds treated with varying amounts of GA. The inclusion of chitosan also enhanced the scaffold's biocompatibility and resistance to collagenase breakdown. The group came to the conclusion that the GA-treated chitosan-collagen scaffold offers potential as a material for making artificial skin that is both durable and compatible with natural skin.[37].

Other scaffold systems, formed from human, animal, and synthetic materials, have also been developed for wound healing. Because of their unique combinations of characteristics, these items may be used in a wide variety of contexts [45, 75].

Although chitosan has several useful qualities for wound healing, its limited mechanical strength might be a hindrance, as recognised by Sun L P and colleagues (2009). To overcome this, they created a three-dimensional scaffold for skin tissue by combining type I collagen and chitosan through a process of freeze-drying and glutaraldehyde cross-linking. They then seeded dermal fibroblasts, which were obtained from neonatal Sprague-Dawley rat skin, into the scaffolds to construct tissue-engineered dermis. Their MTT test results showed that fibroblasts planted in the scaffolds multiplied gradually over the course of 14 days, and their IL-6 analysis showed that the scaffolds did not trigger an inflammatory or immunological response. Flow cytometry results also showed that most fibroblasts seeded on the scaffolds entered a normal cell cycle and that the scaffolds did not trigger cell death. Overall, the researchers concluded that the collagen-chitosan scaffolds cross-linked with GA have the potential as a candidate for tissue repair and regeneration due to their enhanced biostability and good cytocompatibility [76, 77].

HAN C. and colleagues, in 2010, developed a unique scaffold that had distinct pore sizes on each side. They achieved this by incorporating a porous membrane made of collagen and chitosan with fibrin glue, which is a natural polymer that forms a 3D-like-net structure when it's activated. They then used this scaffold to create tissue-engineered skin by seeding it with fibroblasts and a human keratinocyte line called HaCaT. They discovered that epidermal cells expanded smoothly on the scaffold's top surface, whereas dermal fibroblasts grew attached to the scaffold's walls. After three weeks of culture, the constructed composite skin substitute had a similar structure to that of normal skin tissue. Based on the findings of this research, it can be inferred that this asymmetric scaffold has a lot of potential for skin tissue engineering and could have clinical applications in the future [78].

1.6.3 Carbon nanotubes: structure, features, and types

Sumio Iijima was the first person to identify carbon nanotubes (CNTs) in 1991 [79, 80]. These CNTs are incredibly small, measuring at a nanoscale size, and are shaped like cylinders with a needle-like shape, resulting in a large surface area [81, 82]. CNTs are made up of layers of carbon rings consisting of six carbon atoms each, similar to those found in graphite. The sp2-hybridized carbon atoms organise themselves in a hexagonal pattern, and these sheets wind around to form a tube [79, 83-85]. CNTs are unique because of their great tensile strength, which may be up to 63 gigapascals, making them far more powerful than steel [86]. The elasticity of CNTs, as measured in tera pascals (TP), may also vary from 1.0 to 1.8 TP. Because of this, CNTs are very rigid and hard to bend or break [87]. The electrical conductivity of CNTs is likewise extraordinary, being up to a factor of a thousand higher than that of copper cables [88]. For commercial usage, CNTs are typically mass-produced using one of many processes. Three of the most common processes are laser ablation, arc discharge, and thermal chemical vaporization. The carbon-containing substance is vaporized by a high-energy laser beam and subsequently condenses into CNTs in the laser ablation process. CNTs are formed when an electric current is passed between two carbon electrodes in an arc discharge. CNTs are created by the thermal chemical vaporization method by heating a carbon-containing substance in the presence of a metal catalyst [79, 80].

Single-walled carbon nanotubes (SWCNTs), double-walled carbon nanotubes (DWCNTs), multi-walled carbon nanotubes (MWCNTs), and functionalized carbon nanotubes (f-CNTs) are the four major types of carbon nanotubes (CNTs) based on their structural conformation and structure. Single-walled carbon nanotubes (SWCNTs) are either open at one end or closed at the other; their structure is made up of a single layer of folded graphene sheets. However, DWCNTs feature two layers of folded graphene sheets instead of only one. MWCNTs are made by folding two to ten graphene sheets into a complicated shape, or by rolling a single sheet into a cylinder. f-CNTs are CNTs that have been modified by adding hydrophilic functional groups [89, 90]. The diameter of CNTs ranges from a few nanometers for SWCNTs to several hundred nanometers for MWCNTs, while their length can be very long, typically ranging from the nanometer to micrometer scale or even longer [84, 89]. Single-walled carbon nanotubes (SWCNTs) are insoluble in water and tend to aggregate when exposed to sonication, a technique used to agitate a liquid using high-frequency sound waves. On the other hand, multi-

walled carbon nanotubes (MWCNTs) have some solubility in water and tend to create slightly translucent suspensions [89]. CNTs that have not been modified, also known as "pristine" CNTs, don't disperse well in water and tend to clump together and separate and fall out of aqueous solution as solid particles [89, 91, 92]. Pristine CNTs can cause various toxic reactions. However, by adding polar functional groups to these CNTs, it is possible to significantly improve their dispersibility in water, while also greatly reducing their toxicity [93-95]. Previously, Scientists were trying out new tactics to improve the characteristics of carbon nanotubes (CNTs) for drug delivery purposesOne such approach is functionalization, which involves modifying CNTs with pharmacological molecules to improve drug effectiveness, control release, increase solubility, and reduce toxicity. Functionalized CNTs, or F-CNTs, have gained prominence in the field of drug delivery and hold great potential for numerous applications [89, 96, 97]. Functionalizing CNTs with appropriate molecules, whether through covalent or non-covalent methods, has proven effective in enhancing their dispersibility in water, improving biocompatibility, and reducing toxicity levels [98, 99].

Covalent and noncovalent methods are used in functionalizing carbon nanotubes (CNTs). Additionally, researchers employ proteins, DNA, chitosan, polyethylene glycol, and surfactants to achieve the desired functionalization effects [85].

CNTs have been studied as potential carriers for carrying therapeutic compounds because of their ability to quickly permeate cell membranes and be changed with a variety of functional groups. It is not well known whether they enter cells by endocytosis or by penetrating them like a needle. Despite this, it is well-established that CNTs may penetrate many cell types despite the presence or absence of certain surface functional groups [100]. Their large surface area allows for multiple modifications, and there are indications that CNTs possess antimicrobial properties [100].

Finally, carbon nanotubes (CNTs) have garnered significant interest in tissue engineering, the study of developing replacements for diseased or injured tissues [100]. Their mechanical strength, pliability, and excellent heat and electrical conduction are just a few of the many reasons they are so useful in the creation of cutting-edge materials for tissue engineering. Their potential use as key components in the development of innovative materials for tissue engineering is now being investigated by

researchers. CNTs have been shown in prior studies to be biocompatible and to stimulate the development and proliferation of several cell types [84, 100].

One big worry, however, is that CNTs could be hazardous to living things. Research has revealed that CNTs cause oxidative stress and inflammation, the two main causes of toxicity [101]. The toxicity of CNTs is highly dependent on their purity, surface modification, size, number of layers, and dispersion [102]. CNTs may cause inhalation toxicity or cytotoxicity due to their long bio-persistence and needle-like structure [103].

1.6.3.1 CNTs based scaffold

Lalwani, together with collaborators, began on a novel mission in 2015 to investigate the use of carbon nanomaterials in tissue engineering and regenerative medicine. The team's goal was to create 3D structures out of the extraordinary materials at their disposal. Single- or multi-walled carbon nanotubes (SWCNTs or MWCNTs) were joined together using a technique called radical-initiated thermal crosslinking. As a consequence, a series of scaffolds were produced with very high porosity and seamlessly linked pores of varying sizes. The scaffold's ability to foster cell development was evaluated using mouse MC3T3 pre-osteoblast cells (MC3T3 cells), a kind of bone cell developed by the National Institutes of Health. Positive effects on cell survival, adhesion, and proliferation were seen using the scaffold. Cells grown on MWCNT scaffolds were noticeably more elongated than those grown in SWCNT scaffolds, according to the study authors. This finding indicated that the nano topography of the scaffold might be modified to regulate cell morphology. damaging effects on cells when breathed in. Collectively, these findings propose that 3D macroporous scaffolds constructed from SWCNT and MWCNTs possess excellent biocompatibility with cells and hold significant promise for further exploration in the realm of tissue engineering and regenerative medicine applications [104].

Kittana N and colleagues (2018) experimented withwhether a combination of carbon nanotubes and chitosan hydrogel can enhance wound healing in a mouse model. The study involved applying the complex to full-thickness wounds on mice daily and observing the effects on fibroblast survival, extracellular matrix organization, re-epithelialization, and collagen deposit. The results revealed that the complex improved the survival and function of fibroblasts and accelerated the healing process, but also

caused an increase in fibrosis and inflammation [64]. Furthermore, in 2021 the same group researched the impact of adding a combination of multi-wall carbon nanotubes and chitosan (C-MWCNT) on engineered connective tissues (ECTs) in a living organism. The approach they took was to create ECTs using human foreskin fibroblasts (HFF-1) and collagen type I, then incorporating varying amounts of C-MWCNT, specifically 0.025%, 0.05%, and 0.1%. They evaluated the physical properties of the ECTs through mechanical testing and found that a small amount of 0.025% C-MWCNT slightly enhanced the stiffness of the tissue, as shown by Young's modulus. However, when using 0.1% C-MWCNT had notable effects on engineered connective tissues (ECTs). They discovered that when C-MWCNT was introduced, there was a reduction in tissue contraction and an increase in elasticity and extensibility. Increased measures of toughness and resilience, such as the yield point and final strain, showed that ECTs were superior to controls in this regard. It is interesting to note that the addition of C-MWCNT had no effect on the longitudinal alignment of cells inside the ECTs. These results indicate that incorporating C-MWCNT into ECTs may greatly improve their physical characteristics, which may have applications in the field of connective tissue repair Based on these findings, Assali et al. (2022) came up with a novel solution to the problem of carbon nanotubes' (CNTs') poor dispersibility in water. They used a hydrophilic linker produced from polyethylene glycol (PEG) to connect a non-covalent chemical substance called pyrene to the surface of the CNTs. A hydroxyl or carboxyl group might be found in this linker. The purpose of this innovative technique for mixing CNTs in water was to study the effect on certain physical and structural features of synthetic tissues made from collagen. They believed that this method would keep the sp2 nanotube structure and π-conjugated structure of CNTs intact, which would make them more useful for tissue engineering applications that require good electrical conductivity, like creating artificial heart and nerve tissues. The research discovered that the modification of CNTs was successful and that the engineered tissue samples produced using 3T3 cells with varying concentrations of functionalized CNTs displayed a notable increase in the ability to conduct electricity was observed at a concentration of 0.025%. However, it was also noted that cell viability decreased by around 10-20%. The study also found that the samples of engineered tissue that has f-CNTs in them had less fibrosis and lower levels of porousness when compared to the control samples. Overall,

the study implies that the engineered tissue samples have great potential for further examination in living organisms [105].

1.7 Aims and objectives

1.7.1 General aim

Generation of engineered dermis tissue (EDT) consists of a collagen-chitosan-MWCNT complex with and without Ang II for utilization in wound healing.

1.7.2 Specific objectives

- Generation of EDTs based on 3T3 fibroblasts cells and ECM that is composed of a mixture of collagen, chitosan, and different concentrations of MWCNT.
- Transplantation of the EDTs in a mouse model of a full-thickness wound to investigate the potential healing properties.
- Generation of EDTs containing Ang II in the ECM and testing their potential wound healing properties.
- Optimization of primary mouse dermal fibroblast isolation, utilizing them in EDTs generation.

Chapter Two

Materials and Methods

2.1 Materials

2.1.1 Experimental animals

In this investigation, black mice strain C57BL/6 was used. Their ages ranged from 4 to 6 weeks, and they weighed in a range between 18 to 29.8 g. The animals were kept separately in cages that met standard lighting and temperature requirements and they had free access to food and water. The animal handling and treatment protocol was in compliance with the guidelines of the Ethics Committee of the International Association for the Study of Pain. The protocol was also reviewed and approved by the institutional review board (IRB) at An-Najah National University.

2.1.2 Chemicals and reagents

Calcium-free Dulbecco's phosphate-buffered saline (DPBS) (REF # 02-023-1A), Dulbecco's Modified Eagle Medium (DMEM) (REF # 01-055-1A), and penicillin/streptomycin solution (pen/strep) (REF #03-031-1B) were purchased from (Biological industries, Jerusalem). Trypsin-EDTA solution 1X (Catalog # 59417C), Fetal bovine Serum (FBS) (catalog # C8065), DMEM powder (Catalog # 56436C-10L), L-glutamine solution (REF # 03-020-1B), Bovine skin collagen solution (Catalog # C4243-20ML), Angiotensin II human ≥ 93% (HPLC) powder (1mg) (Lot # SLCC4027), Chitosan powder (CAS912764), and Xylazine (Sigma-Aldrich Catalog # X1126-1G) were purchased from (Sigma-Aldrich, USA). Ketamine (as HCL) 50 mg/ml (CA-LA SH110617) was purchased from (Pfizer, USA). Sodium Bicarbonate (NaHCO3) powder (Catalog # 5553360) and Formaldehyde solution (Lot # 210010 No. 015) were purchased from (Frutarom, Jerusalem). Sodium chloride (NaCl) powder (REF # 1G5106384) was purchased from (SDFCL, Mumbai). Sodium phosphate dibasic anhydrous (HO4PNa2) Powder (Lot # 71980) was purchased from (Riedel-de Haën, Germany). Acetic acid glacial 99.85% (CH3COOH) solution (Lot # SSBP.17.01.04) was purchased from (Carlo Erba company). MWCNTs powder was obtained from (Nanostructured and Amorphous Materials, USA). Isoflurane 99.9% solution for inhalation (Lot # 6041795) was purchased from (Abbott company).

Propylene glycol was purchased from Omega Raw Materials Drugstore Company). Normal saline (Batch No. 181168141) was obtained from (Braun company).

2.1.3 Instrumentation and software

Table 1

List of instruments

	Instrument	Model/ Company	Use
1	Hood	Protech AFA 1000 (Biosafety cabinet, MRC company)	To provide an aseptic working area
2	Inverted microscope	XDS2 Company	To observe the cultured cells
3	Digital microscope images	Using the Leica ICC50 HD camera from Leica Camera AG, located in Wetzlar, Germany	For histopathological evaluation
4	Refrigerated centrifuge	Hettich Micro 220 R	Used to precipitate the cells from cell suspension
5	Cell culture CO2 incubator	Esco	Used to incubate the cell line
6	Finnpipette, Micropipette	Thermofisher	Used in pipetting
7	Pipette controller	Heathrow Scientific RF3000®	Allow us to quickly fill and release accurate volumes
8	A hemocytometer		Used to count the cells
9	Electrical Vortex	JP, Selecta	Used for mixing
10	Electrical Balance (0.001g to 150g)	Adventurer	Used for accurate measurement of the weight of materials
11	Sonicator	BRANSONUSA, Model CPX3800HE	Used for evenly dispersing nanoparticles in liquids
12	Hot Air Sterilizing Cabinet	GRX-9053A Model	Used to sterilize small surgical instruments, glass, Petri dishes, etc.
13	Hot plate	Fried Electric	To heat samples

Table 2

List of Software

Software	Version
ImageJ	1.52i
GraphPad Prism®	9.5.1
Microsoft Office®	Microsoft 365

2.2 Methods

2.2.1 Culturing and maintenance of 3T3 cells

2.2.1.1 Preparation of cell culture growth medium with 10% FBS (1X DMEM)

To a 500 ml bottle of DMEM (containing 4.5 g/L glucose), 5 ml of L- glutamine (1%), 5 ml of pen/strep (1%), and 50 ml of fetal bovine serum (FBS) were added and mixed gently.

2.2.1.2 Culturing and maintenance of 3T3 cell line

The content of a vial of frozen 3T3 cells was quickly thawed and transferred to a falcon tube containing 25 ml of the growth medium, after gentle trituration, the cell suspension was transferred to a T-75 flask, which was then incubated in a cell culture incubator (temperature 37ºC, humidity 99% and 5% CO_2) with the medium being changed every other day until the cells were roughly 90% confluent. The time required to achieve this was about two days.

2.2.1.3 Subculturing of 3T3 cells

The old medium was eliminated, and DPBS was used to wash the cells twice. The cells were then treated with 0.05% trypsin. The trypsin solution was added in a volume that was just enough to cover the plate surface (1-2 ml). Afterward, the flask was incubated for 5–10 minutes at 37 °C, 5% CO2, and 99% humidity after most of the cells detached, DMEM growth medium was added to inactivate trypsin. The cell suspension was collected in a 50 ml falcon tube and was then distributed to new culture flasks in a ratio of 1:3.

2.2.2 Generation of engineered dermis tissue (EDT) with 3T3 fibroblasts cell

2.2.2.1 Coating cell culture plates with ٢٠% gelatin gel

Three grams of gelatin powder were mixed with 15 ml of DBPS in a beaker to prepare 20% gelatin gel. The mixture was then heated on a hot plate with stirring until the gelatin powder was entirely dissolved. The beaker was left to cool down to about 60ºC and then approximately 300 µl of the warm gelatin solution was poured into a 48-well plate to create a coat of gelatin gel on the bottom of the wells. The coated plates were then kept in the refrigerator (0–4 °C) for at least 6 hours before use.

2.2.2.2 Preparation of 0.1% NaOH solution

Forty milligrams of NaOH were dissolved in 40 ml of distilled water (D.W) and then it was filtered through a membrane of 0.22 µm pore size to ensure sterilization.

2.2.2.3 Preparation of chitosan solution

First, 1% v/v acetic acid solution was prepared by adding 0.4 ml of glacial acetic acid to 40 ml of 0.9% normal saline (NS). Then, a 1.5% w/v chitosan solution was prepared by adding 300 mg of chitosan to 20 ml of the 1% acetic acid. The mixture was sonicated for 1 hour to entirely dissolve chitosan.

2.2.2.4 Preparation of chitosan-coated multi-wall carbon nanotube (MWCNT) suspension

A volume of 1200 µl of 1.5% chitosan solution was added to an Eppendorf tube containing 50 mg of MWCNT. Then, the suspension was sonicated for at least 2 hours to disintegrate MWCNT aggregates and allow a sufficient interaction of MWCNT and chitosan, so that a dispersable chitosan-MWCNT (c-MWCNT) complex is formed. Afterward, the mixture was transferred to a 15ml-size falcon tube and autoclaved. The cap of the falcon tube was left slightly open. The tube was placed in a beaker containing distilled water during autoclaving to raise the humidity in the autoclave chamber to reduce water evaporation from the suspension. The suspension was allowed to cool down at room temperature. Then, the evaporated water volume was compensated with sterile distilled water.

2.2.2.5 Preparation of double concentration (2X) DMEM

This was done by adding 267.2 mg of DMEM powder, 74 mg of NaHCO3, and 2 ml of FBS to 18 ml of the ready-to-use 1x DMEM to obtain medium with double strength, the mixture was stirred for 30 minutes at room temperature, then it was sterilized by sterile filtration using 0.22 µm filter.

2.2.2.6 Preparation of Angiotensin II (Ang-II) solution

To prepare a 1 mM stock solution of Ang II, 956 µl water for injection was added to a vial containing 1 mg of Ang-II powder and mixed gently. Then it was aliquoted and stored at -20^{o}C.

2.2.2.7 3T3 cell suspension preparation with adjusted cell concentration

When cells were around 90% confluent, the cell suspension was collected as described above. By using a hemocytometer and an inverted microscope, the cell concentration was determined, from which the approximate total cell number was calculated depending on the total volume of the cell suspension. After that, the cell suspension was centrifuged at 1900 rounds per minute (RPM) and at a temperature of 4^{o}C for 7 minutes to precipitate the cells. The supernatant was removed, and then a calculated volume of cold fresh growth medium was added to achieve the desired cell concentration (12x 10^{6}cell/ml).

2.2.2.8 EDT preparation

The master formula required to prepare a single EDT of each of the investigational tissue types is presented in table 3. The method was adopted with modifications from Kittana et. al. and Assail et. al. with modifications [105, 106]. All components were stored in the refrigerator at 4°C and were kept ice chilled throughout the experiment. Each single EDT contained 400,000 cells.

To begin with, the control tissues that consist of collagen and chitosan were made by carefully mixing 50 µl of chitosan solution, 50 µl of collagen solution, and 100 µl of 2X DMEM in a pre-cooled Eppendorf tube (placed in an ice bath) to slow down collagen polymerization. The pH of the acidic mixture was neutralized with 0.1% NaOH as evidenced by turning the color of the mixture from deep purple to bright pink. Then, 33.33 µl of the cell suspension was added to the mixture and mixed gently well. The mixture was rapidly poured into a gelatin-coated well of a 48-well plate (300 µl/well). Tissues containing c-MWCNT were prepared by the same described method, except that the required volume of c-MWCNT suspension was added and homogenized just before adding the cell suspension.

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 seven days when the EDTs were ready for transplantation in a wound mouse model (Scheme1).

Table 3

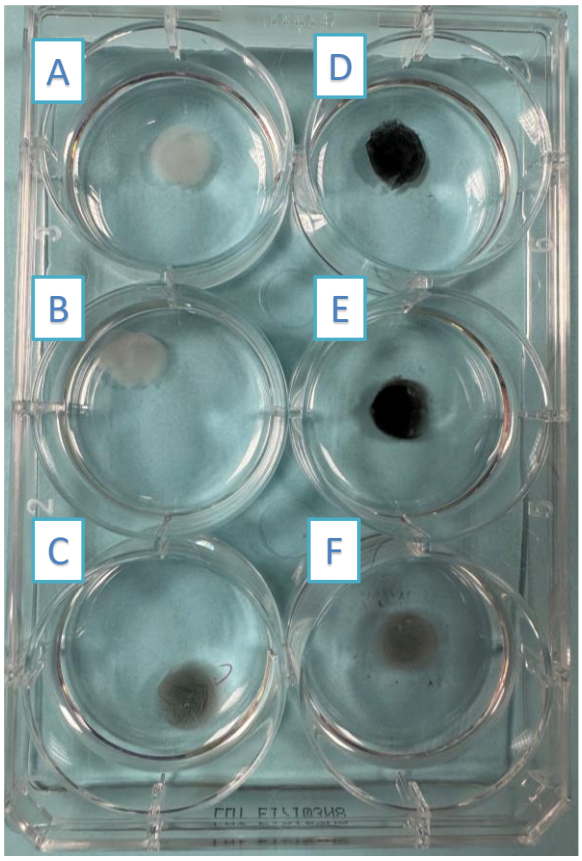
Master formula for a single EDT

	Collagen-chitosan tissue	Collagen-chitosan-0.1%MWCNT tissue	Collagen-chitosan-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
MWCNT	-	~8 µl	~2 µl
0.1% NaOH	90 µl	90 µl	90 µl
Cell Suspension (12x 10^6cell/ml)	33.33 µl	33.33 µl	33.33 µl
Total	~300 µl	~300 µl	~300 µl

For the experiments that investigate the effects of angiotensin-II (Ang-II) on the potential wound healing capacity of the tissue, similar tissues as described in table 3 were generated with the addition of 0.2 µl of a stock Ang-II solution (1 mM) 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 ٦٦٠ nM. Also, we added Ang II (1 µM) to the medium being replaced every other day for seven days (Scheme1).

Scheme 1

Macroscopic image of the EDTs



Note: (A) Control tissue iscomposed of collagen and chitosan. (B) composed of collagen, chitosan, and Ang II. (C) composed of collagen, chitosan, and 0.025% MWCNT. (D) composed of collagen, chitosan, and 0.1% MWCNT. (E) composed of collagen, chitosan, 0.1% MWCNT, and Ang II (F) composed of collagen, chitosan, 0.025% MWCNT, and Ang II.

2.2.3 Generation of engineered dermis tissue (EDT) with primary dermal fibroblasts

2.2.3.1 Preparation of primary dermal fibroblasts culture growth medium with 20 % FBS (1X DMEM)

To a 500 ml bottle of DMEM (containing 4.5 g/L glucose), 5 ml of L- glutamine (1%), 5 ml of pen/strep (1%), and 100 ml of fetal bovine serum (FBS) were added، and mixed gently.

2.2.3.2 Primary dermal fibroblast isolation and EDT generation

Neonatal mice were euthanized by applying cervical dislocation; the fur was removed with a hair-removing cream, and then the skin was sterilized with povidone-iodine and 70% ethanol. Large pieces of full-thickness skin were excised and washed with PBC twice under the hood. Then it was finely cut into small pieces that were collected in a 50 ml falcon tube and digested with 0.25% trypsin (5 ml) in the cell culture incubator (37 ℃ and 5% CO2) for 30 minutes with fine shaking every 5 minutes. The tissues from the previous step were distributed, and it has adhered to the surface of a six-well plate, and the cell culture growth medium was added to cover the tissue under the sterile laminar flow of the hood. Afterward, the six-well plate was maintained in the cell culture incubator (37 °C and 5% CO2, 99% humidity). Then, the next day, we added medium (2 ml) to each well under the sterile laminar flow of the hood. The media was changed every other day until the cells were around 90% confluent.

After that, EDTs were generated by the same methodology described for the 3T3 cells except that the number of cells per EDT was 750,000 cells.

2.2.4 In vivo Experiments: EDT implantation in a mouse wound model

2.2.4.1 Preparation of the anesthetics:

The anesthesia protocol followed the instructions and guidelines of the Vertebrate Animal Research guidelines of the University of Iowa, USA [107]. Firstly, the anesthesia cocktail was prepared (as described in table 4) by combining 800 µl of ketamine (5% w/v solution), 50 µl xylazine (10% w/v), and 1150 µl normal saline. Ketamine is an anesthetic to induce a loss of consciousness and relieves pain. Xylazine is a pharmaceutical drug used for animal sedation, anesthesia, muscle relaxation, and analgesia.

Table 4

The formula of anesthesia cocktails

Ingredient type	Volume
Ketamine (50 mg/ml)	800 µl
Xylazine 10% w/v with 0.9% NaCl	50 µl
Normal saline (NS)	Up to 2000 µl
Total	2000 µl

Secondly, Isoflurane is a volatile liquid; therefore, it was diluted in propylene glycol (20% V/V) to slow down its evaporation. A piece of cotton was wetted with 4 ml isoflurane/propylene glycol solution, then it was wrapped loosely with aluminum foil and was set at the bottom of a one-liter-sized glass jar with a tight lid.

2.2.4.2 Induction of anesthesia

One mouse was placed in the jar, and the lid was tightly closed. The mouse was monitored while in the jar, with particular attention to the breathing pattern. Once the animal has lost the righting reflex, and its breathing has slowed (~50%, i.e., 80–100 breaths/min) but is still regular, a state of anesthesia has been reached. The mouse was removed from the jar, and the mucous membranes' color, respiration rate, and withdrawal reflexes were checked.

2.2.4.3 Maintenance of anesthesia

Anesthesia was maintained by intraperitoneal injections with anesthesia cocktails at a dose of 0.05 ml/20 g. Then a cotton ball was wetted with the isoflurane/propylene glycol solution (0.5–1 ml). The cotton ball was placed at the bottom of a 15 ml falcon tube, which was laid horizontally on the table and fixed with tape. The anesthetized mouse was transferred from the anesthesia jar to the table and was placed on its abdomen in a position where its nose was partially inside the falcon tube to avoid over-anesthesia. Then the EDT transplantation process started after we checked that there were no reflexes and that the mucous membranes and respiration appeared stable.

2.2.4.4 Transplantation of EDTs

In the beginning, the dorsal surface of the mice was shaved with electric clippers, and the remaining hair was removed with a commercial depilatory agent. 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 (6 x 7 cm) to protect the wound bed and to prevent falling and dryness of the transplanted tissue. Then, the mouse was transferred away from the isoflurane tube and was watched until recovered from anesthesia. Then it was housed

individually 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. The skin biopsies were fixed in 10% formalin (Table ٥) for 48 hours and were then kept refrigerated in a PBS solution.

Table ٥

Preparation of 10% Formalin solution

Ingredient type	Volume/weight
35%Formaldehyde	100 ml
Sodium Chloride (NaCl)	9 g
Disodium hydrogen phosphate (HO4PNa2)	12 g
Distilled water	Up to 1000 ml

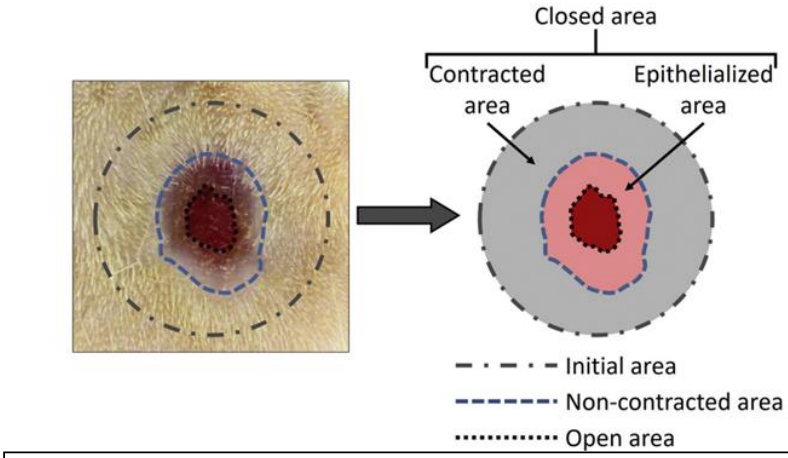
2.2.4.5 Morphometrical studies

We monitored the body weight of the mice during the experiment. The measurements were recorded on the day of the surgical procedure and two weeks later before the animals were euthanized.

For the macroscopic evaluation of the wound-healing process, the wounds were imaged on the day of surgery and the day of euthanasia (the 14^{th} day). The evaluation of the wound healing was performed according to Cifuentes et al. as shown in scheme 2 with modification [108]. ImageJ (NIH, Bethesda, MD, USA) was used to measure the following data; the initial wound area on the day of surgery, the contracted area, the non-contracted area, the epithelized area, the open area, and closed area on the day of euthanasia. These measurements were used to calculate four parameters; The relative change in the wound area is calculated by dividing the non-contracted area by the initial area. The percentage of wound contraction is obtained by dividing the contracted area by the initial area and multiplying the result by 100%. The re-epithelialization is expressed as a percentage of epithelization which is calculated by dividing the epithelized area by the initial area and multiplying it by 100%. Lastly, the percentage of wound closure is calculated by dividing the closed area by the initial wound area and multiplying the result by 100% (Scheme 2).

Scheme 2

Illustration of the parameters used to evaluate the process of wound healing



$$\text{Relative change in the wound area} = \frac{\text{Non- contracted area}}{\text{Initial area}}$$

$$\%\ \text{Contraction} = \frac{\text{Contracted area}}{\text{Initial area}}\ x100$$

$$\%\ \text{Epithelization} = \frac{\text{Epithelialized are}}{\text{Initial area}}\ x100$$

$$\%\ \text{Closure} = \frac{\text{Closed area}}{\text{Initial area}}\ x100$$

Note: the image was adopted from Cifuentes et al. with modification [108].

2.2.4.6 Histological analysis

The collected tissue biopsies were sent to the Histopathology Department at An-Najah National University Hospital, where the tissues were processed and stained with Masson-Trichrome stain according to the followed routine procedure employed for similar clinical samples. Then the tissues were histopathologically imaged using a digital microscope (Leica ICC50 HD camera from Leica Camera AG, located in Wetzlar, Germany). The thickness of the dermis and epidermis in the wound bed and outside the wound was measured by ImageJ software. Then we calculated the ratio of the thickness of the new epidermis at the wound bed to the thickness of the epidermis outside the wound (control). We also calculated the ratio of the thickness of the dermis in the wound bed, to the thickness of the dermis outside the wound (control). These

calculations were performed for both the wounds treated with the transplanted tissue and its corresponding negative control wound on the same mouse. Furthermore, we counted the blood vessels present within the wound beds to evaluate the angiogenesis process.

2.2.4.7 Statistical analysis

The collected data were statistically analyzed by GraphPad Prism version 9.5.1 for Windows, which was developed by GraphPad Software, San Diego, California USA, www.graphpad.com". We used the Two-Way ANOVA statistical test, to compare the means. The results were reported as the mean and the standard error of the mean. The level of significance was set at alpha ≤ 0.05, so a difference is considered significant when the *p*-value is ≤ 0.05 (*); $p \leq 0.01$ (**); $p \leq 0.001$ (***).

Chapter Three

Result and Discussion

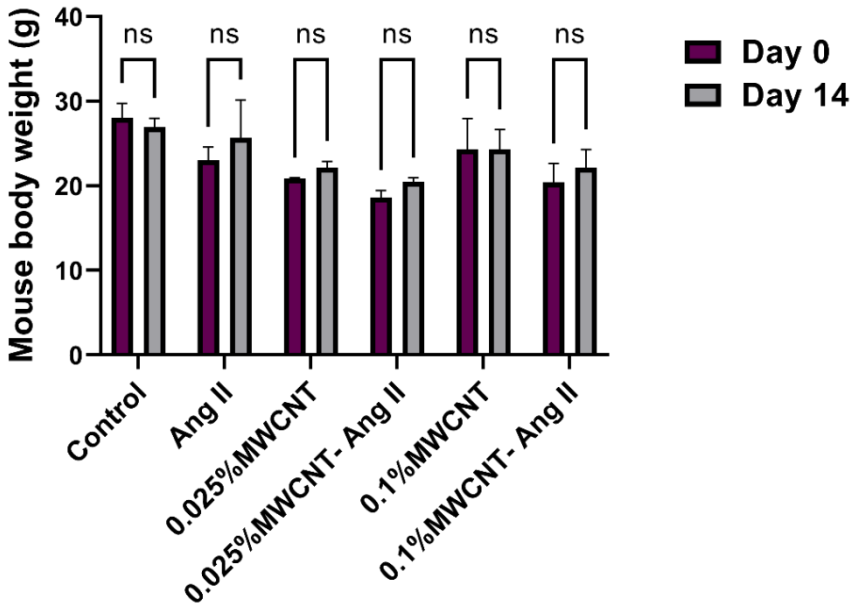
Chronic wounds remain a major burden for the healthcare system, despite advancements in their management. This is why research into novel and more effective wound care strategies continues to be conducted. By combining collagen and chitosan into a hydrogel supplemented with varying concentrations of chitosan-coated MWCNT, with or without the inclusion of Ang II, we hoped to provide a novel treatment alternative for chronic wound healing. Our collagen-chitosan-MWCNT three-dimensional scaffold has several applications in the treatment of chronic wounds. First, collagen mimics the natural dermis and adds mechanical strength to the scaffold. Antibacterial and anti-inflammatory properties of chitosan, a biopolymer produced from chitin, aid in infection prevention and speed up the healing process. The scaffold's mechanical characteristics are improved by MWCNT's incorporation [109]. These characteristics may hasten and improve wound healing by stimulating new cell development and tissue regeneration. Another potential improvement to the healing process is the addition of Ang II, a hormone that may control blood flow and inflammation. Once implanted in the wound, these scaffolds are hypothesized to facilitate the body's natural healing processes and hasten the wound's recovery.

3.1 Assessment of the animal's weight

A vital part of determining an animal's health is to measure and analyse its weight. Checking for weight changes that could suggest health issues or the safety of the test treatments entails measuring the animal's weight at particular intervals, comparing the data, and drawing conclusions. The mice in this study had their weights taken twice: first the day of operation, and again 14 days later, on the day of death. The major goal was to evaluate how the operation affected the mice's general health. According to the findings, the mice's weights did not change noticeably before and after the operation. This indicates that the therapy had no major deleterious effects on the mice's health (Figure 1).

Figure 1

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



3.2 Macroscopic evaluation of wound healing

The macroscopic evaluation of wound healing (figure 2) was carried out to investigate the effect of the transplanted tissues on wound contraction, re-epithelialization, and closure. The methods used to calculate these parameters were previously outlined above in section 2.2.4.5.

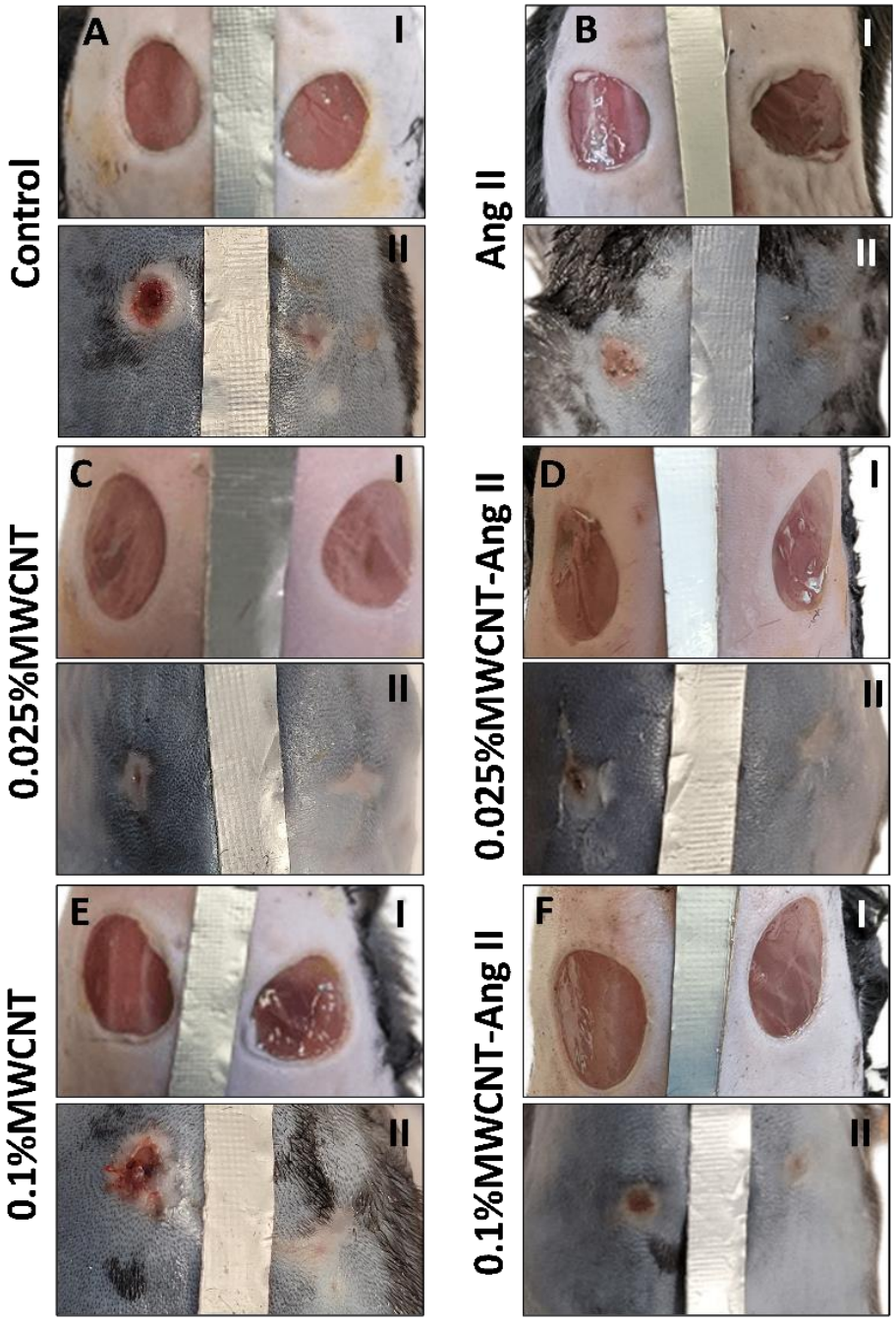
Wound contraction is an important aspect of wound healing, as it helps to reduce the size of the wound and bring the edges of the wound closer. However, excessive wound contraction can lead to scarring and permanent disfiguring of the skin, which can have a negative impact on the appearance and quality of the wound and possible restriction of the underneath organ movement [110]. Therefore, evaluating wound contraction is an essential parameter in evaluating the quality of wound healing under the tested treatment conditions.

The evaluation of the effect of the transplanted tissues on wound contraction was performed by quantifying two aspects of wound contraction; first, by calculating the relative change in the wound area (RCWA), and by determining the percentage of

wound contraction. The difference between these two concepts lies in the information they provide about the wound. RCWA is a useful tool for evaluating the change in wound size (the total area that is not fully healed) over a certain time interval, as it compares the current size of the wound to the original size. On the other hand, The percentage of contraction represents the reduction in wound area due to only wound contraction relative to the original wound size. In essence, the RCWA gives an understanding of the current size of the wound, whereas the percentage of contraction offers insight into the extent to which the wound has contracted compared to its initial size.

Figure 2

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



Note: The composition of the test tissue: (A) collagen and chitosan (control tissue); (B) collagen, chitosan, and Ang II; (C) collagen, chitosan, and 0.025% MWCNT; (D) collagen, chitosan, Ang II, and 0.025% MWCNT; (E) collagen, chitosan, and 0.1% MWCNT; and (F) collagen, chitosan, Ang II, and 0.1% MWCNT.

3.2.1 Relative change in the wound area (RCWA) and percentage of wound contraction

As shown in figure 3A, there was a significant increase in the RCWA between the wound treated with control tissue and its negative control by about 97%. This was close to the RCWA for the wounds treated with the tissues that contain Ang II, 0.025% MWCNT-Ang II and 0.1%MWCNT-Ang II, where the RCWA was 93%, 133%, and 103% respectively.

The results illustrated in figure 3A showed that most wounds treated with transplanted tissue had a higher RCWA compared to the negative control wound on the same mouse. This suggests that the contraction of the wound treated with transplanted tissue was lower compared to the negative control wound.

In line with the data on the RCWA, the percentage of wound contraction was generally lower in the wounds that received transplanted tissues compared to their respective negative control wounds on the same mice (Figure 3B). According to the study's findings, there was a significant decrease in the percentage of wound contraction for the wounds treated with control tissues compared to the negative control, by 11%. Similarly, the wounds treated with Ang II tissue showed a 10% decrease compared to the negative control. In addition, there was a significant difference observed between the wound treated with 0.025% MWCNT-Ang II tissue and its negative control, which showed a decrease of 19%, as well as between the wound treated with 0.1% MWCNT-Ang II tissue and its negative control, which showed a decrease by 11%.

There was a substantial difference between the percentage of wound contraction in the transplanted tissue wound and the negative control wound. Because the dermis layer, which was transplanted in the wound, resists the typical contraction process, it shows that the transplanted tissue may have a favorable influence on the wound-healing process. Because of the reduced pace of contraction, epithelization of the wound may proceed more smoothly, leading to better healing. These findings may have a significant bearing on future efforts to improve the management of wounds, especially those that heal slowly or have the propensity to scar excessively. Finally, the discovery that transplanted tissue shows less wound contraction than negative control wounds is encouraging.

Additionally, it has been suggested that lower levels of wound contraction are related to the presence of Ang II in transplanted tissues. Statistically significant changes in wound contraction were seen between Ang II-containing transplanted tissue and its negative control in our investigation.

Takeda H. and colleagues have previously studied the expression of the Ang II receptors AT1R and AT2R in keratinocytes and fibroblasts, two key cell types in the process of skin wound healing. They found that keratinocytes and fibroblasts from both humans and mice express AT1R, but that only fibroblasts from both species express AT2R. In addition, they used wound healing tests to look at how activating or inhibiting AngII receptor signalling affected the migration of myofibroblasts and the incorporation of BrdU, a cell growth marker. AngII and PD-123319, a selective AT1 signalling agent, were found to increase BrdU incorporation and speed up myofibroblast recovery, while CV-11974, a specific AT2 signalling agent, was found to decrease BrdU incorporation and slow down myofibroblast recovery. These results imply that AT1 signalling promotes myofibroblast development and migration during wound healing, whereas AT2 signalling inhibits these processes. These findings support the idea that harmonious signalling between these receptors is essential for effective wound healing [30].

Wound healing is aided by myofibroblasts, which are specialized cells. They are essential in the process of wound closure and tissue restoration. Stress fibers and contractile machinery are formed when these cells are triggered by substances like TGF- which prompts them to differentiate from fibroblasts. Wound closure and wound bed stabilization are aided by myofibroblasts because of their ability to create and organize collagen and other extracellular matrix components. Furthermore, they release cytokines and growth factors that recruit and activate other cells to aid in the healing process. Fibrosis, the abnormal buildup of extracellular matrix (ECM) proteins that may cause tissue dysfunction, is a pathological condition in which myofibroblasts play a role. In some diseases, myofibroblasts persist beyond the normal healing process and continue to produce ECM, leading to chronic fibrosis and tissue damage. From the information presented, it can be inferred that maintaining a balanced population of myofibroblasts is crucial [111].

Heng-Jun Wu and colleagues conducted a research study to investigate the changes in Ang II and its receptors (AT1 and AT2) during the wound healing process in mice.The findings revealed that Ang II levels increased in the first seven days and then declined, while the expression of AT1 and AT2 receptors followed a similar pattern. Interestingly, AT2 expression increased again after the wound epithelialization phase [32].

Based on this background, we hypothesize the inclusion of Ang II in our tissue samples and media, which were refreshed every other day, potentially facilitated the activation of Ang II type 2 receptor (AT2R). This activation may have contributed to a decrease in the contraction process while still maintaining the balanced signaling between AT1R and AT2R receptors.

In addition, we conducted a visual inspection of the wound contraction and observed that negative control wounds exhibited noticeable deformities, whereas such deformities were much less in wounds treated with transplanted tissue (Figure 2).

3.2.2 Wound closure

Our study aimed to determine the impact of transplanted tissue on the recovery process. To do this, we calculated the percentage of wound closure by comparing the area of the wound on day 14 after surgery to the area of the wound on the day of surgery. This number represents how much the wound has healed at the end of the two-week observation period.

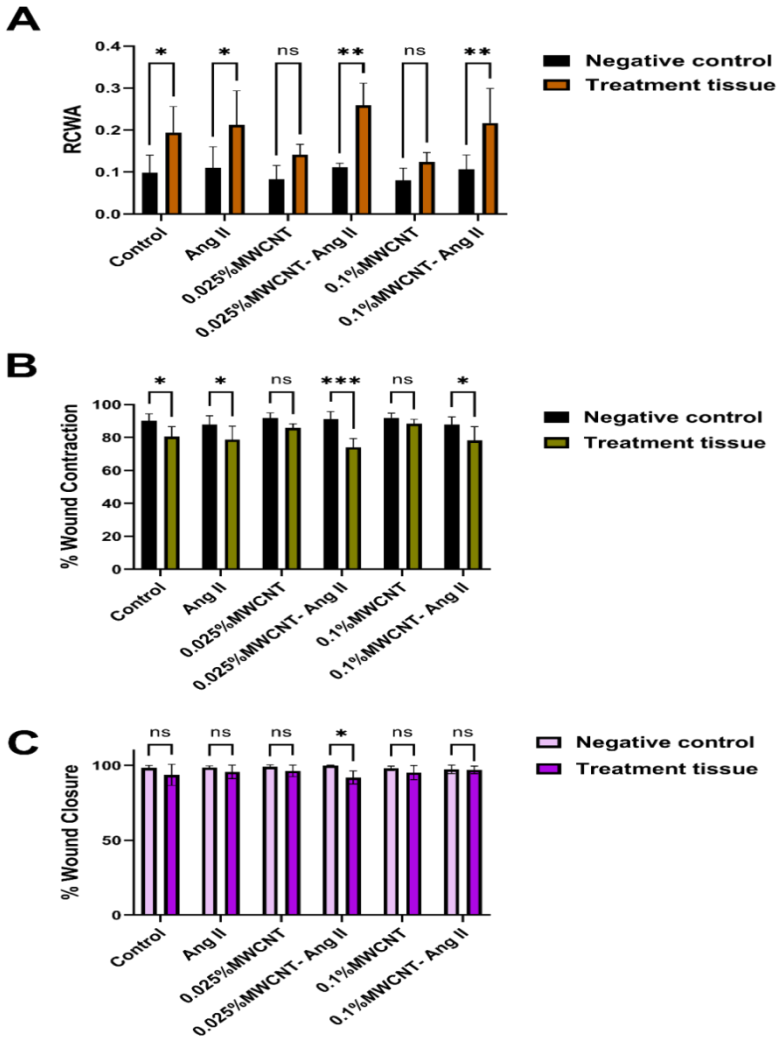
There was no statistically significant difference in the percentage of wound closure for the transplanted tissue group and the control group. Mice treated with 0.025% MWCNT-Ang II showed a modest delay in wound closure compared to controls (Figure 3C).

This indicates that throughout the two-week research period, the presence of transplanted tissue has little effect on the pace at which the incision heals. Wound closure was not significantly altered in untreated mice, although it was somewhat slowed in animals treated with 0.025% MWCNT-Ang II. This shows that this therapy may have a negligible anti-healing impact.

Overall, our investigation suggests that while tissue transplantation may offer benefits in terms of tissue regeneration and healing, it does not significantly alter the overall percentage of wound closure compared to negative control wound.

Figure 3

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



Note: (A) A statistically significant increase in RCWA is shown in wounds treated with the transplanted tissues in four conditions: Control, Ang II, 0.025% MWCNT-Ang II, and 0.1% MWCNT-Ang II tissues. (B) The treatment with the control tissues and the tissues containing Ang II, 0.025% MWCNT-Ang II, and 0.1% MWCNT-Ang II resulted in a significant decrease in the % of wound contraction. (C) No significant differences in the percentage of wound closure between each treatment pair were observed, except for the mice that were treated with 0.025% MWCNT-Ang II tissue. The data is presented as mean ± standard error mean. N= 3-5 for each group. The level of significance was set at a p-value ≤ 0.05.

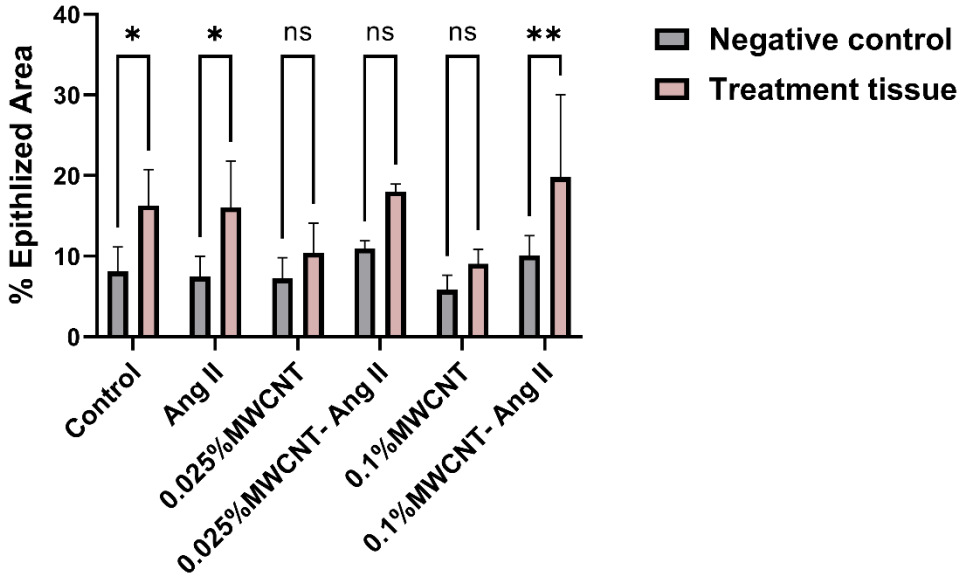
3.2.3 Evaluation of wound epithelialization

Here we studied the impact of the transplanted tissue on the extent of epithelization, which is the formation of a new epithelial layer over a wound to re-establish a functional barrier and promote faster healing. This was done by calculating the percentage of epithelization by taking the percentage of the area that had undergone epithelization on day 14 to the initial area measured on the day of transplantation surgery.

The results showed that compared to the corresponding control wound, the transplantation of the control tissue, Ang II tissue, and 0.1% MWCNT-Ang II significantly increased the percentage of wound epithelization by 100%, 115%, and 96% respectively (Figure 4), which indicates a faster and more effective healing process compared to the negative control.

Figure 4

Comparison of the average percentage of epithelization between wounds treated with the test transplanted tissues and the corresponding negative control wounds. A statistically significant increase was observed by the transplanted tissues labeled Control, Ang II, and 0.1% MWCNT-Ang II tissues. Data presented as mean ± standard error mean. N= 3-5. The level of significance was set at p-value ≤ 0.05



Moreover, Our study showed a significant difference in the process of epithelization between the transplanted tissue containing Ang II, and a negative control, and this indicates that the association between the presence of Ang II and the increase in epithelization is meaningful. As mentioned earlier, Takeda et al.'s study focused on the expression of AT1R and AT2R in keratinocytes and fibroblasts, which are important for skin wound healing. They discovered that both human and mouse keratinocytes and fibroblasts expressed AT1R, whereas only human and mouse fibroblasts expressed AT2R. Activation of the AT1 receptor promotes skin cell repair and regeneration, while activation of the AT2 receptor has the opposite effect. Therefore, the balance between these two receptors is crucial in regulating the skin wound healing process [30].

Furthermore, the researcher previously found that angiotensin AT1 and AT2 receptors are upregulated in human skin wounds within the first week of the injury [31, 112]. Additionally, Yahata Y. and colleagues reported that Ang II boosted the movement of keratinocytes and fibroblasts in a dose-dependent manner by activating AT1R. This led to the release of a growth factor called heparin-binding EGF-like growth factor (HB-EGF), which in turn stimulated another receptor, the epidermal growth factor (EGF) receptor, that is essential for the growth and differentiation of cells [29].

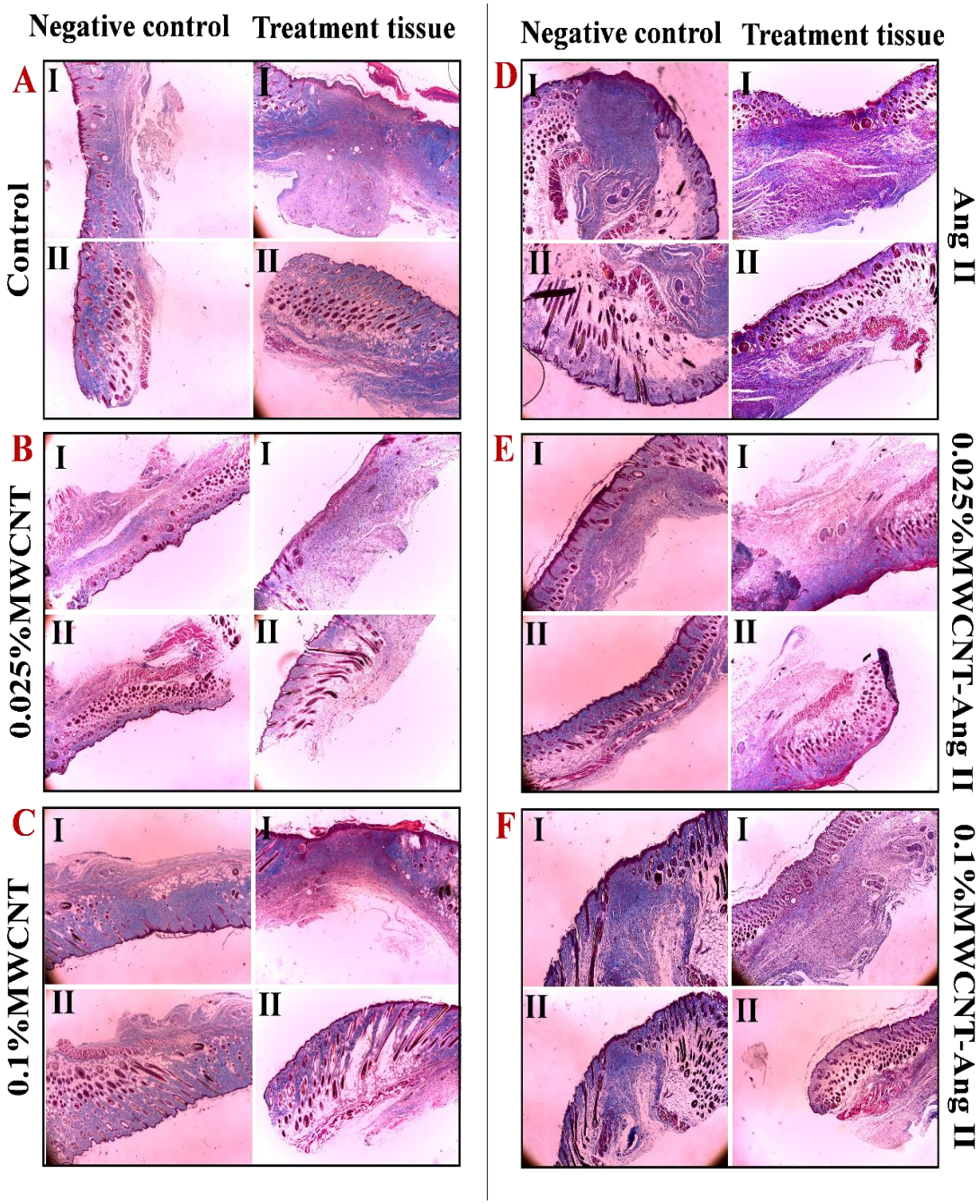
Based on the information from previous studies, we hypothesize that the movement of keratinocytes and fibroblasts toward a wound can accelerate the epithelization process. Keratinocytes form the outer layer of the skin and fibroblasts produce collagen and the extracellular matrix that provide structural support to the skin. Increasing their migration rate helps them reach the wound area faster, thus speeding up the epithelization process. Keratinocytes create a protective layer over the wound, while fibroblasts promote closure and healing by producing matrix and collagen. This elevated cell migration enhances epithelization efficiency, leading to faster wound healing. In addition, we can also attribute the increase in epithelization to the slower rate of contraction, which facilitates more effective epithelization of the wound and ultimately leads to improved quality of the healing outcomes.

3.3 Microscopic evaluation of wound healing

skin tissue samples excised from the wound area were processed, sectioned, and stained with a Masson-trichrome stain (Figure 5). The thickness of the dermis and epidermis at the wound bed and outside the wound was measured using ImageJ software. Two ratios were calculated; First, the thickness of the new epidermis (epidermis formed in the wound bed) relative to the thickness of the epidermis outside the wound (control). Second, the thickness of the new dermis (in the wound bed) in comparison to the thickness of the dermis outside the wound (control). The ratios mentioned above were calculated for both the wound treated with transplanted tissue and the negative control wound.

Figure 5

Histological evaluation of tissue sample sections stained by the Masson-trichrome stain. The images were captured for regions outside the wound bed (I), to serve as an internal negative control for each section and inside the wound bed (II) to evaluate the histological effects of test tissue transplantation



Note: A) Control tissue, B) 0.025% MWCNT tissue, C) 0.1% MWCNT tissue, D) Ang II tissue, E) 0.025% MWCNT-Ang II tissue, F)) 0.1% MWCNT-Ang II tissue.

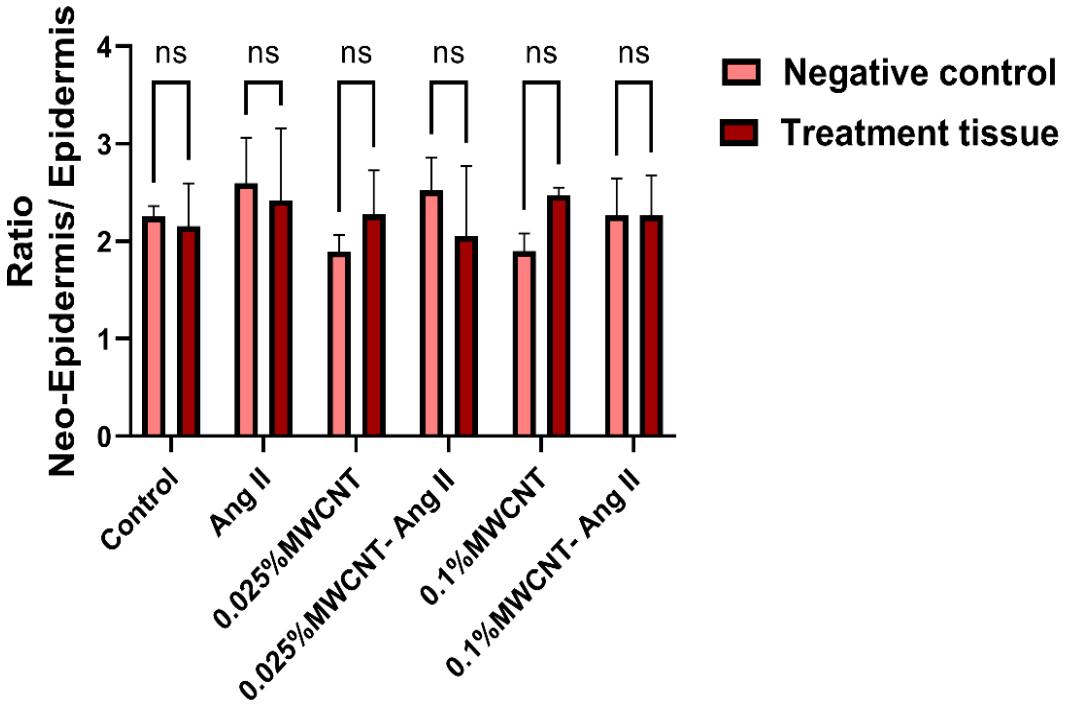
٣،٣،١ Investigating the effects of EDT transplantation on the epidermis thickness

The data indicates that there were no noticeable distinctions between the wounds treated with any of the transplanted tissues and the negative control wounds in terms of the ratio of new epidermis thickness (the epidermis that forms inside the wound) to the thickness of the epidermis outside the wound (Figure 6).

Taken together, the data demonstrate 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.

Figure 6

Effect of test tissues transplantation of neo-epidermis thickness. The data compares the ratios of the newly formed epidermis to the pre-existing epidermis in wounds treated with transplanted test tissues and the corresponding negative control wounds. The results show that there were no significant differences observed between any pair. Data presented as mean ± standard error mean. N= 3-5 for each group. The level of significance was set at p-value ≤ 0.05

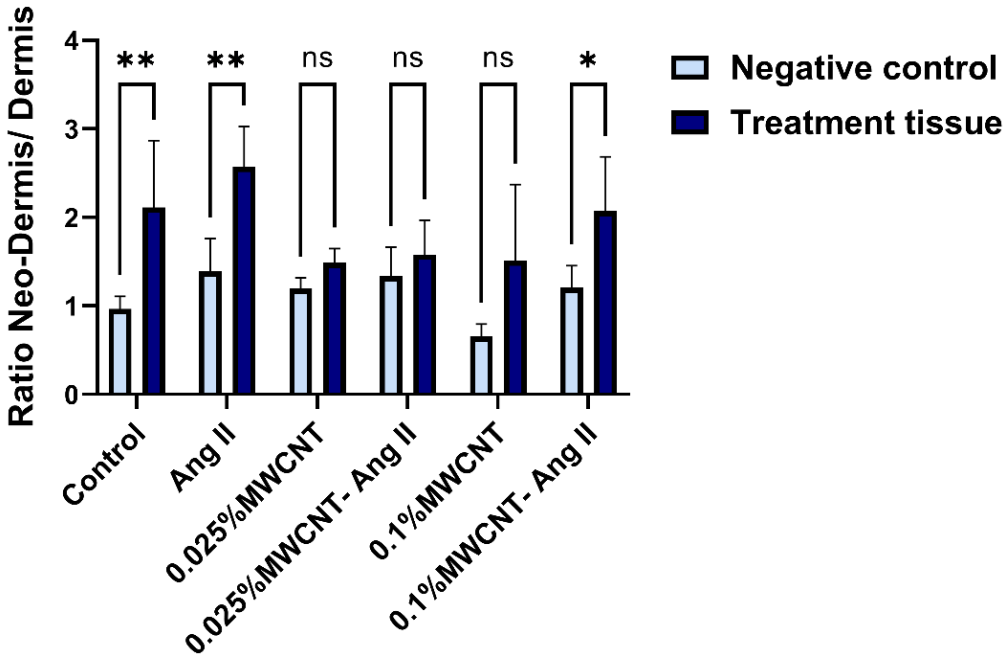


3.3.2 Investigating the effects of EDT transplantation on the thickness of the dermis

The analysis of the data revealed that the ratio of the thickness of the newly formed dermis inside the wound to the thickness of the dermis outside the wound was higher in wounds treated with transplanted tissues compared to the corresponding negative control wounds, as shown in Figure 7. However, this increase was only observed to be statistically significant in three conditions: control tissue, Ang II tissue, and 0.1%MWCNT-Ang II tissue by 119%, 85%, and 72% respectively. This suggests that the observed differences in these conditions indicate that they are unlikely to be due to chance, but rather represent a potential effect of the treatment. We hypothesize that the presence of the transplanted dermis layer contributes to this outcome.

Figure 7

Effect of test tissue transplantation on the formation of a dermis layer in the wound bed. The results show that there was a significant increase in the thickness of the neo-dermis with the test tissues Control, Ang II, and 0.1% MWCNT-Ang II as compared to the corresponding negative control wounds. Data presented as mean ± standard error mean. N= 3-5 for each group. The level of significance was set at a p-value ≤ 0.05



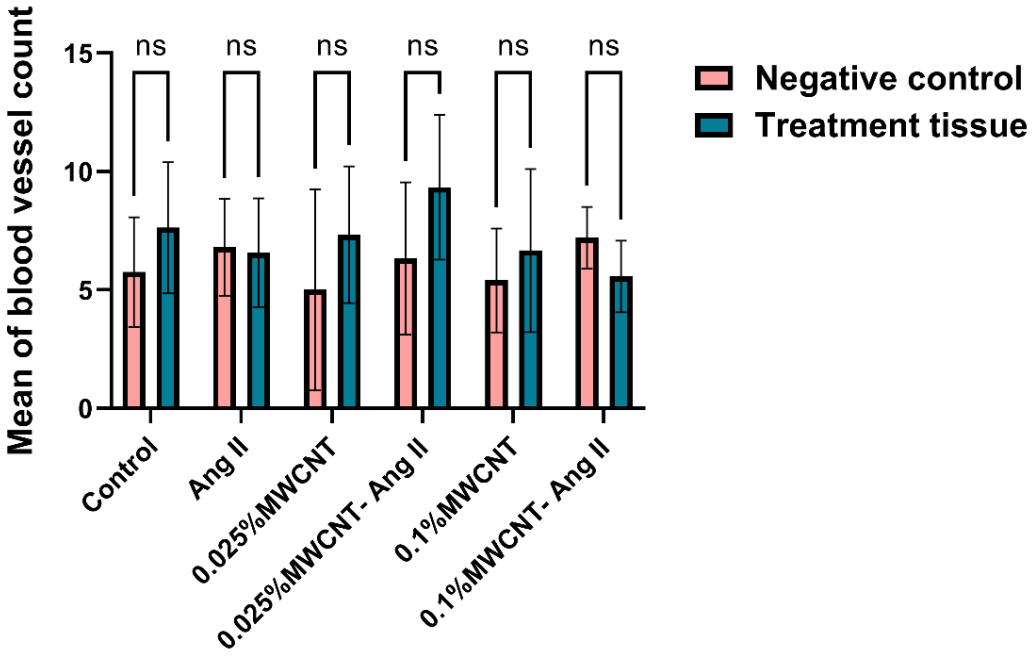
3.3.3 Effect of EDT transplantation on angiogenesis

Ang II is a peptide that possesses several biological functions including the regulation of blood pressure and the promotion of angiogenesis. Kurosaka M. and colleagues studied the role of angiotensin II (Ang II) in wound healing and angiogenesis. they used mice that lacked the Ang II receptor type-1 (AT1-R) to investigate the role of this receptor in Ang II-mediated wound healing and angiogenesis. They found that mice lacking the receptor had significantly reduced wound healing and angiogenesis, and that treatment with an AT1-R antagonist delayed wound healing and decreased the expression of vascular endothelial growth factor (VEGF), which is an important factor for the formation of new blood vessels. They concluded that Ang II-AT1-R signaling plays an important role in wound healing and angiogenesis [113].

Based on these findings, we hypothesized that the enrichment of our EDT with Ang II molecules could promote angiogenesis. However, the results of our investigation showed that there was no significant difference in the number of blood vessels observed in the wound area between the group treated with transplanted tissues (with or without Ang II) and the negative control group (Figure 8). This indicates that our tissues are not expected to affect the angiogenesis process during wound healing. We hypothesize that the failure of Ang II in promoting angiogenesis might be due to the instability of Ang II molecules in the tissues for a period enough to produce a significant effect.

Figure 8

The effect of Ang II on the extent of angiogenesis in the wounds that received tissue transplantation compared to the corresponding negative control wounds. The results indicate that there were no significant differences in the number of blood vessels observed between the group pairs. Data presented as mean ± standard error mean. N= 3-5 for each group. The level of significance was set at a p-value ≤ 0.05



3.4 Isolation and Generation of EDTs based on primary fibroblasts

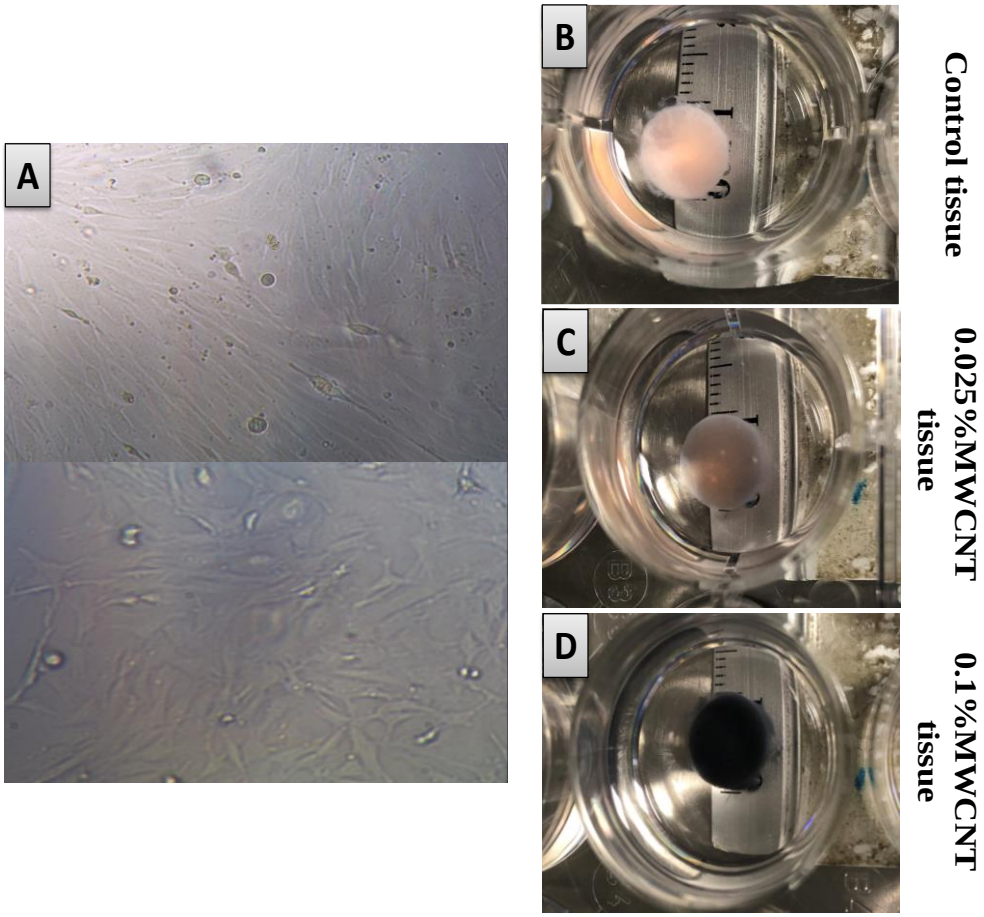
Primary cells are cells that are directly isolated from living tissues. Primary dermal fibroblasts possess natural physiology and characteristics that make them well-suited for generating complex and functional tissues such as engineered dermal tissue (EDT). In addition to their natural physiology and characteristics, primary fibroblasts also offer several other advantages for tissue engineering applications including their ability to be effective in creating functional tissues with the desired architecture and extracellular matrix composition. Moreover, they can control the production of extracellular matrix components more precisely, resulting in the formation of tissues with the desired architecture and composition. This is particularly critical for the formation of functional EDT. Moreover, primary fibroblasts exhibit a more diverse and complex molecular profile compared to 3T3 cells, which are an established cell line that has undergone numerous rounds of subculturing, leading to the accumulation of many genetic changes. In addition, primary fibroblasts have a limited lifespan and are less likely to undergo

spontaneous immortalization, as compared to established cell lines. Also, primary fibroblasts are capable of forming a more natural and integrated tissue architecture compared to established cell lines. This is because primary fibroblasts can interact with other cells more naturally, and they are better able to support cell-to-cell communication and signaling. Another advantage to the primary fibroblasts is their lower tendency to accumulate genetic mutations and chromosomal abnormalities compared to established cell lines. This can reduce the risk of tumorigenesis or other undesired effects in the engineered tissue. In addition, primary fibroblasts are more responsive to environmental signals and can better mimic the in vivo environment of the target tissue. Prospectively, primary fibroblasts can be isolated from patient's own tissues, allowing for the creation of patient-customized engineered tissues that are more representative of the individual's biology and may be more effective for personalized medicine applications. Overall, primary fibroblasts offer many advantages over established cell lines for tissue engineering applications due to their natural physiology, genetic stability, responsiveness to environmental cues, Efficiency of tissue formation, patient-specific biology, and improved tissue integration [114-117].

The primary fibroblasts grew out of the cultured neonatal dermis sections within 3-5 days, and they continued to migrate and proliferate until they reached a confluency of about 90% after 15-21 days (figure ٩A). After that, the cells were collected by trypsinization and were used to generate EDTs as explained before. The generated tissues appeared more condensed and tenacious than those based on 3T3 cells (figure 9B-D), indicating that the fibroblasts were able to contract the matrix much more efficiently than with 3T3 cells. Therefore, we hypothesize that primary fibroblasts-based EDTs would better resemble the natural dermis when transplanted in vivo for future work.

Figure 9

Generation of primary fibroblasts-based EDTs



Note: A) primary fibroblasts that were isolated from neonatal mice and observed under an inverted microscope. B) EDT composed of collagen and chitosan. C) EDTs composed of collagen, chitosan, and 0.025% MWCNT D) EDTs composed of collagen, chitosan, and 0.1% MWCNT.

Chapter Four

Conclusion and Prospects

4.1 Conclusion

The conclusion drawn from our study highlights the significant role of transplanted tissue in improving the quality of wound healing. We observed that the presence of the transplanted tissue, particularly those containing Ang II, had a positive impact on the wound healing process. Notably, it promoted the process of epithelialization, which is crucial for the formation of a protective barrier over the wound site. Additionally, the transplanted tissue demonstrated a reduction in contraction, which is particularly important for wounds that are prone to slow healing or high-risk 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.

4.2 Prospects

We are looking forward to developing a method for a better approach to delivering Ang II in the transplantation area. In addition, future studies are required to investigate the effect of EDT-based on primary skin fibroblasts on wound healing. Moreover, we aspire to optimize the isolation and cultivation of keratinocytes to add an engineering epidermis layer on top of our current EDT to construct an engineered skin tissue, and to assess its impact on wound healing. Such tissue can provide valuable information for the creation of potential therapies for chronic wounds.

List of Abbreviations

Abbreviation	Meaning
ATI	Angiotensin I
ATII	Angiotensin II
AT1R	Angiotensin type 1 receptor
AT2R	Angiotensin type 2 receptor
Brdu	Bromodeoxyuridine
c-MWCNT	Multi-wall carbon nanotubes coated with chitosan
CNTs	Carbon nanotubes
DPBS	Calcium-free Dulbecco's phosphate-buffered saline
DWCNTs	Double-walled carbon nanotubes
ECM	Extracellular matrix
ECTs	Engineered connective tissues
EDTs	Engineered dermic tissues
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
EST	Engineered skin tissue
ESTs	Engineered skin tissues
f-CNTs	Functionalized carbon nanotubes
FGF	Fibroblast growth factors
GA	Glutaraldehyde
HB-EGF	Heparin-binding EGF-like growth factor
HFF-1	Human foreskin fibroblasts
HFP	Hexafluoro propanol
MWCNTs	Multiwalled carbon nanotubes
PDGF	Platelet-derived growth factor
ROS	Reactive oxygen species

RT-PCR	Reverse transcription polymerase chain reaction
SWCNTs	Single-walled carbon nanotubes
TFE	Trifluoroethanol
TGFβ	Transforming growth factor-beta

References

[1] Mathieu, D., *Handbook on hyperbaric medicine*. Vol. 27. 2006: Springer.

[2] Menke, N.B., et al., *Impaired wound healing.* Clinics in dermatology, 2007. 25(1): p. 19-25.

[3] Hopman, W., et al., *Associations between chronic disease, age and physical and mental health status.* Chronic Dis Can, 2009. 29(3): p. 108-16.

[4] Nicholas, M.N. and J. Yeung, *Current status and future of skin substitutes for chronic wound healing.* Journal of cutaneous medicine and surgery, 2017. 21(1): p. 23-30.

[5] Simpson, D.M. and R. Ross, *The neutrophilic leukocyte in wound repair a study with antineutrophil serum.* J Clin Invest, 1972. 51(8): p. 2009-23.

[6] Frykberg, R.G. and J. Banks, *Challenges in the Treatment of Chronic Wounds.* Adv Wound Care (New Rochelle), 2015. 4(9): p. 560-582.

[7] Harries, R., J. Torkington, and K. Harding, *Advances in Acellular Extracellular Matrices (ECM) for Wound Healing.* Skin Tissue Engineering and Regenerative Medicine, 2016: p. 125.

[8] Guo, S. and L.A. Dipietro, *Factors affecting wound healing.* J Dent Res, 2010. 89(3): p. 219-29.

[9] Zhong, S.P., Y.Z. Zhang, and C.T. Lim, *Tissue scaffolds for skin wound healing and dermal reconstruction.* Wiley Interdiscip Rev Nanomed Nanobiotechnol, 2010. 2(5): p. 510-25.

[10] Krafts, K.P., *Tissue repair: The hidden drama.* Organogenesis, 2010. 6(4): p. 225-33.

[11] Reinke, J.M. and H. Sorg, *Wound repair and regeneration.* Eur Surg Res, 2012. 49(1): p. 35-43.

[12] Gosain, A. and L.A. DiPietro, *Aging and wound healing.* World journal of surgery, 2004. 28(3): p. 321-326.

[13] Enoch, S. and D.J. Leaper, *Basic science of wound healing.* Surgery (Oxford), 2008. 26(2): p. 31-37.

[14] Campos, A.C., A.K. Groth, and A.B. Branco, *Assessment and nutritional aspects of wound healing.* Current Opinion in Clinical Nutrition & Metabolic Care, 2008. 11(3): p. 281-288.

[15] George Broughton, I., J.E. Janis, and C.E. Attinger, *The basic science of wound healing.* Plastic and reconstructive surgery, 2006. 117(7S): p. 12S-34S.

[16] Mosser, D.M. and J.P. Edwards, *Exploring the full spectrum of macrophage activation.* Nature reviews immunology, 2008. 8(12): p. 958-969.

[17] Meszaros, A.J., J.S. Reichner, and J.E. Albina, *Macrophage-induced neutrophil apoptosis.* The Journal of Immunology, 2000. 165(1): p. 435-441.

[18] Park, J.E. and A. Barbul, *Understanding the role of immune regulation in wound healing.* The American Journal of Surgery, 2004. 187(5): p. S11-S16.

[19] Swift, M.E., et al., *Age-related alterations in the inflammatory response to dermal injury.* Journal of Investigative Dermatology, 2001. 117(5): p. 1027-1035.

[20] Gawronska-Kozak, B., et al., *Scarless skin repair in immunodeficient mice.* Wound repair and regeneration, 2006. 14(3): p. 265-276.

[21] Mills, R.E., et al., *Defects in skin γδ T cell function contribute to delayed wound repair in rapamycin-treated mice.* The Journal of Immunology, 2008. 181(6): p. 3974-3983.

[22] Jameson, J. and W.L. Havran, *Skin γδ T-cell functions in homeostasis and wound healing.* Immunological reviews, 2007. 215(1): p. 114-122.

[23] Rea, S., et al., *Bone marrow-derived cells in the healing burn wound—more than just inflammation.* Burns, 2009. 35(3): p. 356-364.

[24] Cha, J. and V. Falanga, *Stem cells in cutaneous wound healing.* Clinics in dermatology, 2007. 25(1): p. 73-78.

[25] Liu, Z.-J. and O.C. Velazquez, *Hyperoxia, endothelial progenitor cell mobilization, and diabetic wound healing.* Antioxidants & redox signaling, 2008. 10(11): p. 1869-1882.

[26] Wu, Y., et al., *Bone marrow-derived stem cells in wound healing: a review.* Wound Repair and Regeneration, 2007. 15: p. S18-S26.

[27] Cifuentes, A., et al., *Chitosan hydrogels functionalized with either unfractionated heparin or bemiparin improve diabetic wound healing.* Biomedicine & Pharmacotherapy, 2020. 129: p. 110498.

[28] Morris, D.L., D. Sanghavi, and C.I. Kahwaji, *Angiotensin II.* 2018.

[29] Yahata, Y., et al., *A novel function of angiotensin II in skin wound healing. Induction of fibroblast and keratinocyte migration by angiotensin II via heparin-binding epidermal growth factor (EGF)-like growth factor-mediated EGF receptor transactivation.* J Biol Chem, 2006. 281(19): p. 13209-13216.

[30] Takeda, H., et al., *Effects of angiotensin II receptor signaling during skin wound healing.* Am J Pathol, 2004. 165(5): p. 1653-62.

[31] Steckelings, U.M., et al., *Differential expression of angiotensin receptors in human cutaneous wound healing.* British Journal of Dermatology, 2005. 153(5): p. 887-893.

[32] Wu, H.-J., et al., *The change of angiotensin II production and its receptor expression during wound healing: possible role of angiotensin II in wound healing.* Zhonghua zheng xing wai ke za zhi= Zhonghua zhengxing waike zazhi= Chinese journal of plastic surgery, 2011. 27(2): p. 124-128.

[33] Braza, M.E. and M.P. Fahrenkopf, *Split-thickness skin grafts.* 2019.

[34] Sierra-Sánchez, Á., et al., *Cellular human tissue-engineered skin substitutes investigated for deep and difficult to heal injuries.* NPJ Regenerative Medicine, 2021. 6(1): p. 1-23.

[35] Caplan, A.I., *Design parameters for functional tissue engineering*, in *Functional Tissue Engineering*. 2003, Springer. p. 129-138.

[36] Zhong, S., Y. Zhang, and C. Lim, *Tissue scaffolds for skin wound healing and dermal reconstruction.* Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology, 2010. 2(5): p. 510-525.

[37] Ma, L., et al., *Collagen/chitosan porous scaffolds with improved biostability for skin tissue engineering.* Biomaterials, 2003. 24(26): p. 4833-4841.

[38] MacNeil, S., *Biomaterials for tissue engineering of skin.* Materials Today, 2008. 11(5): p. 26-35.

[39] Kittana, N., et al., *Topical aqueous extract of Ephedra alata can improve wound healing in an animal model.* Chin J Traumatol, 2017. 20(2): p. 108-113.

[40] Rozario, T. and D.W. DeSimone, *The extracellular matrix in development and morphogenesis: a dynamic view.* Dev Biol, 2010. 341(1): p. 126-40.

[41] Kusindarta, D.L. and H. Wihadmadyatami, *The role of extracellular matrix in tissue regeneration.* Tissue regeneration, 2018. 65.

[42] Telis, V., K. Wolf, and P. Sobral. *Characterizations of collagen fibers for biodegradable films production*. in *13th World Congress of Food Science & Technology 2006*. 2006.

[43] Lee, J.E., et al., *Characterization of UV-irradiated dense/porous collagen membranes: morphology, enzymatic degradation, and mechanical properties.* Yonsei medical journal, 2001. 42(2): p. 172-179.

[44] Jorge-Herrero, E., et al., *Influence of different chemical cross-linking treatments on the properties of bovine pericardium and collagen.* Biomaterials, 1999. 20(6): p. 539-545.

[45] Bell, E., B. Ivarsson, and C. Merrill, *Production of a tissue-like structure by contraction of collagen lattices by human fibroblasts of different proliferative potential in vitro.* Proceedings of the National Academy of Sciences, 1979. 76(3): p. 1274-1278.

[46] Bell, E., et al., *Living tissue formed in vitro and accepted as skin-equivalent tissue of full thickness.* Science, 1981. 211(4486): p. 1052-1054.

[47] Rastogi, S., M. Modi, and B. Sathian, *The efficacy of collagen membrane as a biodegradable wound dressing material for surgical defects of oral mucosa: a prospective study*. Journal of Oral and Maxillofacial Surgery, 2009. 67(8): p. 1600-1606.

[48] MacNeil, S., *Progress and opportunities for tissue-engineered skin.* Nature, 2007. 445(7130): p. 874-880.

[49] Seo, Y.-K., et al., *Reinforced bioartificial dermis constructed with collagen threads.* Biotechnology and Bioprocess Engineering, 2008. 13(6): p. 745-751.

[50] Lee, C.H., A. Singla, and Y. Lee, *Biomedical applications of collagen.* International journal of pharmaceutics, 2001. 221(1-2): p. 1-22.

[51] Ng, K.W., et al., *Evaluation of ultra-thin poly (ε-caprolactone) films for tissue-engineered skin.* Tissue engineering, 2001. 7(4): p. 441-455.

[52] Adekogbe, I. and A. Ghanem, *Fabrication and characterization of DTBP-crosslinked chitosan scaffolds for skin tissue engineering.* Biomaterials, 2005. 26(35): p. 7241-7250.

[53] Lanza, R., et al., *Principles of tissue engineering*. 2020: Academic press.

[54] Griffith, L.G. and G. Naughton, *Tissue engineering--current challenges and expanding opportunities.* science, 2002. 295(5557): p. 1009-1014.

[55] Kumbar, S., et al., *Electrospun nanofiber scaffolds: engineering soft tissues.* Biomedical materials, 2008. 3(3): p. 034002.

[56] Tan, W., R. Krishnaraj, and T.A. Desai, *Evaluation of nanostructured composite collagen–chitosan matrices for tissue engineering.* Tissue engineering, 2001. 7(2): p. 203-210.

[57] Zhang, R. and P.X. Ma, *Synthetic nano-fibrillar extracellular matrices with predesigned macroporous architectures.* Journal of Biomedical Materials Research: An Official Journal of The Society for Biomaterials, The Japanese Society for Biomaterials, and The Australian Society for Biomaterials and the Korean Society for Biomaterials, 2000. 52(2): p. 430-438.

[58] Huang, Z.-M., et al., *Electrospinning and mechanical characterization of gelatin nanofibers.* Polymer, 2004. 45(15): p. 5361-5368.

[59] Matthews, J.A., et al., *Electrospinning of collagen nanofibers.* Biomacromolecules, 2002. 3(2): p. 232-238.

[60] Powell, H.M., D.M. Supp, and S.T. Boyce, *Influence of electrospun collagen on wound contraction of engineered skin substitutes.* Biomaterials, 2008. 29(7): p. 834-843.

[61] Rho, K.S., et al., *Electrospinning of collagen nanofibers: effects on the behavior of normal human keratinocytes and early-stage wound healing.* Biomaterials, 2006. 27(8): p. 1452-1461.

[62] Zeugolis, D.I., et al., *Electro-spinning of pure collagen nano-fibres–just an expensive way to make gelatin?* Biomaterials, 2008. 29(15): p. 2293-2305.

[63] Muxika, A., et al., *Chitosan as a bioactive polymer: Processing, properties and applications.* International Journal of Biological Macromolecules, 2017. 105: p. 1358-1368.

[64] Kittana, N., et al., *Enhancement of wound healing by single-wall/multi-wall carbon nanotubes complexed with chitosan.* Int J Nanomedicine, 2018. 13: p. 7195-7206.

[65] Değim, Z., et al., *An investigation on skin wound healing in mice with a taurine-chitosan gel formulation.* Amino acids, 2002. 22(2): p. 187-198.

[66] Kong, M., et al., *Antimicrobial properties of chitosan and mode of action: a state of the art review.* International journal of food microbiology, 2010. 144(1): p. 51-63.

[67] Ueno, H., et al., *Accelerating effects of chitosan for healing at early phase of experimental open wound in dogs.* Biomaterials, 1999. 20(15): p. 1407-1414.

[68] Mi, F.-L., et al., *Fabrication and characterization of a sponge-like asymmetric chitosan membrane as a wound dressing.* Biomaterials, 2001. 22(2): p. 165-173.

[69] Biagini, G., et al., *Wound management with N-carboxybutyl chitosan.* Biomaterials, 1991. 12(3): p. 281-286.

[70] Mao, J.S., Y.J. Yin, and K. De Yao, *The properties of chitosan–gelatin membranes and scaffolds modified with hyaluronic acid by different methods.* Biomaterials, 2003. 24(9): p. 1621-1629.

[71] Cestari, A.R., et al., *Multistep adsorption of anionic dyes on silica/chitosan hybrid: 1. Comparative kinetic data from liquid-and solid-phase models.* Journal of colloid and interface science, 2005. 292(2): p. 363-372.

[72] Gingras, M., I. Paradis, and F. Berthod, *Nerve regeneration in a collagen–chitosan tissue-engineered skin transplanted on nude mice.* Biomaterials, 2003. 24(9): p. 1653-1661.

[73] Germain, L., et al., *Improvement of human keratinocyte isolation and culture using thermolysin.* Burns, 1993. 19(2): p. 99-104.

[74] Sarkar, S.D., et al., *Chitosan–collagen scaffolds with nano/microfibrous architecture for skin tissue engineering.* Journal of Biomedical Materials Research Part A: An Official Journal of The Society for Biomaterials, The Japanese Society for Biomaterials, and The Australian Society for Biomaterials and the Korean Society for Biomaterials, 2013. 101(12): p. 3482-3492.

[75] *Biomaterials Science: An Introduction to Materials in Medicine*. Third ed. 2013: Elsevier.

[76] Sun, L., et al., *Biological evaluation of collagen–chitosan scaffolds for dermis tissue engineering.* Biomedical Materials, 2009. 4(5): p. 055008.

[77] van Tonder, A., A.M. Joubert, and A.D. Cromarty, *Limitations of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay when compared to three commonly used cell enumeration assays.* BMC Res Notes, 2015. 8: p. 47.

[78] Han, C.-m., et al., *Application of collagen-chitosan/fibrin glue asymmetric scaffolds in skin tissue engineering.* Journal of Zhejiang University Science B, 2010. 11(7): p. 524-530.

[79] Kuche, K., et al., *Carbon nanotubes (CNTs) based advanced dermal therapeutics: current trends and future potential.* Nanoscale, 2018. 10(19): p. 8911-8937.

[80] Rashid, M.H. and S.F. Ralph, *Carbon Nanotube Membranes: Synthesis, Properties, and Future Filtration Applications.* Nanomaterials (Basel), 2017. 7(5).

[81] Iijima, S., *Helical microtubules of graphitic carbon.* nature, 1991. 354(6348): p. 56-58.

[82] Cheng, L.-C., et al., *Nano–bio effects: interaction of nanomaterials with cells.* Nanoscale, 2013. 5(9): p. 3547-3569.

[83] Dresselhaus, M.S., G. Dresselhaus, and P.C. Eklund, *Science of fullerenes and carbon nanotubes: their properties and applications*. 1996: Elsevier.

[84] Luo, Z., et al., *Thermal stability of functionalized carbon nanotubes studied by in-situ transmission electron microscopy.* Chem Phys Lett, 2011. 513(1-3): p. 88-93.

[85] Vardharajula, S., et al., *Functionalized carbon nanotubes: biomedical applications.* International journal of nanomedicine, 2012. 7: p. 5361.

[86] Yu, M.-F., et al., *Tensile loading of ropes of single wall carbon nanotubes and their mechanical properties.* Physical review letters, 2000. 84(24): p. 5552.

[87] Meo, M. and M. Rossi, *Prediction of Young's modulus of single wall carbon nanotubes by molecular-mechanics based finite element modelling.* Composites Science and Technology, 2006. 66(11-12): p. 1597-1605.

[88] Collins, P.G. and P. Avouris, *Nanotubes for electronics.* Scientific american, 2000. 283(6): p. 62-69.

[89] Beg, S., et al., *Emergence in the functionalized carbon nanotubes as smart nanocarriers for drug delivery applications*. Fullerens, Graphenes and Nanotubes. 2018:105-33. doi: 10.1016/B978-0-12-813691-1.00004-X. Epub 2018 Jun 8.

[90] Baughman, R.H., A.A. Zakhidov, and W.A. De Heer, *Carbon nanotubes--the route toward applications.* science, 2002. 297(5582): p. 787-792.

[91] Rao, C.N.R., et al., *Nanotubes.* ChemPhysChem, 2001. 2(2): p. 78-105.

[92] Reich, S., C. Thomsen, and J. Maultzsch, *Carbon nanotubes: basic concepts and physical properties*. 2004: John Wiley & Sons.

[93] Ema, M., M. Gamo, and K. Honda, *A review of toxicity studies of single-walled carbon nanotubes in laboratory animals.* Regulatory Toxicology and Pharmacology, 2016. 74: p. 42-63.

[94] Madani, S.Y., A. Mandel, and A.M. Seifalian, *A concise review of carbon nanotube's toxicology.* Nano reviews, 2013. 4(1): p. 21521.

[95] Liu, Z., et al., *Carbon materials for drug delivery & cancer therapy.* Materials today, 2011. 14(7-8): p. 316-323.

[96] Zhang, W., Z. Zhang, and Y. Zhang, *The application of carbon nanotubes in target drug delivery systems for cancer therapies.* Nanoscale research letters, 2011. 6(1): p. 1-22.

[97] Assali, M., et al., *Covalent functionalization of SWCNT with combretastatin A4 for cancer therapy.* Nanotechnology, 2018. 29(24): p. 245101.

[98] Singhai, N.J. and S. Ramteke, *Functionalized carbon nanotubes: emerging applications in the diverse biomedical arena.* Current Nanoscience, 2020. 16(2): p. 170-186.

[99] Galassi, T.V., et al., *Long-term in vivo biocompatibility of single-walled carbon nanotubes.* Plos one, 2020. 15(5): p. e0226791.

[100] Bosi, S., L. Ballerini, and M. Prato, *Carbon nanotubes in tissue engineering.* Making and exploiting fullerenes, graphene, and carbon nanotubes, 2013: p. 181-204.

[101] Figarol, A., et al., *In vitro toxicity of carbon nanotubes, nano-graphite and carbon black, similar impacts of acid functionalization.* Toxicology in Vitro, 2015. 30(1): p. 476-485.

[102] Liu, Y., et al., *Understanding the toxicity of carbon nanotubes.* Accounts of chemical research, 2013. 46(3): p. 702-713.

[103] Fujita, K., et al., *Cytotoxicity profiles of multi-walled carbon nanotubes with different physico-chemical properties.* Toxicology Mechanisms and Methods, 2020. 30(7): p. 477-489.

[104] Lalwani, G., et al., *Porous three-dimensional carbon nanotube scaffolds for tissue engineering.* Journal of Biomedical Materials Research Part A, 2015. 103(10): p. 3212-3225.

[105] Assali, M., et al., *Noncovalent functionalization of carbon nanotubes as a scaffold for tissue engineering.* Sci Rep, 2022. 12(1): p. 12062.

[106] Kittana, N., et al., *Modulating the Biomechanical Properties of Engineered Connective Tissues by Chitosan-Coated Multiwall Carbon Nanotubes.* Int J Nanomedicine, 2021. 16: p. 989-1000.

[107] Iowa, I.V.A.R.a.t.U.o. *Anesthesia (Guideline)*. 2020 02/12/2020 [cited 2021; Available from: https://animal.research.uiowa.edu/iacuc-guidelines-anesthesia.

[108] Cifuentes, A., et al., *Chitosan hydrogels functionalized with either unfractionated heparin or bemiparin improve diabetic wound healing.* Biomed Pharmacother, 2020. 129: p. 110498.

[109] Kittana, N. and M. Assali, *Modulating the Biomechanical Properties of Engineered Connective Tissues by Chitosan-Coated Multiwall Carbon Nanotubes.* 2021. 16: p. 989-1000.

[110] Correia, M.I.T.D., *4 - Wound healing*, in *The Practical Handbook of Perioperative Metabolic and Nutritional Care*, M.I.T.D. Correia, Editor. 2019, Academic Press. p. 47-55.

[111] Hinz, B., *The role of myofibroblasts in wound healing.* Current Research in Translational Medicine, 2016. 64(4): p. 171-177.

[112] Wu, H.J., et al., *[The change of angiotensin II production and its receptor expression during wound healing: possible role of angiotensin II in wound healing]*. Zhonghua Zheng Xing Wai Ke Za Zhi, 2011. 27(2): p. 124-8.

[113] Kurosaka, M., et al., *Reduced angiogenesis and delay in wound healing in angiotensin II type 1a receptor-deficient mice.* Biomed Pharmacother, 2009. 63(9): p. 627-34.

[114] Sorrell, J.M. and A.I. Caplan, *Fibroblasts—a diverse population at the center of it all.* International review of cell and molecular biology, 2009. 276: p. 161-214.

[115] Wong, T., J. McGrath, and H. Navsaria, *The role of fibroblasts in tissue engineering and regeneration.* British Journal of Dermatology, 2007. 156(6): p. 1149-1155.

[116] Khan, M. and S. Gasser, *Generating Primary Fibroblast Cultures from Mouse Ear and Tail Tissues.* J Vis Exp, 2016(107).

[117] Cole, M.A., et al., *Extracellular matrix regulation of fibroblast function: redefining our perspective on skin aging.* J Cell Commun Signal, 2018. 12(1): p. 35-43.

جـامعــة النجاح الوطنية

كليــة الدراســات العليــا

هندسة أنسجة جلدية مدعمة بمعقد الشيتوزان – أنابيب نانوية كربونية متعددة الجدران للاستخدام في التئام الجروح

إعداد

أمل جعفر القطو

إشراف

د. نعيم كتانة

د. محي الدين عسالي

قدمت هذه الرسالة استكمالا لمتطلبات الحصول على درجة الماجستير في علم الأدوية، من كلية الدراسات العليا، في جامعة النجاح الوطنية، نابلس– فلسطين.

٢٠٢٣

هندسة أنسجة جلدية مدعمة بمعقد الشيتوزان – أنابيب نانوية كربونية متعددة الجدران للاستخدام في التئام الجروح

إعداد

أمل جعفر القطو

إشراف

د. نعيم كتانة

د. محي الدين عسالي

الملخص

مقدمة وخلفية: عملية التئام الجروح هي عملية معقدة وتتضمن أربعة مراحل دقيقة: التخثر، الالتهاب، التكاثر، وإعادة البناء. عندما تنقطع هذه العملية، يمكن أن تؤدي إلى جروح مزمنة تشكل عبئًا صحيًا واقتصاديًا كبيرًا. تم اقتراح أنسجة الجلد المهندسة بمكونات مختلفة كعلاج محتمل.

هدف المشروع: إنتاج أنسجة الأدمة المهندسة كبديل لطبقة الأدمة لتعزيز التئام الجروح.

المواد والطرق: استندت دعامات أنسجة الأدمة المهندسه المتولدة على الكولاجين، وهو مشابه للأدمة الطبيعية، وقد تم دعمه بالشيتوزان الذي هو بوليمر طبيعي متوافق حيوياً وقابل للتحلل الحيوي ويمتلك خصائص داعمة لالتئام الجروح، وتضمنت الانسجة كذلك تراكيز مختلفة من الأنابيب النانوية الكربونية متعددة الجدران، التي يمكن أن تعزز الخواص الميكانيكية لانسجه الادمه المهندسه. كما تمت دراسة تأثير دمج برونين أنجيوتنسين ٢ في الأنسجة على تكوين الأوعية الدموية. إضافة لما سبق تم تزويد جميع الأنسجة بخلايا ٣ت٣. تم زرع انسجه الادمه المهندسه في نموذج جروح في الفئران. بعد ١٤ يوماً من عملية الزراعة جرى تقييم لجودة التئام الجروح. وذلك بتحليل صور مواقع الجروح على مستوى المقاييس

الكبيرة، وكذلك تم فحص الانسجة مجهريا بواسطة صبغة ماسيون تراخوما وجرى تحليل رقمي للصور الملتقطة.

النتائج: بشكل عام، وجدت دراستنا أن الأنسجة المزروعة ليس لها تأثير سلبي على صحة الحيوان. وأنها قللت من انقباض الجرح وعززت تكون النسيج الظهاري، لكنه لم يؤثر على النسبة المئوية لإغلاق الجرح. لم يؤثر زرع أنسجة الأدمة المهندسه على سمك البشرة الجديدة، لكنه زاد من سمك الأدمة. كما ان دمج أنجيوتنسين ٢ في أنسجة الأدمة المهندسه لم يؤثر على درجة تكوين الأوعية الدموية.

الاستنتاجات والتوصيات: عززت الأنسجة المزروعة جودة التئام الجروح من خلال تعزيز تكون النسيج الظهاري وتقليل الانكماش. تعتبر هذه النتائج مهمة لتطوير العلاجات المحتملة للجروح بطيئة الالتئام أو الجروح عالية الخطورة.

الكلمات المفتاحية: الشيتوزان، الأنابيب النانوية الكربونية متعددة الجدران، الأنجيوتنسين ٢، الأنسجة الجلدية المهندسة، التئام الجروح.