Muhammad Mohtasim Raza

Investigation of steroids and keratinocytes impact on wound healing and mTOR, AKT, PI3KCA pathways related genes obtained from mouse

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APPROVAL

We certify that we have read the thesis submitted by Muhammad Mohtasim Raza titled **'Investigation of Steroids and Keratinocytes impact on Wound Healing and mTOR, AKT, PI3KCA Pathways-Related Genes Obtained from Mouse Model'** and that in our combined opinion it is fully adequate, in scope and quality, as a thesis for the degree of Master of Educational Sciences.

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DECLARATION

I hereby declare that all information, documents, analysis, and results in this thesis have been collected and presented according to the academic rules and ethical guidelines of Institute of Graduate Studies, Near East University. I also declare that as required by these rules and conduct, I have fully cited and referenced information and data that are not original to this study.

Muhammad Mohtasim Raza

27/06/2022

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All praise be to **ALLAH** the almighty, nourisher, and the cherisher of the whole world, who has bestowed knowledge and wisdom endowed to all mankind. Countless salutations on the noble personage of **Hazrat Muhammad** ﷺ, and peace and mercy Upon His noble companions' eminent members of the family and true followers till the Day of Judgment.

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Muhammad Mohtasim Raza

ABSTRACT

Investigation of Steroids and Keratinocytes impact on Wound Healing and mTOR, AKT, PI3KCA Pathways-Related Genes Obtained from Mouse model

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Background: The skin is the largest organ in the human body and is involved in a variety of functions including hydration, protection from toxins and pathogens, vitamin D production, excretion, and heat control. As a result, severe skin injury might be life-threatening. The healing of a skin wound demonstrates an exceptional cellular function mechanism that is unique in nature. Closing the lesion requires the interaction of cells, cytokines and growth factors. Injury-associated difficulties, especially for chronic wounds, are mostly connected to treatment and maintenance practices that impede wound recovery rather than tissue integrity restoration. Keratinocytes play important role during proliferation phase in which damaged ECM are replaced and structural and functional integrity of damaged tissue is restored. Keratinocyte activation through the macrophages *via* growth factor release and cytokines cause the production of collagen, ECM, angiogenesis and epithelialization. Steroids play anti-inflammatory role in wound healing. Cells constituting the newly formed tissue secreted the TGF-β and IGF-1, and they act as mediators of cellular response and play vital role to regulate the interactions between cells on the basis of the nature of their receptors. *Pi3k/Akt/mTor* pathway is initiated as a result of wound injury. Any defect in this pathway results in the reduction in epithelial mesenchymal transition (EMT), no proliferation of cells and subsequently affects the wound healing. Insulin is polypeptide known for regulation of blood sugar especially in case of hyperglycemia. Because of its ability to heal wound, its interest is increased in the field of wound healing and tissue regeneration. Therefore, focus of

study was to determine the role of keratinocyte and steroids in wound healing by analyzing the gene express of five different gene *Akt1, Akt2, Pi3k. mTor and Pi3kca.*

Methods: The mouse models were established in the Manisa Celal Bayar University, Turkey by Prof. Seda Vatansever's research group. Briefly, four groups of mouse models were established RNA was extracted from samples. Purity of RNA was checked using Nano-drop spectrophotometer. cDNA was synthesized using the extracted RNA. Gene expression level in each sample was evaluated using real-time polymerase chain reaction (RT-PCR). Furthermore, a statistical analysis was performed on the RT-PCR data to evaluate the expression of the desired genes.

Results: The conducted study depict that differentiated keratinocytes and steroids are involved in wound healing, these supplements cause the activation of several pathways like mTor/Akt/Pi3kca that take part in wound healing. Furthermore, it was observed during the study that keratinocytes and steroids regulate the gene expression of *mTor, Akt1, Akt2, Pi3kca* and usually these genes are up-regulated by the differentiated keratinocytes and this activation speed up the process of wound healing.

Keywords

Wound healing, gene expression, *mTor, Akt1, Akt2, Gadph, Pi3k*, Secrin, Genipin, Real time PCR, cDNA synthesis, RNA Extraction, keratinocytes, steroids

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mTor	Mammalian/mechanistic target of rapamycin
mTORC	Mammalian target of rapamycin complex 1
Akt	Ak strain transforming/ the AKT family of proteins
Akt1	The gene for the first member of the AKT family
Akt2	The gene for the second member of the AKT family
Gadph	Glyceraldehyde-3-phosphate dehydrogenase
Pi3k	Phosphoinositide 3-kinases / phosphatidylinositol 3-kinases,
PCR	Polymerase Chain Reaction
RNA	Ribonucleic acid
cDNA	Complementary Deoxyribonucleic acid
mRNA	messenger RNA
EGF	Epidermal growth factor
FGF	Fibroblast growth factor
PDGF	Platelet-derived growth factor
KGF	keratinocytes growth factor
ECM	Extra-Cellular Matrix
TGF-β1	Transforming growth factor βeta 1
TGF- β2	Transforming growth factor βeta 2
AMPK	Adenosine monophosphate activated kinase
CES	Cultured epithelial substitutes
3T3 cells	3-day transfer, inoculum 3 x 10^5 cells.

IGF-1 Insulin growth factor-1

FKBP12 FK506 binding protein

Colla2 Collagen Alpha-type 2

REDD1 Regulated in Development and DNA damage 1

FKBP51 FK506 Binding proteins 5

Thr308 Threonine

Ser473 Serine 473

PHLPP1/2 PH domain and Leucine rich repeat Protein Phosphatases) are a pair of protein phosphatases

EMT Epithelial mesenchymal transition

PDK1 3-Phosphoinositide-dependent kinase 1

p53 Tumor suppressor protein

IRS Insulin receptor substrate (IRS) proteins.

ERK Extracellular signal-regulated kinase

MEF Mouse Embryonic Fibroblasts

Raf Rapidly Accelerated Fibrosarcoma.

eNOS Endothelial nitric oxide synthase

VEGF Vascular endothelial growth facto

Pik3ca Phosphatidylinositol 4-5 bisphosphate 3 kinase catalytic subunit alpha

ACTB Beta-*actin* human gene and protein

HUR Human Antigen R Protein.

ELAV Embryonic lethal abnormal vision

SiRNAs Small interfering RNAs

tRNAs	Transfer RNA
CGR8	The germ-line competent cell line

CHAPTER I

Introduction

This chapter contains the general overview of the wound healing and the different aspects of drugs used to treat the wound healing. It also explains the different techniques used for wound healing, currently used therapeutics treatment, background, applications, functions and the side effects. This section elaborates the genes that are involved in wound healing and the genetic pathways that regulate the expression of these genes like *mTor, Akt1* and *Akt2.*

1.1 An Overview of Wound Healing

The skin is the largest organ in the human body and is involved in a variety of functions including hydration, protection from toxins and pathogens, vitamin D production, excretion, and heat control. As a result, severe skin injury might be life-threatening. The healing of a skin wound demonstrates an exceptional cellular function mechanism that is unique in nature (Borena et al., 2015). Wound healing process consist of four stages wound injury, inflammation, proliferation and remodeling. Closing the lesion requires the interaction of cells, cytokines and growth factors. Injury-associated difficulties, especially for chronic wounds, are mostly connected to treatment and maintenance practices that impede wound recovery rather than tissue integrity restoration (Schiavon et al., 2016). As a result, various research studies are focused on developing more effective wound remedies in order to save healthcare expenses while also providing long-term alleviation and eventually, successful scar repair (Boyce & Lalley, 2018).

There are two types of skin wound treatments "conventional" and "regenerative". Scars emerge as a result of traditional therapy, regardless of the cosmetic or functional changes that may occur. Regenerative wound treatment is a novel and fast evolving field of biomedical research that attempts to restore skin to its original function by regenerating damaged cells and skin tissue without leaving scars (Tottoli et al., 2020). Surgery, traumas, extrinsic factors (burns, cuts and pressure) and pathologic disorders such as diabetes or vascular illnesses can all produce wounds. Depending on the

underlying causes and outcomes, these forms of injury are classed as acute or chronic wounds (Karimi et al., 2017). Acute wounds are typically repaired in a systematic and suitable manner, resulting in the long-term restoration of anatomical and functional integrity. Chronic wounds, on the other hand, are unable to acquire maximum anatomical and functional integrity. Healing is influenced and governed by both pathogenic processes, as well as the kind, degree, and condition of the host and environment (Gerald et al.,2015). Systemic variables such as patient age, the existence of autoimmune illnesses, metabolic and vascular diseases as well as current pharmacological therapy, may all have an impact on wound healing. An optimally healed wound is one that has been restored to normal anatomical structure, function, and appearance following an injury; a minimally healed wound is one that has been restored to anatomical continuity but has not produced long-term functional consequences; hence, the wound can reoccur. Between these two situations, an acceptable healed wound is distinguished by the restoration of sustained functional and structural continuity (Guo & DiPietro, 2010).

1.2 Current Therapeutics in Wound Healing: Advantages and Disadvantages

Currently there are multiple therapeutics for treatment of wound. Therapeutics involve the surgical treatment of wound, non-surgical procedures and use of pharmaceuticals products. According to World Health Organization all these therapeutic procedures cost about 12 billion dollar annually and will reach up to 35 billion dollar at end of 2023 (Monavarian et al., 2019). Therapeutic options for treating the wound depend on the nature of wound. There are some pros and cons of current therapies for treatment of wound. Current therapies for treatment of wounds along with their advantages and disadvantages are discussed (Rennert et al., 2013).

Surgical options of treating wound depend on type and nature of wound. Skin grafts are common surgical operation to treat the chronic and burn wound. Skin grafts are divided in to three groups; autografts, xenografts and allografts (Ferrario et al., 2020). Autograft is the gold standard procedure for restoration of wound. Procedure involves the transplantation of skin taken from healthy part of the same patient to wounded area. Autologous skin taken from the same person have advantages of local vasculature

development, restoration of tissue structural and functional integrity and furthermore there is no chance of immune rejection. However, there are some limitations in the use of autografts, such as less availability of skin sites for transplant in case of bigger wounds, scar development and painful healing. Allografts are alternative to autografts and require skin to be grafted from another person while xenografts require the skin taken from another species for grafting (Varkey et al., 2015).

Another surgical procedure for treating the wound is use of skin flaps. A flap consisted of skin and partially detached subcutaneous tissue that still have connection with major blood vessels i.e. arteries or vein (Honrado & Murakami, 2005). This flap is then moved to cover the nearby wound. Main advantage of this procedure over the skin grafts is that it provides the nutrients required for cell proliferation, growth factor and cytokines because of its connection to blood stream (Ii et al., 2019). However this process is anesthetic dependent and requires expertise in biomechanics and physiology for higher success (Lucas, 2017).

Non-surgical therapies involve the use of topical formulation, dressing of wound and skin substitutes. Topical formation can be stated as appliance of formulated drugs to treat wound (Powers et al., 2019). As compared to various systematic drugs topical formulation have several advantages. Application of formulations is through local route so there is no involvement of first pass metabolism and emergence of systematic side effects (Yadav et al., 2017). Furthermore, these formulations are easier to apply on wound and are suitable for the self-medication. Some complication may rise such as irritation of skin, allergic reaction and discomfort. This all depends on the formulation used in the treatment (Ohnstedt et al., 2019).

Topical formulation included the use of sprays, gels, lotion, creams, emulsions and pastes. There are also advancement in the use of topical formulations like nanoparticle application on wound and use of liposome and biopolymers (Bhowmik et al., 2012). Antibiotics are also being used as a topical formulation. Topical formulation containing antibiotics are useful in treating the bacterial infections present in the wounds. Antimicrobial formulations accelerate the healing of wound by containing microbial infections, water loss in wounds and maintain the moisture in the wound (Bonomo et al.,

2007). Application of antibiotics through local route decreases the chance of antimicrobial resistance as in the case of systematic delivery of antibiotics. However, antibiotic formulation is useful during infectious phase of wound healing and cannot be used afterwards (Ward & Saffle, 1995; Smack, 1996).

Dexpanthenol (alcoholic analogue of D-pantothenic acid) is the most common used topical formulation in treating the skin wounds and it is used as an active agent for the formation of healthy epithelium and to prevent vitamin deficiency. It helps in fibroblast proliferation and formation of granulation tissue. It also expedites the re-epithelization by promoting proliferation and migration of keratinocytes toward the wound bed. For proper re-epithelization there must be delicate balance between the growth factors, nutrients and cytokines (Ebner et al., 2002). So, dexpanthenol is an excellent molecule that provides environment for proliferation of keratinocytes and fibroblast. Furthermore, it supports the migration of keratinocytes towards the wound bed which is crucial in wound healing (Gorski et al., 2020).

Remodeling phase of wound healing is very important because it restores the structural integrity of the skin tissues. Usually after the wound healing, the scar tissue remains on the skin tissue. Collagenase formulations are common debriding agent used to breakdown denatured collagens, extracellular proteins and necrotic tissue present in the wounds. Collagen formulations also play important role in proliferation of keratinocytes and development of endothelial layer in the wound bed (Palmieri & Magri, 1998).

Growth factors regulate the process of wound healing. Growth factors initiate coordinated healing response by several cells like keratinocytes, fibroblasts, macrophages of endothelial cells and platelets. Furthermore, they play important role in proliferation and migration of keratinocytes and fibroblast to injury site (Pan et al., 2018). Application of growth factors in topical formulations is emerging therapeutics to treat the wound. Epidermal growth factor (EGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF) family have been widely used in topical formulations to heal the wounds (Li et al., 2019). A recombinant PDGF based formulation known as Becaplermin (PDGF-BB) proved its effectiveness in the treatment of diabetic wounds because it induces fibroblast proliferation and differentiation, and fibroblasts are the

cells that are involved in formation of connective tissues. Moreover, they secrete collagen protein that is involved in wound healing (Heldin & Westermark, 1999). Studies showed that the keratinocyte growth factors (KGF) conjugated with gold nanoparticles help in keratinocyte migration and proliferation which result in subsequent re-epithelization (Waycaster et al., 2012). Another study revealed that FGF10-loaded microspheres initiate angiogenesis and collagen formation, which is an important process in wound healing (Xu et al., 2020).

Prior to both surgical and topical formulations treatment for healing wound, another therapeutic is known as dressing of wound. Dressing helps in protecting wounds from exogenous agent and external mechanical disruption of wound. This results fast healing of wound and also decrease the risk of inflammation (Psimadas et al., 2012). The dressing was designed in such a way that it leaves the wound dry and remove the wound exudate. That was a drawback in the use of dressing. Recent research showed that moist environment plays an important role in wound healing. So, recent dressing is more different than the previous one. It helps to the keep wound environment moist, provide good gas exchange and remove the extensive exudate from the wound (Souto et al., 2020). Dressing classification is based on material used. Polymeric dressings are considered more important because of their good effect on wound healing. Polymeric dressings include hydro-fibers, foams, films, alginate, hydrocolloids and gels. Polymeric dressing helps in proliferation of keratinocyte and deposition of collagen to enhance the process of wound healing (Chen et al., 2018).

There is another type of dressing synthesized by combination of biomolecules, drugs and growth factors. These dressings reduce the extracellular matrix in the wound and increase the process of cell proliferation, differentiation and migration. Biological compound can be added to these dressings. Most of the natural compounds that are associated with biological dressing are honey, silk fibroin, propolis and quercetin (Jian et al., 2020). These biological dressings along with natural compound help in moisturizing the wound environment (the balanced moist surface helps the growth factors, cytokines and chemokines to perform their action efficiently and promoting the cellular growth at the wound site), antibacterial activity and removal of excessive

exudate. Antibiotics can also be added to these types of dressing. Studies showed that the antimicrobial dressing involving doxycycline (tetracycline-class antibiotic) enhances the process of wound healing, improves re-epithelization and antimicrobial activity in the wound bed (Oba et al., 2020; Chen et al., 2020).

Skin substitute is the most advanced therapeutic technique for healing chronic wound. Substitutes consist of heterogeneous group of biopolymers, growth factors, cells and scaffolds that are used to temporarily or permanently cover the wound (Girirajan et al., 2011). There are also acellular substitutes that are just scaffold to provide the template for fibroblast and keratinocytes present in the wound. Polymers used in synthesis of substitutes are collagens, alginates, fibronectins, hyaluronic acids and chitosan. Growth factors can also be added to substitutes to increase proliferation, differentiation and migration of keratinocytes and endothelial cell (Lakhani et al., 2017).

Cellular skin substitutes include the cell that are required for healing wound. Cell included in cellular substitutes are keratinocytes, fibroblasts, macrophages and stems cell. Addition of these cell help the in the control of regenerative mechanisms. As analogues to the skin tissue, they are temporarily used to cover the wound especially in case when patient has lost big part of their skin. However, there are some disadvantages of skin substitutes that they require time for their synthesis and scar developments (Lakhani et al., 2017).

1.3 Role of Keratinocytes in Wound Healing

Skin barrier is affected by a variety of circumstances, including trauma, hormonal impacts, skin diseases and personal well-being. Upon damage the skin barrier goes through the constant maintenance and development. Wound healing process involves four phases; hemostasis, inflammation proliferation and remodeling (Rowan et al., 2015). Normally the skin is repaired through these four-step mechanism but loss of extensive skin requires the intervention to heal the wound and tissue restoration. Upon complex injuries, there is initiation of cascade of events which involves the recruitment specialized cells, molecules and pathways. Keratinocytes play important role during proliferation phase in which damaged extracellular matrix (ECM) is replaced and structural and functional integrity of damaged tissue is restored. Keratinocyte activation

through the macrophages *via* growth factor release and cytokines cause the production of collagen, ECM, angiogenesis and epithelialization (Gurtner et al., 2008).

Restoration of circulatory system, i.e. vascular network, in skin wound is very important, because it involves the transportation of oxygen and nutrients to the damaged skin tissue. Upon activation of the endothelial cell by macrophages, their cell-to-cell junction start to part up and start to migrate. This process is encouraged by the acidotic and hypoxic environment that is present in the wound. Once the revascularization is established, it neutralizes that environment and subsequently results in the decrease of endothelial cell migration and proliferation. After angiogenesis the re-epithelization migration starts in which important role is played by keratinocytes. Regeneration ability of damaged skin tissue is determined by the quantity of epidermal stem cell present in stem cell niches. After determination of epithelial stem cell, the macrophages release the growth factors which involve the migration of keratinocytes to the wounded part and fill the deficit caused by an injury (Kiwanuka et al., 2017).

For migration to fill the deficit in skin bed the keratinocytes undergo modification and hence there is alteration in their phenotypic expression. This phenotypic expression helps them to loosen their intracellular interaction and freely migrate towards the wounded bed. Upon expression of integrins, it results in flattening of keratinocytes and then altered keratinocytes to move towards the granulation tissue and form mono-epithelial cell layer. During the migration, they also secrete the proteolytic enzymes that degrade the provisional matrix in the skin wound and help them move freely. Once the uniform layers of epithelial cell are formed in the wound bed, they start to mature under the influence of *TGF-β1* and *TGF- β2* (Kiwanuka et al., 2017).

1.9 Aims / Objectives

The aims of this study were to determine the gene expression of the specific genes involved in the wound healing, to determine the role of keratinocytes and steroids treatment applied on the wounded mice, to determine the genetic regulation of these treatments in wound healing.

CHAPTER II

Literature Review

Keratinocytes play vital role in wound healing, as it makes up the 90-95% of the epidermis and in skin homeostasis. It acts as an innate immune cell. It has been observed that the activation of keratinocytes leads to the rapid wound healing *via* the Pi3k (phosphatidylinositol 3-kinase/Akt (protein kinase B)/mTor (mammalian target of rapamycin) pathway. This pathway is up-regulated by the keratinocytes, and inhibition of keratinocyte migration at the wounded site causes the inhibition Pi3k/Akt/mTor pathway (Misiura et al., 2020).

Furthermore, to determine whether the Pi3k/Akt/mTor signaling pathways is upregulated or down-regulated in the wound healing, a study was conducted by using the mice model. The results showed that the proliferation and migration of the keratinocyte is controlled by the activation of *mTor*. Moreover, the activation of *mTor* leads to the activation of Pi3k/Akt pathway, indicating that Pi3k/Akt/mTor pathway is upregulated in wound healing. It has been also observed that the *Pten* excision also leads to the upregulation of Pi3k/Akt/mTor pathway and accelerate the epithelial migration at the wound site. *Pten* negatively regulates the Pi3k pathway by converting phosphatidylinositol 3,4,5-triphosphate (Pip3) into phosphatidylinositol4,5-bisphosphate (Pip2), thus further restraining the *Akt* activation. This leads to impairment of basal epithelial layer causing deficient activation of mTor (Squarize et al., 2010).

Activation of AMPK (adenosine monophosphate activated kinase) inhibits the mTor pathway which suppresses the migration and proliferation of keratinocytes at the wound site, showing that the locomotion of keratinocytes is upregulated by the mTor pathway. Diabetic drug metformin (metformin hydrochloride) was used to block the Pi3k/Akt/mTor pathway and reduce the proliferation of keratinocytes at the injury site (Crane et al., 2021)

1.5 Application of Keratinocytes in Wound Healing

Since early 1980s, the cultured epithelial substitutes (CES) have been analyzed for their role in skin cell regeneration. Cultured keratinocytes were first cells used in tissue

engineering to treat the wounds. According to previous studies, keratinocytes were isolated from cultured flask and directly engrafted on skin wounds (Sierra-Sánchez et al., 2021). CES are usually cultured from patient skin. Culturing requires 2-3 weeks that can cause life threatening problems for patients. Therefore, allo-CES are prepared which involved culturing of epithelial cell taken from other donors. These CES are prepared initially and can be stored for subsequent use (Sakamoto et al., 2020). Allo-CES of cultured keratinocyte sheet is used to treat the chronic wound, burn and diabetic ulcers in the form of dressing. However, success rate depends on immune response of the host, i.e. immune rejection. Kaloderm® is cryopreserved Korean allo-cultured substitute used in the treatment of burn wounds. Similar to this Korean product, all CES are cryopreserved and stored for later use in the treatment of wound. However, cryopreservation results in depletion of viable cell. This depletion in viable cells definitely affect the tissue regeneration (Marsch-moreno & Ocaz, 1990).

First cultured substitute was developed by Rhienwald and Green. They cultured the autologous keratinocytes to form coalescent sheet of epithelial cell. In this process, they cultured the keratinocytes along with irradiated mouse 3T3 cells, which is fibroblast cell line that was isolated from a mouse NIH/Swiss embryo. Culturing was performed in medium containing growth factors required for epithelial cell proliferation and fetal calf serum. Such type of cultured epithelial grafts can help in restoration of epidermal layer in patients with burn wounds. Take of such type of confluent sheet is poor and may result in blistering (Rheinwatd & Green, 1975). Pre-confluent sheet of keratinocytes are alternative to these confluent sheets. Their take ratio is better than confluent sheets. Moreover, these sheets require less time to build and are cost effective. Clinical studies showed that these pre-confluent sheets can be transferred through a variety of carrier systems (Leigh & Navsaria, 1998). These carrier systems include aerosol sprays, fibrins and polyurethane membranes. Furthermore, such systems are easier to apply as compared to epidermal confluent sheets. For treatment of bigger wound pre-confluent sheet can be used along with meshed skin autografts, which provides keratinocytes and fibroblast stem cells. In one of study, the keratinocytes cultured on polymer plasma surface enhance the re-epithelization when they were grafted along with meshed split

skin. Another study also showed that the cultured keratinocyte accelerated the healing in the donor sites (Coolen et al., 2007).

New advances in tissue engineering involve use of keratinocytes with wound dressings. Cultured grafting is time consuming and expensive. Furthermore, there is a risk of organ rejection by host immune response in case of allografts and xenografts (Seet et al., 2012). Use of biomaterials to transfer non-cultured keratinocytes to wound site is an efficient technique to treat wound. Biomaterial based non-cultured autologous skin is easier to apply and it is cost and time effective as compared to cultured skin substitutes. Hydrogel as keratinocyte carrier is successfully used for delivery of non-cultured keratinocytes to wound area. Hydrogels are hydrophilic polymeric compounds capable of carrying of big amount of water without changing their 3D structure. Due to their ability of carrying high water, they can be used as scaffolds because of their resemblance to human skin tissue (Mohd Amin et al., 2012).

Study conducted by Nicholas et al (2016) demonstrated the use of pullulan as hydrogel. They described pullulan as combination of gelatin and collagen that have antioxidative properties and can carry large amount of water. They used this hydrogel to act as a bilayer skin substitute (Nicholas et al., 2016). A freeze-dried scaffold consisting genipin (fruit extract aglycone) and secrin (antidiabetic agents) seeded with fibroblast and keratinocytes was used as skin analogue in another study. Mazylyzam and his co-workers (2007) created a skin substitute containing non-cultured keratinocyte and fibrin as carrier system for delivery to wound site. Fibrin (non-globular protein used in blood clotting) used as a carrier system was derived from patient's plasma thus reducing the chance of immune rejection (Mazlyzam et al., 2007).

In short, keratinocytes have broad applications in wound healing. It involves the direct application of cultured keratinocytes and grafting on skin and use in different formulations, dressing and carrier system. Application of each technique to deliver the keratinocyte to wound bed is dependent on the type and nature of the wound. Furthermore, it's dependent on the regeneration ability of recipient host and immune response of host (ter Horst et al., 2018).

1.6 Role of Steroids in Wound Healing

Steroid is an organic compound, present in the animals, plants and the fungi. Inside the body steroids play two main functions, as it is the main component of cell membrane. It has an important role in the alteration of membrane fluidity and secondly, steroids are signaling molecules in the body. Steroids have the several examples like lipid cholesterol, sex hormones, i.e. estradiol and testosterone, and dexamethasone (derivative of glucocorticoid), which is an anti-inflammatory drug used in skin diseases. Dexamethasone decreases the expression of cytokines including transforming growth factors (TGF- β), tumor necrosis factors (TNF), PDGF and interleukin in wounded tissue. Moreover, it is also used as a drug to treat asthma, severe allergies and lung diseases (Rhen & Cidlowski, 2005).

Animal steroids have sub class of corticosteroids known as glucocorticoids and actually these glucocorticoids play anti-inflammatory role in wound healing (Ehrlich et al.,1968). Cells constituting the newly formed tissue secreted the TGF-β and IGF-1, and they act as mediators of cellular response. They play vital roles to regulate the interactions between cells on the basis of the nature of their receptors. From the previous studies, it has been assumed that corticosteroids lower the level of TGF-β (transforming growth factor β) and IGF-1 (insulin growth factor-1). This leads to delay tissue deposition in wounds while the retinoids (a class of vitamin A) stimulate the TGF-β and IGF-1, leading to speed up the tissue deposition in wounds. No doubt, that the glucocorticoids have beneficial effects in rheumatoid arthritis and bronchospasms but the inflammatory and immunosuppressant action of the steroids leads to the delay in wound healing (Vukelic et al., 2011, Feeser et al., 2009).

1.7 Steroid Regulated Gene Expression in Wound Healing

According to the previous studies, it has been analyzed that expression of keratinocyte growth factor (KGF) was suppressed in the wounded mice treated with glucocorticoids. It was observed that the glucocorticoids have direct effect on the *Kgf* expression in mesenchymal cells. The anti-inflammatory drug, dexamethasone (glucocorticoids),

reduced the *Kgf* mRNA levels in the wounded mice, which leads to the reduced level of *Kgf* expression in wound healing (Brauchle et al., 1995).

In the kidney transplantation, it was observed that corticosteroids are immunosuppressive agents that cause inhibition in wound healing (Flechner et al., 2003). Actually, these steroids (corticosteroids) bind to its cytosolic immunophilin FKBP12 and inhibits the kinase activity of the specific cell cycle regulatory protein known as *mTor.* Inhibition of *mTor* leads to stop the cell cycle progression from G1 phase to S phase (Sehgal et al.,1998).

During the epithelization of wound healing, the glucocorticoid is present throughout the epidermis but it is expressed mainly in the basal layer. It represses the expression of basal cell -specific keratins (K5 and K14) and the wound healing associated keratins (K6, K16 and K17) leading to the inhibition of epithelization in wound healing (Vukelic et al., 2011).

Brassinosteroids, are naturally produced plants' steroids and they are involved in the growth of plants, cell division and differentiation. These brassinosteroids were used in the animals for the wound healing, and it was found that the application of brassinosteroids significantly reduced the size of wound and accelerate the process of wound healing. Actually, protein kinase *B* (Akt) is the main factor in the phosphoinositide 3- kinase (Pi3k/Akt) network and previous studies suggested that this pathway plays a vital role in wound healing. The inhibition of *Pi3k* leads to decreased basal expression level of *Colla2* gene (producing collagen type 2 protein) by reducing the stability of mRNA (Asano et al., 2004) and the activation *of Pi3k* causes the up-regulation of *Akt w*hich leads to increase in the *Colla2* gene expression that ultimately results in accelerating the wound healing. The brassinosteroids activate the Pi3k/Akt pathway with low androgenic effects (Esposito et al., 2011).

Recently, it has been reported that *REDD1* (Regulated in Development and DNA damage 1) and *FKBP51*(FK506 Binding proteins 5) are negative regulators of mTor/Akt signaling pathway, because FKBP51 inhibits the mTor activity by binding to it FK506-rapamycin binding domain. REDD1 and FKBP51, inhibitors are induced by the

glucocorticoids in the human and mouse skin. The activation of *Akt* requires the phosphorylation at Thr308 and Ser473, while the FKBP51 increases the interaction between phosphatases PHLPP1/2 and Akt, which leads to dephosphorylation of Akt at Ser473 (Manning & Cantley, 2007).

1.8 Genetic Pathways and Wound Healing

1.8.1 *Akt/mTor Pathway*

Upon injury the network of coordinated genetic pathways is initiated by signaling peptides. This stimulation is not fully regulated by intrinsic signaling between the cells but are dependent on the wound environment (Castilho et al., 2013). Study conducted showed the different level of phenotypic expression in *P-Pi3k (Phosho-Pi3k), P-Akt (Phospho-Akt), Pi3k* and *Akt* (Gao et al., 2017). Expression of both *p-Akt* and *Pi3k* were at peak during proliferation and inflammatory phases. The levels of *Akt* and *Pi3k* expression were high during remodeling phase (Xiao et al., 2017). This showed that there is significant association between the expression of these two genes and wound healing. In family of Akt kinases there exist three isoforms, Akt1, Akt2 and Akt3. Akt1 is the most important isoform and crucial in cellular growth. However, the combined action of three isoform is necessary in cell growth and development. Similarly the wound healing pathways are initiated by kinases belonging to different families (Maurer et al., 2014).

Several factors, including insulin, glucose and a number of growth hormones and cytokines, can cause PI3K/AKT/mTOR pathway activation (Bodine, 2006). Usually, these substances stimulate receptor tyrosine kinases (RTKs) and G protein-coupled receptors (GPCRs), which stimulate PI3K and cause the synthesis of phospholipids. These signals cause PI3K downstream effectors like AKT and mTORC1 to become active. The activity of protein kinase B (AKT) is necessary for PI3K signaling. The regulatory hydrophobic region of AKT, which interacts with it to phosphorylate at Ser473, is where the AKT signaling pathway is activated by mTORC2. The translocation of activated AKT into various cell compartments results in the activation of a number of downstream substrates, including small G protein factors (Sun et al., 2020). Any defect in this pathway results in the reduction in epithelial mesenchymal

transition (EMT), no proliferation of cells and subsequently affects the wound healing (Yang et al., 2017). Two different complexes are formed by *mTOR, mTORC1 and mTORC2. MTORC1* is involved in the synthesis of different proteins which are involved in the migration, proliferation and differentiation of cells (Zarogoulidis et al., 2014). *MTORC2* plays an important role in activation of *Akt*. Phosphorylation of amino residues of *Akt* by *MTORC2* and *PDK1 (*Pyruvate Dehydrogenase kinase 1) activates the *AKT. AKT* is involved in activities related to cell cycle progression. Any dysregulation in this pathway results in emergence of several diseases like cancer, diabetes, obesity and cardiovascular diseases (Porta et al., 2014). Despite controlling the expression of growth factors; i.e. epithelial growth factor, fibroblast growth factor and vascular epithelial growth factor; this pathway is involved in the synthesis of collagen and angiogenesis in wounded tissue (Huang et al., 2015).

Expression of *p-AKT* in wound tissue indicates the *mTor/Pi3k/Akt* pathway activation. As this cycle progresses the proliferation of cell is down regulated by tumor suppressor protein p53.This suppression prevents the further proliferation by this pathway and hence reduces the development of tumor in wound tissue (Strozyk & Kulms, 2013). Hoke et al (2016) reported that there is increase in the expression of mTor/Pi3k/Akt pathway on both protein and mRNA level in different stages of wound healing in murine wound models (Hoke et al., 2016). Thus, wound healing pathway constitutes of different kinase families and depends on each other to activate the pathway. Any deficit in one of the kinase family involved in pathway lead to no healing and development of serious diseases (Gu et al., 2020).

Research studies were carried out on model organisms to analyze the role of *Akt/mTor* pathway in wound healing. Study conducted by the Zhang et al (2016) investigated the possible role of Akt/mTor pathway in wound healing in diabetic mouse models. This study aimed to investigate the role of saponin molecule Ft-1 in the treatment of diabetic foot ulcer. This study revealed that the Ft-1 molecule accelerates the wound healing by increasing the expression of Akt/mTor pathway. This increase in the expression level resulted in the higher production of collagen and fibroblast proliferation. Formulation bases application of Ft-1 on diabetic mouse model results in higher gene expression

relation to this pathway and accelerates the wound more than five times than the controls. This is clear evident that Akt/mTor pathway plays a significant role in wound healing (Zhang et al., 2016).

In similar study role of *mTORC1* was investigated in wound healing in mice model. Research analysis shows that activation of *mTORC1* results in the active migration and proliferation of fibroblast cell to injury site. It is to be remembered *that mTORC1* is one of two complexes formed by mammalian recipient of rapamaycin (*mTOR)*. Activation of this complex results in cell migration, proliferation and differentiation. Before it was thought that *mTORC1* only activates the keratinocytes. In this study, researchers concluded that *mTor* pathway is involved in wound healing not by activating keratinocytes but also by activating fibroblast. Study results showed that activating *mTORC1* accelerates the wound healing by excessive collagen production and fibrosis (Selvarajah et al., 2019).

Angiogenesis is important phenomena observed during wound healing. It involves in synthesis of vascular network in wounded area. Once the network is established, it helps in the movement of epithelial cells, growth factors and hormones to injury site. It has been observed that Akt/mTOR pathway plays an important role in angiogenesis within the injury site. Angiogenesis was observed in human brain vascular cells when Akt/mTor pathway was up regulated by ginsenoside Rg1 (Chen et al., 2019). Another study was conducted to investigate the inhibition of Akt/mTOR pathway. Study was aimed at knocking down the genes involved in the pathway by microRNA-218–5p in cultured human pterygium cells (epithelial cells). Results indicate that inhibition of this pathway leads to no epithelial cell migration and proliferation. Inhibition of migration and proliferation result in low wound healing, since it is a crucial step in tissue regeneration (Han et al., 2019).

1.8.2 *Irs/Akt/Pi3k Pathway*

Insulin is topic of decade for its involvement in tissue regeneration and wound healing. Insulin is polypeptide known for regulation of blood sugar especially in case of hyperglycemia. Because of its ability to heal wound, its interest is increased in the field of wound healing and tissue regeneration. From past decades, insulin is being used in

many topical formulations, dressing and creams to apply on injury site. All the studies conducted on insulin concluded that it is an activator of angiogenesis at wound site (Liu et al., 2021) . Increase expression of insulin receptors like *Irs-1 and 2,* in wound tissue comparing of its expression level in intact skin indicates the role of insulin in wound healing (Brem & Tomic-canic, 2007). *Akt* have the ability to phosphorylate various proteins that help in cell survival, production of lipid and other macromolecules. Research analysis showed that *Akt* activation is necessary for release vascular epithelial growth factors for development of vascular network and angiogenesis (Saltiel & Pessin, 2002). This important step is the most important in wound healing. Study conducted by Lima et al (2012) showed that inhibition of this pathway in diabetic mouse model resulted in delay of wound healing process as compared to the control group. Application of topical insulin on mice wounds accelerated the wound healing which indicates the insulin initiates the cascade of Pi3k/Akt pathway involved in wound healing. That is the reason *Irs1, Irs2 and Akt* expression are on its peak at wound site as compared to control group (Lima et al., 2012).

*ERK s*timulation by insulin results in phosphorylation of IRS. Phosphorylated IRS interact with growth factor receptor bound protein (Grb2) to recruit SOS protein into plasma membrane to activate Ras. Once Ras is activated, it starts to acting as a molecular switch. This molecular switch in turn activates ERK, MEF and Raf (Skolnik et al., 1993). Activated *ERK* moves to nucleus and phosphorylate the various kinases which result in initiation of transcriptional programs involved in the cell differentiation and proliferation. Thus, the expression of *ERK* enhancing at wound site as compared to intact skin. It was also noted that *ERK* plays an important role in the migration of keratinocytes to wound site which is a crucial step in wound healing (Cheng et al., 2007).

Regulation of the migration of epithelial cells involves a complex mechanism in which eNOS (endothelial nitric oxide synthase) activation by VEGF (vascular endothelial growth factor) is required. This activation results in the migration of epithelial cell to the wound site. Non-availability of insulin to *IRS* results in low expression of *VEGF* and no vascularization in wound site. Katagiri et al (2016) investigated the effect on insulin

receptor on wound healing and angiogenesis in deficit mice model. Genetically induced insulin resistant mice were subjected to the experiment. Study results showed that high rates of angiogenesis in mice model with elevated expression of *Irs* as compared to insulin resistant mice. This study demonstrated that insulin is a potential stimulator of wound healing (Katagiri et al., 2016). However, a slow wound healing is observed in patients with diabetes, insulin resistance and obesity. The main phenotypic expression observed at wound site in insulin resistant organism is the development of gangrene at damaged tissue. Main cause of gangrene development is loss of vascular network and blood supply to the wound site. Therefore, any deficit in insulin signaling pathway results in loss of angiogenesis because it is involved in the development of vascular network at the injury site (Nakamura et al., 2020).

Study conducted by Takashi Shimoaka et al (2004) showed that the deficiency of *Irs1* leads to the slowdown of bone impairment or un-union of the fracture site (Shimoaka et al., 2004) . This is due to the suppression of callus formation and decreased chondrocytes proliferation. The chondrocyte proliferation is regulated by the phosphatidylinositol 3-kinase/Akt pathway. While the presence of *Irs1* at the fracture site leads to the rapid reunion of the bones, because the activation of *phosphatidylinositol 3-kinase/Akt* leads to the increase in chondrocytes proliferation and this increase in chondrocyte proliferation causes the restoration of callus formation (Ogawa, and Terauchi, 2004). Insulin receptor substrates (IRSs) play an essential role as a substrate of the receptor tyrosine kinases and has great impact on the pleiotropic effects of *IGF1*and insulin on cellular function. This study proposed that the *Irs1* can be used as a target molecule for the bone regenerative medicine (Kon et al., 2003).

1.8.3 *Pik3ca Pathway*

Pik3ca is the phosphatidylinositol 4-5 bisphosphate 3 kinase, catalytic subunit alpha and it is also called p110-α protein. This protein is encoded by the *Pik3ca*. This gene plays several essential roles. It is involved in the phosphorylation of signaling molecules and these molecules then trigger different reactions that transmit the chemical signals inside the cell. Pik3ca signaling plays a vital role to carryout important cell activities, such as cell growth, cell division, migration of cells, production of new proteins, locomotion of

the materials between the cells and within the cells, cell survival and the regulation of some vital hormones that are involved in the maturation of fat cells (Phillips et al.,1998).

A study was conducted to determine role of *Pik3ca* that showed that it plays a role in wound healing and it was observed that mutations in *Pik3ca* activates the Pi3k/Akt pathway and this pathway stimulates the movement and differentiation of keratinocytes to the wound site, and this triggers the process of wound healing (Ross et al., 2013).

1.8.4 *Housekeeping Genes (ACTB/GAPDH)*

ACTB gene is involved in the formation of protein called the Beta-actins. Beta actin belong to actins proteins family. Actin is divided into six groups. Four types of actins are present in muscle cells whereas the remaining two are present in all cells throughout the body. Actin proteins play a crucial role in determining the cell shape and the migration of the cell (Cuvertino et al., 2017).

Normal expression of *ACTB* is important in cellular process. Any dysregulation in *ACTB* expression subsequently affects the wound healing. Expression of *ACTB* is regulated at both transcriptional and post transcriptional stages (Olave et al., 2002). HUR (Human antigen R protein) is expressed in all proliferating cell and is considered as the most important regulator of gene expression of *ACTB* (Kislauskis et al., 1993). Study conducted by Dormoy-Raclet and colleagues (2013) showed that depletion of human antigen R have significant effect on cytoskeleton functioning and furthermore it influences the cell migration, invasion and adhesion. It was also observed that loss of HUR antigen results in loss of beta actin fiber within the cell. Thus, HUR binding the AU rich element stabilizes the messenger RNA of *ACTB* and hence, *ACTB* plays a crucial role in wound healing (Dormoy-Raclet et al., 2013).

To check whether decreased expression level of *ACTB* affects the wound healing, Joseph et al (2014) demonstrated that silencing of HUR by small interfering RNAs (siRNAs) resulted in low level of *ACTB* expression. This low expression resulted in the decreased fibroblast proliferation and wound healing (Joseph et al., 2014). In another study conducted by the Helms et al.(2010), the expression of Beta actin gene was observed in earthworm during wound healing (Helms & Coker, 2010). Actin genes are considered as housekeeping genes. The three forms of actin genes known as alpha, beta and gamma.

Alpha-Actin genes are present only in vertebrates while *Beta-Actin* and *Gamma-Actin* forms are found in both vertebrates and invertebrates. *Beta-Actin gene* (*ACTB)* is used as control for genetic expression studies (Singh et al., 2009).

Glyceraldehyde-3-phosphate dehydrogenase (Gadph) is an enzyme widely known for its role in glycolysis. In addition to glycolysis, it is also involved in other processes, such as DNA repair, replication, exportation of tRNAs, endocytosis and cytoskeleton development. Studies showed that *Gapdh* works as sensor in case of any intra or extra-cellular stresses and involved in triggering of cell death or apoptotic pathways (Zhang et al., 2015).

Both *Actb and Gadph* are used as housekeeping genes. Housekeeping genes are often considered as the most stable gene and there is no variation observed in the expression of these gene. Housekeeping genes are stable because of their function, i.e. maintenance and cellular functions. Therefore, they require to be stable for survival of the cells. Both *Gadph and Actb* are used as internal control for measuring relative gene expression especially in wound healing studies (Turabelidze et al., 2010).

CHAPTER III

Materials and Methods

This chapter contains the information regarding the materials and the processes that were used to perform the experiment. Experiments were performed to study the expression of *mTor, Akt1, Akt2, Irs,* and the housekeeping gene *Gadph* in the mouse models.

2.1 Sample Collection and Sample Size

The mouse models were established in the Manisa Celal Bayar University, Turkey by Prof. Seda Vatansever's research group. Briefly, four groups of mouse models were established. The first group contained only wounded skin without any treatment and this group was considered as control group, the second group was composed of mouse models of wounded skin with differentiated keratinocyte treatment, third group was composed of mouse models of wounded skin with steroid treatment and the fourth group was composed of mouse models of wounded skin with a combination of steroids and differentiated keratinocyte treatment. In all groups, a 0.5x0.5 cm-wound model containing the epidermis and dermis were created on the area cleaned of hair in the nape region using a sterile surgical set. After the wound model was created, dexamethasone was applied to group 3 and group 4 every day for 3 weeks. Keratinocytes, differentiated from CGR8 mouse embryonic stem cells, was transferred to the wound site of the subjects belonging to group 2 and group 4. A 5μm sections were obtained from six models in each group with a total of 24 samples. These samples were embedded in paraffin blocks.

2.2 Gene Expression Analysis

The gene expression studies, involving RNA extraction, measuring RNA concentration and purity, cDNA synthesis and real time PCR analyses were conducted in Near East University, DESAM Research Institute laboratory.

2.3 RNA Extraction

RNA Extraction was performed using the NORGEN FFPE purification Kit (Norgren Canada) following the manufacturer protocol. The first step in RNA extraction was deparaffinization. In this step, 1000µl Xylene was used to wash the sample followed by addition of 1000µl ethanol, centrifugation was performed at 14000 RPM for 2 minutes and at end of this step pellet was formed. The second step in RNA extraction was lysate preparation. In this step, 300µl Digestion Buffer A, 10µl proteinase K, 300µl Buffer RL and 100% ethanol were used. Third step in RNA extraction was binding RNA to column. In this step, spin columns along with the collection tubes were used and 600µl lysate was passed through the spin columns to the collection tubes after centrifugation for 1 minute at 6000 RPM. The fourth step in RNA extraction was the washing of columns. In this step, 400µl Wash Solution A was used to wash the column. The fifth step in RNA extraction was RNA Elution. In this step 30µl Elution Solution A and 1.5ml Elution tubes were used to collect the purified RNA. The purity of extracted RNA was evaluated by using the Nano-Drop Spectrophotometer following manufacturer's instructions.

2.4 cDNA Synthesis

cDNA synthesis was performed using the ABM cDNA Synthesis Kit (ABM) according to the manufacturer's protocol. Briefly, 10µl of RNA, 1µl of oligo(dt) primers, 1µl of reverse transcriptase enzyme, 2µl of deoxyribonucleotides triphosphate (dNTPs), 4µl of buffer solution and 2µl of nucleases free water were used for the reaction. The synthesis was performed at 55° C for 15 minutes, concentration of the newly synthesized cDNA was estimated using the Nano-Drop Spectrophotometer**.**

2.5 Real Time PCR

Expression of *mTor, Akt1, Akt2 and Pik3ca* were investigated. *Gapdh* was used as a housekeeping gene. For real time PCR, light-cycler® 480 SYBR green-1 master kit (Roche) was used following manufacturer's protocol. Primers were designed by using NCBI database and the Primer 3 Plus software. Sequence and the concentration of the primers used during the real time PCR are shown in Table 1.

Real-time PCR system (Q96) was used to observe the expression of the targeted genes *via* the use of cDNA samples. Real-time PCR was set up according to the conditions listed in Table 2. A negative control with no cDNA template and SYBR green mix was used.

2.6 Statistical Analysis

The statistical analysis of samples was performed by using the GraphPad prism v8.4. One-way Anova analysis and student's T-test was performed to analyses the difference in the expression levels of *mTor*, *Akt1*, *Akt2* and *Pi3kca* in different groups, P-value ($P < 0.05$) was considered as statistically significant.

2.7 Gel Electrophoresis

During the real-time PCR, the gene expression can be observed by analyzing the different sigmoid graphs on the screen, but to check the primer dimers, gel electrophoresis was performed. In this process, 250 ml Tris-borate EDTA was used**,** 6.75gram agarose, 6 µl ETBR, 3-4 µl dye and 10 µl PCR sample were used. Once the agarose gel was solidified, the gel was placed in the electrophoresis unit, ladder was loaded in the first lane of gel following the manufacturer range, samples were loaded in the additional wells of gel along with blue dye. The gel was run for 1 hour at 120-V and the fragments were visualized under the UV light.

Table 1

Tm and Concentrations of the Primers Used in Real Time PCR

Primers	**Forward primers**	**Reverse primers**	**Reaction chemicals**	**Concentration of primers**	**Tm °C**
Akt1	GGCAGGA AGAGACG ATG	CCTGTGGC CTTCTCTTT CAC	SYBR Green, F. primers, R. primers, cDNA	0.125 µM	62 °C
Akt2	GAAGACT GAGAGGC CACGAC	CTTGTAAT CCATGGCG TCCT	SYBR Green, F. primers, R. primers, cDNA	0.125 µM	62 °C
mTor	CGCTTCTA TGACCAG CTGAA	TGACAACT GGATCGCT TGAG	SYBR Green, F. primers, R. primers, cDNA	0.125 µM	62 °C
Pik3ca	CACCTGA ACAGACA AGTAGAG GC	GCAAAGCA TCCATGAA GTCTGGC	SYBR Green, F. primers, R. primers, cDNA	0.125 µM	62 °C
Gapdh	CATCACTG CCACCCA GAAGACT G	ATGCCAGT GAGCTTCC CGTTCAG	SYBR Green, F. primers, R. primers, cDNA	0.125 µM	62 °C

Table 2

Real-Time PCR Conditions for mTor, Akt1, Akt2, Pi3kca and Gadph

	PCR Steps	**Temperature ^{0}C/Time**	**Cycles**
Steps	Initial Denaturation	95 ^{0}C / 10 minutes	1
	Denaturation	95 ^{0}C / 10 seconds	40
	Annealing	56 ^{0}C / 20 seconds (Depending on the primer used)	
	Elongation	72 ^{0}C / 25 seconds	

CHAPTER IV

Results

This chapter includes the results of the conducted experiment. The results that are mentioned in this chapter are obtained from the Nano-Drop Spectrophotometer and real-time PCR. Moreover, these results were statistically analyzed using the one-way ANOVA and student's T-test. This method was helpful in creating the numerical and graphical results that are presented in this chapter.

The total sample size was 24 and the samples were collected from the mouse animal models and divided into four groups. The first group involved samples obtained from the mice with wounded skin without any treatment and this group was considered as the control group. The second group included samples obtained from mouse models of wounded skin with differentiated keratinocyte treatment. The third group consisted of samples from mouse models of wounded skin with steroid treatment and the fourth group included samples from mouse models of wounded skin with a combination of steroids and differentiated keratinocyte treatment. RNA extraction was successfully performed from these samples. The purity of RNA was evaluated by using the Nano-Drop Spectrophotometer. The results are shown in the table 3. The RNA purity was determined by measuring the ratio of the absorbance at 260 and 280 nm (A260/280). The A260/280 ratio of pure RNA is 2.0, and the A260/280 ratio were obtained for all the RNA samples. In this experiment, the mean ratio was approximately 1.78. The average range of concentration of extracted RNA during this experiment was 2000-2500 ng/µl (Table 3). The levels of expression of *mTor, Akt1, Akt2, Pik3ca and Gapdh* genes were determined by real-time PCR analysis and the results were evaluated using the one-way ANOVA statistical analysis. The CT values were obtained from the real-time PCR equipment. These values were used to obtain the log ΔΔCT fold change (this fold change shows the up- and down-regulation of the genes in the graph) values for comparative ΔΔCT analysis (Table 4).

Table 3

Nano-Drop Spectrophotometer Results Showing the Purity and Concentration of Extracted RNA.

Treatment group	**Sample ID**	**RNA concentration (ng/µl)**	**A260/280 nm ratio**
Group 1 Control (wounded mice without any treatment)	1	3171.7	1.79
	2	2302	1.74
	3	2106	1.74
	4	2201.4	1.73
	5	2101.4	1.76
	6	2234.2	1.76
Group 2 (wounded mice treated with Steroids)	7	2013.1	1.74
	8	2096.7	1.75
	9	2398.1	1.74
	10	4025.8	1.78
	11	2860.3	1.49
	12	2358.3	1.75
Group 3 (wounded mice treated with differentiated Keratinocytes)	13	2244.4	1.73
	14	2636.0	1.73
	15	2098.5	1.76
	16	2815.2	1.73
	17	1590.0	1.73
	18	2240.1	1.75
	19	2781.8	1.70
	20	2570.7	1.74

Group 4 (wounded mice treated with combination of Steroids + Keratinocytes)	21	2286.7	1.75
	22	2498.0	1.76
	23	1556.9	1.73
	24	2326.4	1.77

Table 4

The Results of Mean CT Values Following Real Time PCR for mTor, Akt1, Akt2, Pik3ca and Gapdh genes.

Groups	Sample ID	*Gapdh*	*mTor*	*Akt1*	*Akt2*	*Pik3ca*
Ct value of Group 1	1	25.6	28.1	23.9	29.6	24.7
	2	22.9	28.9	22	27.1	25.7
	3	19.6	23	19.8	23.5	22.5
	4	19.2	22.9	19.4	22.9	21.8
	5	20.1	24.2	18.6	15.3	20.9
	6	19.7	22.7	18.6	21.2	20.9
Ct value of Group 2	7	22.1	27.9	23.4	26.4	25
	8	22.7	28.5	24.7	27.7	24.9
	9	22	22	18.9	22	22.3
	10	22	22.5	17.6	22.3	21.7
	11	18.9	22.1	17.6	17.3	20.9
	12	25.8	28.5	24.1	26.5	25
Ct value of Group 3	13	22.5	28.5	23.6	25.7	25
	14	19.2	22.4	18.8	21.7	20.9
	15	19.9	22.5	18.4	24.4	18.7
	16	17.9	21.8	17.9	16.5	19.6

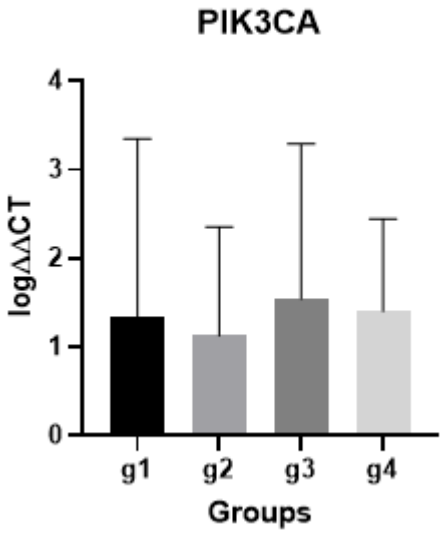
	17	19.8	22.7	18.7	21.8	20.5
	18	22.5	25.5	23.7	25.4	24.7
Ct value of Group 4	19	24.6	26.4	23.6	25.7	25.1
	20	18.9	22.5	25.4	23	21.8
	21	18.4	21.1	25.6	21.5	19.3
	22	18.2	22.6	29.9	18	18.8
	23	19.3	23.6	19.5	22	18.9
	24	19.7	22.3	16.7	23	21.2

3.1 *Pik3ca* Expression Analysis

The expression levels of Pik3ca were investigated in four groups. The average CT value of group 1 (the first group contained only wounded skin without any treatment and this group was considered as the control group) was 22.7, group 2 (the second group was composed of mouse models of wounded skin with differentiated keratinocyte treatment) was 23.3, group 3 (the third group was composed of mouse models of wounded skin with steroid treatment) was 21.5 and group 4 (the fourth group was composed of mouse models of wounded skin with a combination of steroids and differentiated keratinocyte treatment) was 20.8, respectively. The results of CT values showed that the fold change of *Pik3ca* in each treatment group is slightly above than the control group, showing that this gene is up-regulated relative to the controls. However, the differences between the groups were not statistically significant. Student's T-test was also performed and the p-value was greater than 0.05. These analyses also showed that there was no significance difference in the expression levels of *Pik3ca* in different groups (Table 4, figure 1).

Figure 1

The Statistical Analysis of the Log ΔΔCT Value of Pik3ca in Each One of the Mice Groups

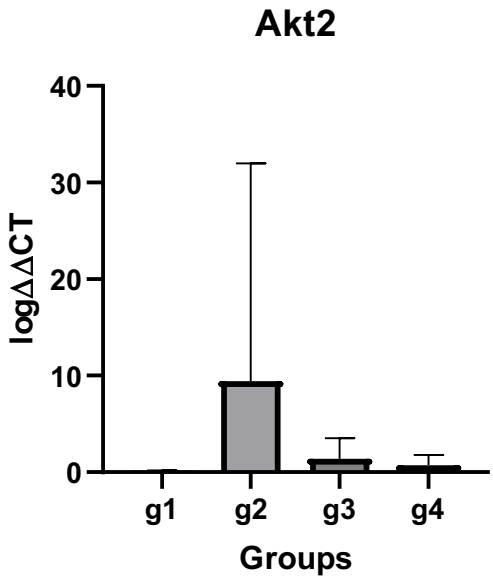


3.2 *Akt2* Expression Analysis

The expression levels of *Akt2* were investigated in four groups. The average CT value of group 1 was 23.2, group 2 was 23.7, group 3 was 22.5 and group 4 was 22.2, respectively. The results of CT values showed that the fold change of *Akt2* in each treatment group is slightly above than the control group, showing that gene is up-regulated. However, the differences between the groups were not statistically significant. Student's T-test was also performed and the p-value was greater than 0.05. These analyses also showed that there was no significance difference in the expression levels of *Akt2* in different groups (Table 4, figure 2).

Figure 2

The Statistical Analysis of the Log ΔΔCT Value of Akt2 in Each One of the Mice Groups

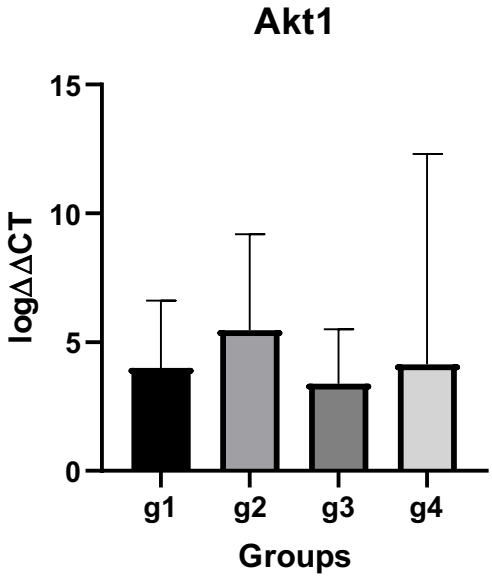


3.3 *Akt1* Expression Analysis

The expression levels of *Akt1* were investigated in four groups. The average CT value of group 1 was 20.3, group 2 was 21.0, group 3 was 20.1 and group 4 was 23.4, respectively. The results of CT values showed that the fold change of *Akt1* in each treatment group is slightly above than the control group, showing that gene is up-regulated. However, the differences between the groups were not statistically significant. Student's T-test was also performed and the p-value was greater than 0.05. These analyses also showed that there was no significance difference in the expression levels of *Akt1* in different groups (Table 4, figure 3).

Figure 3

The Statistical Analysis of the Log ΔΔCT Value of Akt1 in Each One of the Mice Groups

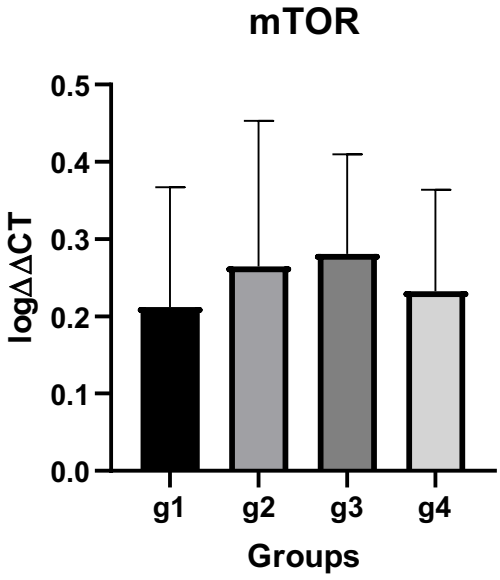


3.*4 mTor* expression analysis

The expression levels of *mTor* were investigated in four groups. The average CT value of group 1 was 24.9, group 2 was 25.2, group 3 was 23.9 and group 4 was 23.0, respectively. The results of CT values showed that the fold change of *mTor* in each treatment group is slightly above than the control group, showing that gene is up-regulated. However, the differences between the groups were not statistically significant. Student's T-test was also performed and the p-value was greater than 0.05. These analyses also showed that there was no significance difference in the expression levels of *mTor* in different groups (Table 4, figure 4).

Figure 4

The Statistical Analysis of the Log ΔΔCT Value of mTOR in Each One of the Mice Groups



CHAPTER V

Discussion

The skin is an essential as well as the largest organ in the human body. It is involved in performing various functions, such as hydration, excretion, production of vitamin D, heat control and protection from foreign agents to enter the body. As skin is performing these all-essential activities, a severe skin injury can be life-threatening. Wound healing process have four main stages, the first is wound injury, the second is inflammation, the third is proliferation and the fourth is remodeling. Currently different therapeutic treatments are used for wound healing. For instance, dexpanthenol topical formulation is very commonly used in wound healing where it expedites the re-epithelization by triggering the proliferation and migration of keratinocytes at the wounded site (Gorski et al., 2020).

Previously published studies, hypothesized that keratinocytes play vital role in the wound healing, because up to 90-95% of the epidermis is made up of keratinocytes, and it acts as an innate immune cell. It was observed that keratinocytes were activated *via* the Pi3k/Akt/mTor pathway. Inhibition of the keratinocyte migration at the wounded site causes the inhibition of Pi3k/Akt/mTor pathway. Results of the different studies showed that the *Pi3k, Akt and mTor* genes are up-regulated by activating the keratinocytes, and these genes or their pathways is involved in the wound healing (Misiura et al., 2020).

The treatment of skin wound was performed by different applications, but the most commonly keratinocytes and steroids (corticosteroids) are being used for the treatment. The use of keratinocytes depends on the injury type and in some cases cultured keratinocytes can be used and they can be grafted on the injury site. The use of keratinocytes is determined by the regeneration ability of the recipient and the immune response of host (Kanapathy et al., 2016).

In wound healing not only the keratinocytes are used, but some of the studies revealed that steroid-corticosteroids also play vital role in the treatment of skin wounds. Steroids are the organic compounds produced in the body and they are involved in several activities like alteration of membrane fluidity and as well as, acting as a signaling

molecule in the body. Dexamethasone, is an example of steroids, and it is an anti-inflammatory drug used in skin diseases, asthma and severe skin allergies. Moreover, glucocorticoids are the sub-class of steroids and they play anti-inflammatory role in wound healing (Ehrlich et al.,1968). According to the previous studies, it was assumed that the glucocorticoids suppress the expression of keratinocytes growth factors (KGF) (Hoyle & Rubin, 1996). Furthermore, a study was conducted on wound healing following kidney transplantation and it was noted that corticosteroids were immunosuppressive agents because they caused inhibition in wound healing. These steroids (corticosteroids) inhibit the kinase activity of *mTOR* by binding to its cytosolic immunophilin *FKBP12*. This inhibition of mTOR pathways showed that steroids down-regulated the *mTOR* gene expression (Sehgal et al., 1998).

Another study was conducted to investigate the treatment possibility of wounds in mouse models using brassinosteroids, which is a steroid that is naturally produced by plants. In this study, samples were divided into two groups; one was the control group (wounded mice without any treatment) and the second group consisted of wounded mice with brassinosteroids treatments. Samples were examined after 3-4 days of injury and it was found that size of the wound treated with brassinosteroids was reduced rapidly as compared to the control group. After gene expression analysis, it was analyzed that these steroids activated the *Pi3k a*nd the activation of Pi3k leads to the up-regulation of *Akt.* The up-regulation of *Akt* increases the expression level of *Colla2,* that ultimately accelerated the wound healing. The results of the study showed that Pi3k/Akt pathway is up-regulated by brassinosteroids treatments (Asano et al., 2004).

Gene expression analysis, that was performed during the previous studies, shows that the level of expression of *Pi3k* and *Akt* were at peak in the proliferation and inflammatory phase of the wound healing, indicating that there is significant link between these two kinases and the wound healing (Li et al., 2016). It was noticed that as a result of skin injury, Pi3k/Akt/mTor pathways are activated in the body, and then these pathways lead to the wound healing. An increase in the expression level of *Pi3k*, *Akt and mTor* during the different stages of wound healing in murine wound model was reported (Hoke et al, 2016). This increase in the expression level of these genes speed up the wound healing

process, while any deficit in one of these kinases can cause no healing and also can develop severe diseases. *Pik3ca* is also involved in wound healing, the mutation in *Pi3kca* activates the Pi3k/Akt pathway and this pathway triggers the migration of keratinocytes at the wound site to heal the wound (Ross et al., 2013).

Akt1 is main stimulator of angiogenesis, vascular maturation, and matrix decomposition during wound healing. Angiogenesis is one of main step involved in wound healing process that involve the movement of nutrient material and growth factors to injury site. *Akt2* plays an important role in cell migration, proliferation and adhesion of keratinocyte to the injury site (Jiang & Liu, 2008). The previously published studies showed that *mTor* was upregulated by keratinocytes and was down-regulated by steroids. But in this study, *mTor* was up-regulated by differentiated keratinocytes as well as by steroids. The previous studies revealed that *Akt* and Pi3kca were up-regulated by using the steroids (brassinosteroids) (Asano et al., 2004). But this study shows that a number of genes within the Pi3k/Akt pathway is down-regulated by steroids. The difference in the results might be due to the types of steroids used (Sehgul et al., 1998).

Gapdh plays a vital role in glycolysis and in addition to this, it is also involved in performing different functions like, DNA repairing, replication, exportation of TRNAs, endocytosis and cytoskeleton development. Recent study showed that *Gapdh* act as a sensor for any intra or extra-cellular stress, and work in triggering the cell death and apoptotic pathways (Zhang et al., 2015). *Gapdh* is house-keeping gene, house-keeping genes are stable and show no variation in the expression level, they are stable because of their function, i.e. maintenance and cellular functions. These genes are used as internal control for measuring the relative gene expression level especially in wound healing studies. (Turabelidze et al., 2010).

Taking together the literature and the results of this study, a hypothesis was developed that, differentiated keratinocytes and steroids play essential in wound healing, Moreover, these treatment strategies are involved in the up and down-regulation of genes involved in Pi3k/Akt pathway, such as *mTor, Akt1, Akt2* and *Pi3kca.*

CHAPTER VI

Conclusion

This section contains the conclusion of the study and future prospects of the study. The results of this study show that differentiated keratinocytes and steroids are involved in wound healing, and these supplements cause the activation of several pathways, such as mTor/Akt/Pi3k that take part in wound healing. Furthermore, it was observed that the use of keratinocytes and steroids regulate the gene expression of *mTor, Akt1, Akt2, Pi3k* and usually these genes are up-regulated by the differentiated keratinocytes and this activation speed up the process of wound healing.

Recommendations

Still further studies are required to confirm the effect of steroids and differentiated keratinocytes on the wound healing and also it is required to determine the detailed molecular mechanism of the steroids and keratinocytes in wound healing process and how they direct target the expression *of mTor, Akt1, Akt2, and Pi3k.* This study may help to develop new clinical strategies to accelerate the healing of critical-sized, chronic and life-threatening wounds in patients.

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Hayvan Deneyleri Yerel Etik Kurulu

KARAR TARİH / NO	16/ 02 / 2021 / 77.637.435
ARAŞTIRMANIN ADI	Uzun Süreli Steroid Baskısı Altındaki Farelerde Oluşturulan Yara Modelinde Fare Embriyonik Kök Hücrelerinden Farklılaştırılan Keratinositlerin Yara İyileşmesi Üzerine Olan Etkisi
SORUMLU ARAŞTIRMACI	Prof. Dr. Hasan Aydede - Genel Cerrahi Anabilim Dalı
ARAŞTIRMA EKİBİ	Araş.Gör. İlayda Gençer,- Prof.Dr. H.Seda Vatansever,- Araş. Gör. Damla AKOĞULLARI
ARAŞTIRMANIN NİTELİĞİ	**UZMANLIK TEZİ** ☒ | **YÜKSEK LİSANS--DOKTORA TEZİ** ☐ | **AKADEMİK AMAÇLI** ☐ | Eğitim ☐
DEĞERLENDİRİLEN BELGELER	11 / 12 / 2020 / Tarih ve 40422 sayılı; araştırma dosyası
KARAR BİLGİLERİ	Araştırma dosyası incelenmiş, bilimsel ve etik açıdan **UYGUN** olduğuna oy birliği ile karar verilmiştir.

Unvanı/Adı/Soyadı		Araştırma İle İlişkisi Olan Üye	Toplantıya Katılmayan Üye	Unvanı /Adı /Soyadı		Araştırma İle İlişkisi Olan Üye	Toplantıya Katılmayan Üye
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Etik Kurulumuzun kararı yukarda belirtilmiştir. Araştırma Başvuru Formunun Taahhütname kısmında belirtilmiş olan hususların dikkate alınarak istenilen bilgilerin Etik Kurulumuza zamanında iletilmesi konusunda bilgilerinizi ve gereğini rica ederim.

Ercüment ÖLMEZ
Başkan

Thesis

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