THE EFFECT OF WHEAT (*TRITICUM AESTIVUM L.*) DERIVED EXOSOMES ON WOUND HEALING

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THE EFFECT OF WHEAT (*TRITICUM AESTIVUM L.*) DERIVED EXOSOMES ON WOUND HEALING

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ABSTRACT

THE EFFECT OF WHEAT (*TRITICUM AESTIVUM L.*) DERIVED EXOSOMES ON WOUND HEALING

Wound healing is the main physiological process which undergoes homeostasis, inflammation, proliferation, migration and tissue remodeling. Acute injuries usually heal in a short period of time but chronic illnesses associated with infection and prolonged inflammation, may resulted with the life-threatening infections, limb amputations that presents a serious challenge for the current technology. Although wound healing has been studied for decades, improved drugs are not still effective enough due to their high cost, low efficiency, having patient specific response, requiring wound care facilities and severe side effects. In this sense, developing new, safe, self-applicable, effective and cheap wound care products is still an attractive area of international research. Cell to cell communication is compulsory in order to sustain tissue homeostasis but most communication mechanisms between cells requires a coordination of the receptors and the signaling molecules. Recently, nano vesicle mediated cell communication become prominence because of the ability to shuttle different molecules from producing cells to the target cells via extracellular vesicles called exosomes. Previous studies reported that, exosomes released from plants such as grape, grapefruit, ginger and carrot have anti-inflammatory and anti-cancer properties. Although the underlying mechanisms of wound healing process of plant exosomes are still unknown. For this reason, in this study, for the first time, rate limiting factors of wound regeneration such as cell proliferation, migration, angiogenesis, gene and growth factor expression cell migration and proliferation was stimulated by using wheat derived exosomes by using both in vitro and in vivo approaches. According to the results, wheat exosomes increased cell proliferation, migration and angiogenesis, have anti-inflammatory effect, increased essential growth factor, and gene expression levels of HDF, HUVEC and HACAT cells in vitro. Moreover, wheat exosome-containing hydrogel formulations improved wound healing and histopathological rates in vivo. All in all, this research indicates a brand-new scope for wound healing therapeutics using wheat derived exosomes for the first time.

ÖZET

BUĞDAY (*TRITICUM AESTIVUM L.*) KAYNAKLI EKZOZOMLARIN YARA İYİLEŞMESİ ÜZERINE ETKİSİ

Yara iyileşmesi, homeostaz, inflamasyon, proliferasyon, göç ve doku yenilenmesinden oluşan bir dizi fizyolojik süreçtir. Akut yaralanmalar genellikle kısa bir süre içinde iyileşir, ancak kronik yaralar, yaşamı tehdit eden enfeksiyonlara, mevcut teknoloji için ciddi bir sorun olan ekstremite amputasyonlarına neden olabilir. Her ne kadar yara iyileşmesi onlarca yıldır çalışılmış olsa da, ilaçların yüksek maliyeti, düşük etkinliği, hastaya özel yanıtı, yara bakım tesisleri gerektirmesi ve ciddi yan etkileri nedeniyle hala yeterince etkili değildir. Bu anlamda, yeni, güvenli, kendi kendine uygulanabilir, etkili ve ucuz yara bakım ürünleri geliştirmek hala uluslararası araştırmaların çekici bir alanıdır. Doku homeostazını ve organizmaların bütünlüğünü korumak için hücreden hücreye iletişim zorunludur, ancak hücreler arasındaki iletişim mekanizmalarının çoğu reseptörlerin ve sinyal moleküllerinin koordinasyonunu gerektirir. Son zamanlarda, nano vezikül aracılı hücre iletişimi, eksozomlar olarak adlandırılan hücre dışı veziküller yoluyla farklı molekülleri üreten hücrelerden hedef hücrelere aktarma yeteneği nedeniyle öne çıkmaktadır. Önceki çalışmalar, üzüm, greyfurt, zencefil ve havuç gibi bitkilerden salınan ekzozomların anti-inflamatuar ve anti-kanser özelliklerine sahip olduğu bildirilmektedir. Bitki ekzozomlarının yara iyileşme sürecinin altında yatan mekanizmaları hala bilinmemektedir. Bu nedenle, bu çalışmada ilk kez, hücre proliferasyonu, migrasyon, anjiyogenez, gen ve büyüme faktörü ekspresyonu, hücre migrasyonu ve proliferasyonu gibi yara rejenerasyonu etkileyen faktörleri, hem in vitro hem de in vivo yaklaşımlar kullanılarak buğday eksozomları kullanılarak uyarılmıştır. Buğday ekzozomları dermal hücre proliferasyonunu, migrasyonunu ve anjiyogenezini arttırmıştır, anti-inflamatuar etkiye sahiptir, yara iyileşmesinde görevli sitokin ve genlerin seviyesini arttırmıştır. Ayrıca, buğday ekzozom içeren hidrojel formülasyonları in vivo yara iyileşmesini ve histopatolojik skor seviyelerini yükselmiştir. Sonuç olarak, bu araştırma, yara iyileştirici özellikleri nedeniyle buğday kaynaklı eksozomları kullanarak akut ve kronik yara iyileşmesi tedavisi stratejisi için yepyeni bir bakış açısı önermektedir.

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LIST OF SYMBOLS/ABBREVIATIONS

ADSC	Adipose derived stem cells
AKT	Protein kinase B
bNGF	Beta nerve growth factor
DHEA	Dehydroepiandrosterone
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
ECM	Extracellular matrix
EGF	Epidermal growth factor
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
H&E	Hematoxylin and eosin
H_2O_2	Hydrogen peroxide
HA	Hyaluronic acid
HDF	Human dermal fibroblast
HGF	Hepatocyte growth factor
HUVEC	Human umbilical vein endothelial cells
IGF	Insulin like growth factor
IL	Interleukin
KGF	Keratinocyte growth factor
LPS	Lipopolysaccharide
MMP	Matrix metalloproteinase
MSC	Mesenchymal stem cell
MTS	3-(4,5-dimethyl-thiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfo-phenyl)-2H-tetrazolium
NADPH	Nicotinamide dinucleotide phosphate oxidase
NaOH	Sodium hydroxide
NGF	Nerve growth factor
NO	Nitric oxide
PF4	Platelet factor 4
PI3K	Phosphoinositide 3-kinase

PLGA	Poly (lactic acid-*co*-glycolic acid)
PSA	Penicillin, streptomycin, and amphotericin B
ROS	Reactive oxygen species
SDF	Stromal-derived factor
TGF-β1	Transforming growth factor beta1
PDGF	Platelet-derived growth factor
TNF-α	Tumor necrosis factor alpha
VEGF	Vascular endothelial growth factor
WST-1	Water-soluble tetrazolium salts

1. INTRODUCTION

Early and late complications of wounds and wound infections can be a serious problem in clinical practice, as they can result in high rates of disease and death [1]. Cutaneous wound healing comprises a well-organized physiological process which undergoes homeostasis, inflammation, proliferation, migration and tissue remodeling [1]. The entirety of this process is under a tight regulation that is conducted by a concerted participation of different cell types, chemokines, and hormones, utimately culminating in the proper repair the wounded area and restoration of tissue integrity. Today, studies about the physiology and molecular mechanism of the wound healing process contributes to the development of new therapeutic agents and therapeutic strategies to reduce wound healing and its complications [1]. Although wound healing has been studied for decades, improved drugs are not still satisfactory enough due to their high cost, low efficiency and side effects [2]. Therefore, recently therapy strategies based on plant derived agents have stirred up interest due to the safity, reliance, and low cost properties they offer.

Cell to cell communication is compulsory in order to sustain tissue homeostasis and integrity of the organisms but most communication mechanisms between cells requires a coordination of the receptors and the signaling molecules [3]. Recently, nano-vesicle mediated cell communication become prominence because of the ability to shuttle different molecules from producing cells to the target cells via extracellular vesicles called exosomes [4]. Previous research found that plant-derived nanovesicles had properties identical to mammalian exosomes [5-6]. In other words, there is evidence that plant-derived exosomes have comparable properties to mammalian exosomes. Furthermore, the primary secretion process of plant exosomes is believed to be multivesicular body-mediated extracellular release [7]. Studies demonstrated that exosomes can be isolated from various plants such as lemon, ginger and carrot etc [8-11].

Exosomes, nano scaled vesicles with 30-120 nm in diameters, are released from multiple cell types including mesenchymal stem cells, immune cells such as B & T cells, dendritic cells, epithelial cells, endothelial cells and cancer cells. [11]–[16]. They are lipid bilayer vesicles and also contains multiple proteins, lipids, and 3’-5’ exonucleases that work well-orchestrated [17]. Exosomes are found in both the nucleus and the cytoplasm. They have

ability to shuttle between cells due to their small nanometric sizes (30-120 nm) and also have function in the RNA processing [18], [19]. The ability of exosomes to access the target cell is an excellent companion for drug delivery. According to the studies conducted, exosome treatment seems to be very promising for the treatment of various diseases such as cancer and neurodegenerative disorders [20]–[23].

1.1. THE ANATOMY OF THE SKIN

The skin is the largest organ found in the body, which functions as the protective barrier against harmful environmental factors. The skin is composed of two major layers which are known as the epidermis on the upper layer consisting of epithelium and dermis on the lower layer formed from connective tissue, and additionally this layers are connected each other via dermal epidermal junctions [1]. Main function of the epidermis is to provide a protective surface of the body, and there are several sublayers of epidermis known as Stratum Basale (SB), Stratum Spinosum (SS), Stratum Granulosum (SG), Stratum Corneum (SC) and lastly the thinnest layer called Stratum Lucidum (SL). Epidermis consists of different types of cells such as Langerhans cells, melanocytes, inflammatory cells, merkel cells and keratinocyte cells with a different differentiation stages. Non-differentiated cells remain close to the dermal layer, while keratinocytes that differentiate migrate outward. Keratinocytes, which are nondividing and differentiated cells, are important for the function of the epidermis as they are replenished from stem cells located in the epidermis layers. Additionally, hair follicles, sebaceous glands and sweat glands are the appendages of the skin and aids in the thermoregulation of the body [2].

Between the epidermis and dermis layers, a basement membrane is found which is also known as an extracellular matrix (ECM) structure. Hyaluronic acid (HA) is one of the principal extracellular matrix (ECM) element in the epidermal layer. Below the basement membrane, dermis is located consisting of papillary and reticular layers. Fibroblasts are the main cell type within the dermis, found mostly at the papillary layer which provides ECM proteins such as collagen and elastin. Another cell types found in the dermal layer is endothelial cells which forms vascular network, mast cells, dendritic cells, neurons, lymphocytes and macrophages. At the bottom of the dermis, another layer called hypodermis

composed of adipocytes are located. Overall, dermis provides the mechanical strength of the skin [2].

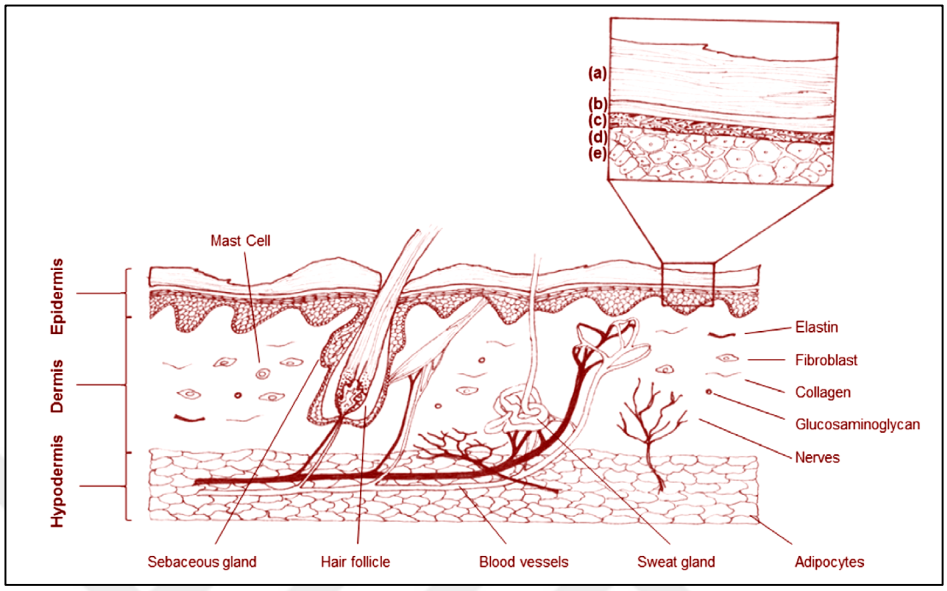


Figure 1.1: Mammalian skin structure [2].

1.2. WOUND HEALING

The injury of the body that results in disruption of the continuity of the body structure can be named as wound. In other words, a disturbance in the normal organization of the skin anatomy which leads to the loss of function can be defined as a wound. Wounds are divided as acute or chronic wounds due to the time that the wounds have been present. There are different variations of wounds that can be mainly classified as incisions, burns, abrasions, lacerations, avulsions and punctures. Wounds can be graded in various ways. The most important discrimination is that the wound is open or closed wound. In close wounds, the maintenance of the superficial layer integrity protects wounds against contamination. On the other hand, classification of open wounds can be classified due to deterioration in skin or mucous membrane in open wounds in other words differs according to degree of contamination [24].

The wound described as disrupting the continuity of the skin, cellular function, and anatomical structure resulting in partial or complete loss of function [25]. Cutaneous wound healing includes the organization of the cells both located within the skin and those which transferred via blood vessels upon molecular signals, and the ECM. Wound can occur due

to the trauma, infection, inflammation, harsh conditions such as extreme cold or hot, hypoxia and metabolic dysfunctionality [25]. To provide homostasis, one of the most essential primary functions of the skin is to protect the body against different environmental effects such as temperature control and insulation [26-27]. Skin disruption can also cause significant pathological complications, including infections, inflammation, loss of body fluid and electrolyte imbalance. In addition, large wounds that is caused by a disease or an injury can also resulted with serious dysfunctions or even death [28].

Wounds that occur after the skin is torn, cut or pierced (called open wounds), or those that are formed by such as a traumatic force called closed wounds, immediately respond to the injury which is called as wound healing process. The wound healing process starts the functional regeneration. Mainly, the wound healing process occurs within three phases known as hemostasis and inflammation, (ii) epithelialization, angiogenesis and the granulation of the tissue, and (iii) tissue remodeling. [1].

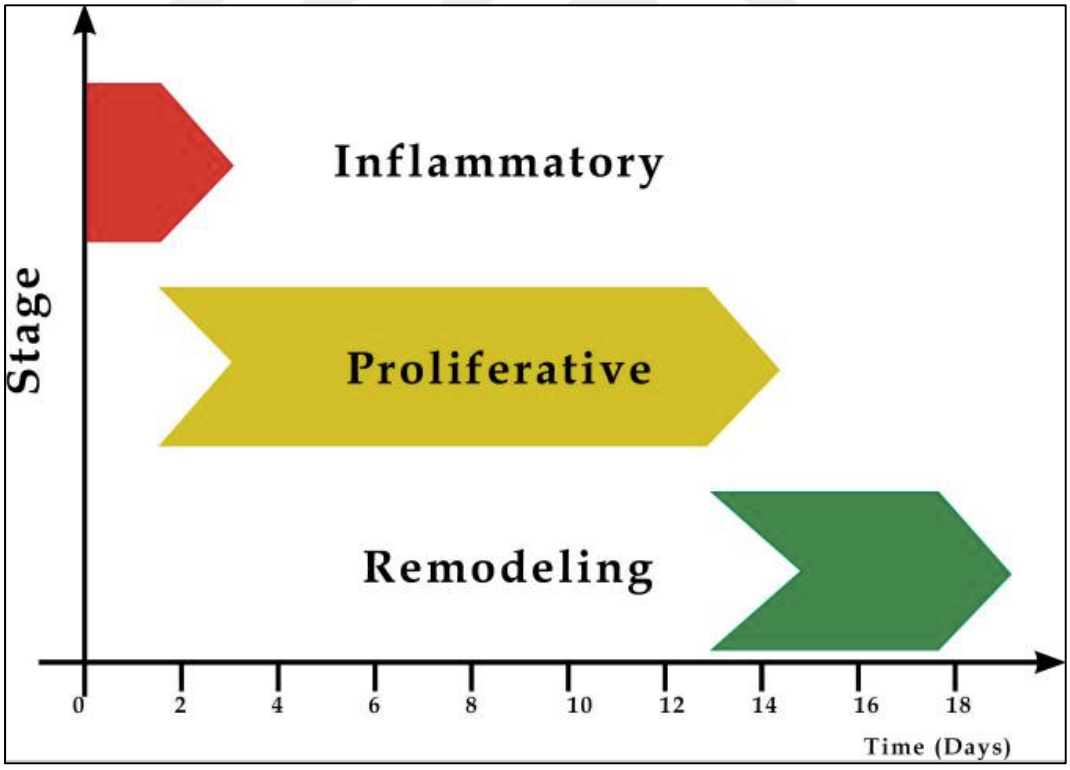


Figure 1.2. The stages involved in tissue repair [1].

1.2.1. Homeostasis and Inflammation

Main goal of establishing homeostasis after tissue damage is to prevent blood loss to protect the vascular system ever harming the remaining organs. Another goal is to create a matrix environment composed of fibrins for the invasion of cells which are required for the other stages of wound healing.

During wound formation in the dermis layer within the case of vascular network injury, blood and lymph from damaged vessels infuses to the wound bed. In healthy body, bleeding is halted instantly by functional and physical events, named homeostasis, which consist of coagulation and fibrin clot formation [26]. In healthy homeostasis progress prevents bleeding in order to affect other organs negatively. Bleeding from the wound promotes the synthesis of extracellular matrix proteins such as collagen, fibronectin and vitronectin, which has a role in providing a suitable environment for cellular invasion into the wound bed [27], [28]. In other words, straight after an injury, as the blood leaks to the damaged area, platelets and other components present in the blood interact with the ECM components at the area to provide homeostasis. This interaction allows the blood clotting agents to be released by the platelets which includes fibrin, fibronectin, thrombospondin and vitronectin [28]. The coagulation results with the formation of a fibrin network. This fibrin network acts as a barrier for protection against microorganisms and creates a matrix for the cell migration [28]. In addition, within 5 to 10 minutes of wound formation, vasoconstriction of damaged vessels begins which mediated by catecholamine, serotonin, prostaglandin, and histamine that helps to minimize bleeding from the wound [29]. At the injury site then, vasodilation starts mediated by vasoconstrictors known as thromboxane A2 and prostaglandin 2-α which have role in cellular trafficking. Vasoconstriction and vasodilatation cause coagulation, leading to an increase in capillary permeability, which is following by a leakage of matrix proteins [27]. Among these matrix proteins, soluble fibrinogen transforms into insoluble fibrin matrix fibers, creating a crosslink that prevents the blood out and the entry of pathogens into the wound bed. This fibrin crosslink structure does not only form a shield against microbial invasion, but also maintains the storage of growth factors which are essential for wound healing phases known as transforming growth factor β1 (TGF -β1), platelet derived growth factor (PDGF) and platelet factor 4 (PF4) [30].

After around three hours, swelling, pain and redness occur and the next stage of wound healing starts which is known as inflammation. Due to the activating cells are carried during the hemostasis, inflammatory phase is started. In this phase, the innate immune system, neutrophils and monocytes have role as a key player to the injured skin. The inflammatory phase is started by the mediators which are released by the resident cells in the wound bed and by leucocytes and platelets which are transmitted by the circulation after wound formation. These factors trigger a series of events that try to stabilize the wound, destroy invasive organisms by producing and regulating inflammatory mediators. Inflammatory mediators are soluble factors and known as cytokines and growth factors. Cytokines are very potent and classified predominantly into groups such as lymphokines, chemokines, interleukins (ILs), monokines, and interferons (IFNs) and growth factors in hematopoietics. Platelet derived growth factor (PDGF), fibroblast growth factor (FGF), epidermal growth factor (EGF) and vascular endothelial growth factor (VEGF) which are known as connective tissue growth factors have a significant role in this stage of tissue regeneration [31].

More specifically, towards the end of the coagulation formation, inflammatory response begins when leukocyte uptake into the wound bed is initiated. Immune cells are activated by pro inflammatory molecules that are involved in tissue damage, which increases levels of inflammatory signaling molecules, including cytokines, interferons and lipopolysaccharides and hypoxic conditions. Additionally, these molecules direct the neighboring immune cells to the site of injury. Resident platelet cells and macrophages attract neutrophils by secreting vital signal molecules which are interleukin (IL) -1, tumor necrosis factor (TNF) -α, TGF-β and platelet four (PF4). They enter the wound bed and undergo phagocytosis of microbial invaders and debris by reactive oxygen species (ROS) and proteolytic enzymes within the first 24 hours. After the neutrophils provide security in the body from microbial infection, growth factors and chemokines, mentioned above, they appeal circulating monocytes into the wound area [30]. After that, with the attachment of monocytes to the fibrin clot using their integrin receptors, they become major macrophages which are essential for the inflammatory phase. Macrophages have role in destroying bacteria and cleaning bacterial residue and cell debris [32].

Lymphocytes are the final contributor of the inflammation phase which are activated by Interleukin 1 and IgG by products [32]. The inflammatory response is suppressed to shift the second stage of the wound healing process at the end of the inflammation phase. When there

are no more bacteria or cell debris in the injured area, additionally, platelet plug and fibrin matrix are well disposed, the pro-inflammatory status of macrophages change into the anti-inflammatory status called as alternative phenotype and they start secreting interleukin 1 and interleukin 10 antagonist to decrease the inflammation [33]. In addition, in order to stimulate the cell proliferation and granulation tissue formation, macrophages start to secrete insulin like growth factor (IGF)-1, vascular endothelial growth factor (VEGF) and transforming growth factor beta (TGF-β) [34].

1.2.2. Proliferative Phase

A few days after injury, the inflammation subsides, proliferative phase which is called as a repair or rebuild of the wound begins. In this phase, the wound begins to recover the wound surface with healthy tissue, restore the vascular network and start forming the tissue granulation. There are four main subgroups of the proliferative phase known as, re-epithelialization, angiogenesis, granulation tissue formation and contraction [35].

1.2.2.1. Re-epithelialization

Briefly, the proliferation, migration and differentiation of keratinocytes at the injured area is required for re-epithelialization. In epidermal wounds, keratinocytes which located at the injured area are firstly activated, known as free edge effect, and stimulating the keratinocyte migration to the wound bed. Within up to 1 day of injury, keratinocytes start migration around the wound area. To close the wound with inducing cell proliferation, the keratinocytes at the basal layer of the wound edge and stem cells from neighbor hair follicles or sweat glands start proliferating around 2-3 days after injury. With the loss of contact inhibition and physical tension at desmosomes and hemidesmosomes, which are cell structures specialized for cell to cell adhesion, cell migration is stimulated by activated membrane associated kinases that is resulted with an increase of membrane permeability for calcium. In addition, in order to change the adhesion characteristics, keratinocytes release proteases which have role in the breakdown of matrix proteins. After the new adhesion structures are constituted, cell migration stops and the keratinocytes start to restore the basement membrane by secreting proteins. The migration of keratinocytes, mainly the part of the re-epithelization process, can be stimulated by various growth factors and cytokines such as epidermal growth factor (EGF), KGF, TGF-α, IL-1α, TNF-α, and IL-6 [36].

1.2.2.2. Angiogenesis

Nutrients and oxygen that is needed for migrated cells in wound healing is provided by vasculature. Therefore, reorganizing the network of blood vessels is significant in wound repair process. Angiogenesis, known as the formation of new blood vessels from the pre-existing vessels around the wound, occurs correspondingly with fibroblastic activation. Angiogenesis is initiated by pro-angiogenic factors secreted by keratinocytes, fibroblasts and macrophages. The growth factors which activate the angiogenesis are primarily vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF). Additionally, angiopoietin, angiogenin, angiotropin, TGF-β, TNF-α, IL-1, IL-8 and serine protease thrombin have also role in angiogenesis.

As a result of low oxygen stresses, the amount of VEGF, considered to be the stimulator of angiogenesis, rises at the time of injury. Furthermore, two specific VEGF receptors have role in the activation of endothelial cells known as VEGF receptor 1 (VEGFR1/Flt1) and VEGF receptor 2 (VEGFR2/KDR/Flk1) [37]. Active endothelial cells migrate to the wound bed, start proliferating and tube formation occurs which provides the blood flow [37]. In addition, once angiogenesis is started, activated endothelial cells disrupt the interactions associated with the nearby endothelial cells, digest the ECM components and the basement membrane, proteolytic proteases secreted from the endothelial cells such as matrix metalloproteinases (MMPs) degrade the basal lamina which allows entering new blood vessels to the wound bed, that is called sprouting. Released MMPs first digest the basement membrane, followed by the digestion of ECM to provide a clear path for endothelial cells to migrate. At the site of neovascularization, endothelial cells continue to proliferate and form immature tubules which later forms the functional blood vessels.

The motility of active endothelial cells for migration and proliferation is strongly dependent on an organized ECM [38]. Laminin, one of the ECM proteins has been shown to play the most important role in endothelial cell proliferation and tubule formation [39]. Additionally, it has been demonstrated that the αvβ3 integrin receptor exerts exclusively in the wound healing process and has a regulatory role in angiogenesis as well as accumulation of MMP-2 on the endothelial cell surface.

1.2.2.3. Granulation Tissue Formation

The transient wound matrix produced during hemostasis is replaced by granulation tissue consisting of fibroblast, granulocyte, macrophage, blood vessels in complex with bundles of

collagen partially restoring the role and structure of the wounded skin in the proliferation process. Since the epidermal layer of the injured tissue is almost covered with migrated keratinocytes and shaped as a zipper-like, the phenotypes and expression profiles of keratinocytes return to the standard condition [40]. The fibroblasts at the edge of the healthy dermal layer begin to progress inward when the keratinocytes form a safe barrier during the re-epithelialization process.

The main function of migrating fibroblasts is to synthesize and model extracellular matrix proteins, such as collagen, which is called as granulation tissue formation. In other words, fibroblasts migrate from nearby dermis to the wound, start proliferating and generate ECM proteins, in response to cytokines and growth factors mainly PDGF, TGF-β, bFGF connective tissue growth factor (CTGF) which are produced by platelets, resident fibroblasts and macrophages [41]. These growth factors have role in ECM deposition which is crucial in wound healing, by facilitating cell attachment and migration.

There are two main stimulator of the granulation tissue formation phase known as PDGF and TGF-β. PDGF has role in activating fibroblasts in autocrine/paracrine manner. Yet another, Pro-TGF, the inactive form of TGF-β found in the fibrin clot at early granulation phase, activated by proteinases which are secreted by resident cells. In order to produce ECM constituents, integrin receptors and tissue inhibitors of matrix metalloproteinase (TIMP), TGF-β stimulates fibroblast cells [42].

1.2.2.4. Contraction

After the formation of granulation tissue, wound fibroblasts produce actin microfilaments and undergo myofibroblast differentiation [43]. Differentiated myofibroblasts then start expressing myosin, desmin and α-smooth muscle actin [44]. Differentiated myofibroblasts make a complex, by altering integrin receptors profiling and link to specific ECM components and also link each other, which allows myofibroblast contraction to be delivered to all the wounds and edges of the tissue [45]. These cells accumulate on the borders of the wounds, cause contraction, and retract the borders of the wounded tissue towards the center.

There are two main growth factors that have role in providing wound contraction and myofibroblast differentiation such as PDGF and TGF-β.

1.2.3. Remodeling

During the proliferative phase, *de novo* synthesized components are randomly arranged and should be correctly aligned during the remodeling phase. This phase can last even weeks, years or longer depending on the type of the wound, and environmental conditions. Remodeling of the collagen fibrins takes place in this phase to ensure the tensile strength, mechanical strength as in intact skin prior to injury [46].

The remodeling phase is characterized by an increase in the scar strength due to the tissue remodeling. During the remodeling or maturation phase, Collagen type III, which is found as one of the components of granulation tissue, is replaced by stronger collagen type I and non-essential cells, most of neutrophils, macrophages, fibroblasts from the wound area undergo apoptosis or emigration [47]. This case causes the scar formation with the reduction of the cell number. In addition, to regulate vessel network, revascularization occurs in remodeling phase [48].

As mentioned above, remodeling phase can take several weeks even years after the injury, but the original tensile strength of the tissue would not be regained as in intact wound [29]. In healthy tissue, hair follicles, sweat and sebaceous glands, the moving ability and the elasticity of the tissue was found, but after the significant injury these cannot be recovered as in intact wound [48].

The exact molecular mechanisms of the remodeling phase are still largely unknown, but the effect of blocking TGF-β activity resulted with an extreme scar formation which suggest that TGF-β may have role in scar formation [48]. Despite that, in vitro and in vivo studies should have done to investigate the molecular mechanisms of the remodeling phase.

1.3. FACTORS AFFECTING WOUND HEALING

Since the process of wound healing comprises of many interconnected stages, wound healing process may also intersect with many systemic and local factors [24]. Although the local complications are directly related to the wound itself, the systematic conditions are connected to the individual's overall health status. Despite, local variables impacting the wound healing are governed by the systemic variables that lead to the pathology of chronic

wounds [24]. The next part will present the key factors Our current understanding about the key factors affcting the wound healing process will be discussed based on the most recent findings from experimental and therapy-based studies.

1.3.1. Local Factors Affecting Wound Healing

Local factors such as oxygenation, infection, dehydration, necrosis and also edema can adversely affect and disrupt the regular scheme of wound healing processes.

1.3.1.1. Oxygenation

In a natural healing process, oxygenation is a significant aspect as it is involved in almost all wound healing phases. It is necessary for the prevention of infection, activation of wound cells, cell proliferation and cell migration, contraction of wounds and remodeling [29]. The metabolism of the cells during wound healing process and maintenance cellular energy levels through the sustained production of ATP is strongly reliant on oxygenation.In addition, tissue oxygenation is required to prevent inflammation through increased expression of those enzymes that catalyze generation of superoxides, a commonly recognized prerequisite for the killing of pathogens. The successful removal of pathogens is therefore significantly dependent on adequate oxygen level during wound healing [29]. Oxygenation also encourages neovascularization which stimulates the cells needed for wound healing process, and ensures the requisite migration of the cells to the wound area [29].

Hypoxia is mostly expected in wounds at a time when nearby metabolically active cells need high levels of oxygen, considering that the vascular system is compromised during the injury. In fact, due to the distrupt in vascularization, many systemic conditions, such as age or a diabetic condition, can lead to improper inflow of oxygen during healing that is recognized as a contributing factor to the formation of chronic wounds. In fact, highly hypoxic environments induced due to both wound formation and ill defined blood flow promoting the grade of the wound to chronic. Hypoxic wounds generate a gradient of oxygen between the wound and the undamaged tissues that are adjacent. This gradient facilitates the diffusion of oxygen through the hypoxic tissue. The gradient of oxygen begins from the wound center where hypoxia is most intensely found and decreases progressively towards the edge of the wound [29].

Cytokine production and growth factors are also triggered through hypoxia. VEGF, TNF-alpha, PDGF and TGF-β are formed in response to hypoxia and promote chemotaxis, cell proliferation and migration. In earlier phases of wound healing, wound cells generate ATP via glycolysis for metabolic functions. But in later stages, much more ATP is required for protein synthesis, cell proliferation and migration. Cells should also shift their energy-producing state to oxidative phosphorylation. In fact, as predicted, oxygen is required during the inflammatory process for the production of ROS. The induction of ROS is affected not only by hyperoxia, but also by hypoxia [27].

All in all, prolonged hypoxia caused by systemic conditions which leads to delays in wound healing, while temporary hypoxia stimulates wound healing via stimulating angiogenesis, cytokine secretion, and cell proliferation and migration.

Systemic factors in wound healing such as capillary density in the skin, peripheral vascular disorders (main signs of diabetes), anemia, atherosclerosis, heart failure are mainly responsible for ischaemic wounds. In addition to systemic problems, continuous pressure can cause a decrease in blood flow in the area under pressure, as in bedridden patients. This ultimately induces insufficiency of oxygen and hypoxia around damaged tissue that leads to ischemic tissue and chronic wound consequently [27].

1.3.1.2. Infection

Upon disruption of the skin integrity, subcutaneous skin become vulnerable to the microorganisms. Although the wound healing inflammation process removes any residual invaders from the wound site, this phase sometimes fails and microorganisms may proliferate and trigger infection within the wound [34]. During invasive infections, the infection can spread into other uncontaminated tissues. As described in the preceding chapters, the inflammatory process usually takes care of microbial infection under wound - healing conditions by neutrophils, macrophages and their substances such as proteases and ROS. Contamination (non-replicative microorganism), colonization (biofilm development and small number of microorganisms), critical colonization/local contamination (microorganism infecting granulation tissue only) and invasive infection (additional surrounding tissue infecting microorganism) are defined as microorganism contamination in wounded tissue [34]. The microbial activity in the wound depends on the microorganism's host reaction, inflammatory cell behavior and number, virulence and pathogenicity. In particular, healthy intact skin carries approximately 10^5 CFU/gr tissue and a wound infection

accounts for more than this amount [35]. Just several microbial species, however, such as β-hemolytic *Streptococci* spp. leading to having a high virulence factor, harmful wound infections under 10^5 CFU/gr tissue are induced In particular, healthy intact skin has a microbial load of approximately 10^5 CFU/gr tissue and wound infections accounts for more than this amount. Therefore, a good way to diagnose infection is not always to assess microbial numbers at the wound site. *Pseudomonas* and *Staphylococcus* spp are prevalent in both chronic and acute wound tissues, apart from β-hemolytic *Streptococci* spp. *Pseudomonas* spp interacts with each other and creates biofilm layers consisting of proteins, nucleic acid, polysaccharides and cellular debris containing extrapolymeric substances. This biofilm prevents existing therapies and acts as a bacterial barrier. Increased protease and endotoxin release contributes to the degradation of proliferating cell and ECM proteins induced by extended microbial survival in the wound bed [35]. Furthermore, resident cells are stimulated by proliferating microorganisms in order to generate growth factors and cytokines such as TNF-a and IL-1 that stimulate the chronic inflammatory process. This high response often causes MMPs to be continuously expressed and TIMPs to unexpectedly decrease, resulting in continuous deterioration of the surrounding tissue [27].

Preventing the development of chronic wounds arising from infections and excessive inflammatory responses is important. Therefore, either by using antibiotics or other therapeutic strategies, such as the use of silver including additives, microbial contamination can be eliminated from the wound site. Significant side effects, including antibiotic resistance or silver toxicity, of these therapies are challenges which have to be treated in order to reverse microbial infection. In addition to the bacterial infection in burn wounds, other contaminations caused by fungal infections may also present. The fungus infection mortality rate is very high. The number of antifungal agents available, on the other hand, is not sufficient. Therefore, the need for novel antifungal-microbial agents is needed [47].

1.3.2. Systemic Factors Affecting Wound Healing

There are also several personal factors such as age and gender, eating patterns, and chronic conditions are directly linked to systemic factors that are neither related to the injury nor position.

1.3.2.1. Age

As age-related changes at the cellular level lead to delays in wound healing, older age is a significant risk for delayed healing. These delays, indeed, are only time-dependent, so they do not impact the efficiency of healing. The main suppressing factor is known to be the inflammatory response because it results in suppressed penetration of T cells into the wound region, decreased macrophage function, and increased development of chemokine with increased age.

According to the literature, Increased age also hinders the synthesis of collagen, angiogenesis, and re-epithelization. In this study, old mice suffered from weak skin tensile strength with a decreased cell proliferation rate and poor growth factor release, moreover delayed cell activation in compared to younger animals, where old and young mice with no other systemic differences were seen in compared in their wound capabilities [33]. While each stage of wound healing drastically changes with increased age, contradictory findings for the inflammation phase have been published. According to the study, with age, the release of alpha granules including TNF-a, TGF-β, and PDGF increases as the release of nitric oxide synthesis and the vasoactive facilitator amounts decrease [34]. The adherence of both platelets to the endothelium and their aggregation is therefore increased with age. While more inflammatory mediators are secreted by leukocytes with increased age, the proliferation of leukocytes with the same parameter decreases [33]. Besides, keratinocytes, endothelial cells and fibroblasts have a very reduced rate of proliferation in aged animals during the proliferative stage. With increasing age, collagen synthesis and reepithelization, angiogenesis and wound strengthrates are either decreased or delayed.

1.3.2.2. Chronic Diseases

Chronic diseases such as diabetes, obesity, hearth diseases, vascular problems and cancer may affect the wound healing. People with diabetes struggle with acute wound healing disorders and almost 15 percent of such patients are vulnerable to chronic diabetic foot ulcers (DFUs). Numerous extrinsic factors contribute to the development of DFUs, such as pain, inflammation, neuropathy, trauma, callus formation and vasculopathy. In addition, hyperglycemia and the subsequent development of ROS from hypoxic conditions often lead to significant damage to cells and the tissue [151]. In diabetics, disrupted wound healing is also associated with the development of advanced glycation end-products (AGEs) and increased expression in their receptors (RAGEs), especially under hyperglycemic

conditions. AGEs created under hyperglycemic conditions lead to inadequate ECM formation, thus preventing cell migration and collagen formation. Some more tissue damage occurs through the formation of excessive protein kinase C, which facilitates the development of DFU by stimulating further production of ROS through nicotinamide dinucleotide phosphate oxidase (NADPH) [151]. Diabetic ulcer fibroblasts have reduced proliferative and migratory properties, in comparison to the acute wounds. VEGF secretion, PDGF, IL-10 and IL8, IL-10, iNOS and IGF-1 levels are significantly suppressed in keratinocytes and endothelial cells in DFUs. The difficulty of wound healing among diabetic patients leads to all of these aspects described above.

Venous insufficiency is a significant systemic factor contributing to impaired wound healing. The leading factor contributing to smaller ulcers is persistent with venous deficiency. Defects in the direction of blood pressure and flow decrease the efficacy of the vessels, which eventually results in blood retention in the lower leg as the blood can no further flow away from the site. In deep veins, excessive blood mistreats the muscles in the region and the resulting chronic hypertension occurs, which is also a pathway to the formation of venous ulcer. At the end, ischemia, necrosis and ulcer could eventually develop [111].

In the past several years, obesity has been an escalating health problem. Obesity raises the risk of developing co-morbidities such as diabetes, heart disease, stroke, cancer and hypertension. Many complications including infections, ulcers and hematoma are suffered by obese patients. The skin begins to fold on itself with increased weight, and the friction between these folds offers an excellent environment for microorganisms and damages the skin. In obese patients, the incidence of infections is very high because of the cytokine release. Due to the ischemia and vascularization problems, there are significant numbers of pressure related ulcers and injuries in obeses.

1.4. CURRENT TECHNOLOGIES IN WOUND HEALING

The process of wound healing should not be underestimated, and wound management (particularly chronic wounds) should be properly cared for. Even though typical wound healing may not entail considerable care, extensive studies have been carried out in the evolution of the wound healing strategies because a significant health and life risk is caused

by non-healing wounds or delayed wound healing challenges. Recent molecular and cellular findings in wound healing cascades have provided our minds with a better comprehension that leads to new clinical practice technologies and strategies [34].

1.4.1. Wound Dressings and Scaffolds

Mostly as similar approach, many techniques have been used in the care of wounds, such as cotton, plastic bandages, gauzes. The main aim of these procedures is to avoid infection and keep the region of the wound moist. With the aid of technical developments, innovative dressing techniques offer ideal parameters for the circulation of oxygen, antimicrobial surface, and moisture for the wound area [111].

Scaffolds have recently recently become very popular because they are highly compatible with skin cells. There are many biocompatible types of systems for scaffolding. Dressings based on chitosan, hyaluronic acid, collagen, and silicon are commercially available, and other potential biomaterials such as gelatin, cellulose, and hydrocolloid dressings are being tested for possible application [112]. Developments in wound dressings have led to problems faced during skin engraftment as they are used as carrier and often ease wound ulceration, contraction, and scarring at the wound area. Hyaluronic acid (HA) is present in the ECM and is composed of disaccharide units, as described previously. While HA is used for various therapeutic strategies for drug delivery systems, hyaluronic acid biodegradation and biocompatibility help cellular proliferation. Research findings have shown that HA must be degraded to facilitate cellular proliferation, since the biodegradation by-products of HA stimulate the proliferation of epithelial cells [112]. When using HA as a wound dressing, its molecular weight is another factor to remember. Although low molecular HA has been shown to be pro-inflammatory, the wound healing mechanism is also prevented by high molecular weight HA. For the optimal wound care, medium molecular weight HA is best advised because it upregulates adhesion molecules during the healing process. While treatment with HA is highly effective for about 79 percent of patients with DFU, its use among high-risk patients is limited [112].

For wound dressing, collagen is used as well. In order to improve re-epithelization, the function of this dressing enables blood clot formation around the injury. Despite multiple benefits of collagen based dressing, including a reduction in healing time for up to 5 days,

the use of biobrane dressing which is a collagen based dressing used for burn wounds, is clinically limited because the inflammation does not decrease. Additional studies have shown that biobrane is also associated with elevated infection rates in addition to its high costs [74].

As an essential form of dressing, hydrogels are composed of water and hydrophilic polymers. They can also activate different parts of the process of wound healing and are effective for wounds. Hydrogels are introduced to provide elastic properties or solidity in the amorphous gel form or film. In order to stabilize dressing, a supplementary dressing like bandages should be used if they are applied in gel forms. On either side, hydrogel formulations on wounds are effective and have both moisture and water permeability. Hydrogels have been stated to be effective stimulators for all wound phases and effective dressing for all types of wounds except for infected wounds [111].

As the dressing products commonly used in the clinic only provide an appropriate condition and minimize waste from the wound area, innovative technologies for chemicals and substances are increasingly being developed.

1.4.2. Skin Autographs

Tissue engineering, in accordance with research bias, is a promising trend in wound care theapeutic strategies. In tissue engineering research, the first effort to recover healing is skin transplant. However, there are some important drawbacks that it is only possible to apply full thickness skin transfer in up to 2% of patients lost in the total skin surface. It is also not sufficient for patients with deep burns which is around 50 percent of total skin surface area or bigger. Other than that, some abnormal healing issues have been identified in partial or full-thickness skin grafting applications. Some other problem with skin alternatives occurs when the skin of the donor is unbalanced with the age of the patient and the rate of cell growth. Split-thickness skin graft, mostly known as a gold standard, involves harvesting a donor's full thickness tissue. According to the experiments reported on this topic, healing time lasted for a total of 6 weeks, apart from the 4 weeks needed for autograft split thickness preparation. Cultured autologous grafts have been developed in order to remove problems created by split skin autographs. Keratinocytes seem to be of significant help for this function because they both react and secrete their own growth factors and other signalling and

emerging cytokines [137]. Autografts composed of cultured autologous keratinocytes were used clinically in 1981 as a treatment tool for chronic wounds for the first time. In this process, the cells were isolated by biopsy and cultivated in vitro with other growth factors in the conditioned medium. The average wound healing time is approximately 3 to 4 weeks, and these engineered autologous grafts could be used for the healing of both acute and chronic wounds without any further issues, including rejection [137]. However, the effectiveness of the grafts depends on the attachment to the extracellular matrix of the exogenous keratinocytes, since non-attached grafts are still unable to proliferate and differentiate. While infection was generally seen among 40 percent of patients, this treatment supported almost 30 percent of patients with leg ulcers.

All in all, tissue engineering therefore seeks to build skin such as structures that in all types of wounds can increase functional healing ability in a sensible manner.

1.4.3. Growth Factors and Other Molecules

Growth factors are important molecules of wound healing because in each healing process various growth factors function various roles. In a closely regulated and organized manner, they stimulate cells and each other. Any deficiency in the growth factor due to inadequate formation or significant deterioration affects the cascade and leads in abnormal tissue repair or chronic wounds. Therefore, it is a very common research field among scientists to provide missing growth factors that plays a role in many processes [27].

Platelets and their growth factor secretion are regulated by macrophage and neutrophil mobilization to the site of injury. They are converted into macrophages once they link to ECM and begin to secrete molecules such as interleukins, FGF-2, TNF-al, IGF-1, PDGF, VEGF and TGF-β. Gels containing up to three PDGF dosages have been approved by the FDA for the treatment of DFU. The use of PDGF gel has triggered an interest in growth factor enriched dressing/scaffolds despite its controversial findings. In wound healing, there are several different growth factors involved [28]. For instance, the FGF family has twenty three various members and FGF1, FGF2, FGF7, FGF10, and FGF22 are connected with this in the wound healing process by triggering the pathways of Ras/MAPK, PI3K/Akt pathways. For chronic traumatic ulcers, the FGF embedded collagen sponge increased healing time by twenty four percent and the sponge also improved the wound closure duration within 21 days

by sixty eight percent. Moreover, another study shows that FGF-2 was embedded into chitosan film an found to be effective in wound healing stages, such as reepithelization and granulation tissue formation [28]. On the other hand, the TGF-β family has approximately 30 members, in wound healing cascade, TGF-β1, TGF-β2, and TGF-β3 have been shown to be involved and also showing contrary scar tissue consequences [28].

In chronic wounds, inflammatory mediators are also regulated, in addition to growth factors. Gene delivery seems to be more efficient and works better, in comparison to growth factor delivery. The delivery of genes is a more effective choice because, the delivery of growth factors affected due to the breakdown and metabolic repression. Gene transmission is carried out either by transient expression with episomal vector which are non-viral vectors or by incorporating the target gene into the more stable host chromosome. There are some threats to gene transmission when conducted with viral vectors, including stimulating and encouring carcinogenesis and mutagenesis, and also resulted with the immune responses. However, conversely non-viral vectors that are commonly used in clinical studies, viral vectors have better infectivity rates. Through the use of small interfering ribonucleic acid (siRNA) sequence specific gene delivery models, regulation of inflammatory cytokines can be achieved. For instance, to facilitate healing, P53 which is a tumor suppression gene and SMAD3 which is a pro-inflammatory gene can be suppressed around the wound site. Besides that, as a potential therapeutic, microRNA-based targeting is now being investigated. Small non-coding RNA molecules which can manupilates gene expression in a post transcriptional level are known as microRNAs [39]. Albeit, the gene delivery technology in wound healing has advantages and benefits such as the controlling location of gene delivery, this system still needs optimization and needs to increase the safety levels for the clinical use.

1.4.4. Stem Cells

Stem cells have the ability to self-renew and proliferate and can be differentiated into many groups of cells. Theoretically, stem cells are good candidates for wound healing since they can, by paracrine signaling, replace missing or damaged tissue together with supporting cellular processes [71]. There are many types of stem cells such as bone marrow stem cells, umbilical cord blood derived stem cells, foreskin derived stem cells, and peripheral blood derived stem cells are mentioned to be ideal candidates for the treatment of wound healing

as they have stimulate tissue regeneration. Stem cells are known to promote wound healing stages, such as angiogenesis and granulation tissue formation. Studies have suggested that the key activators of wound healing are signaling molecules presented in the conditioned medium of stem cell culture [71]. Other studies show the direct application of stem cells to the wound region, and scaffolds with stem cells have a much more efficient rate of regeneration in wound healing process. Various stem cell types have been reported as possible alternatives for wound healing and skin regeneration, such as mesenchymal stem cells (MSCs), embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) [72]. There is a high incidence of pluripotent behavior in embryonic stem cells at their both early and late passages. ESCs were proposed to facilitate tissue regeneration through the paracrine signalling and differentiation into dermal/epidermal lineage which may contribute the regeneration of the skin due to ESCs high pluripotency. There are some limitations for using ESCs in clinical use because of their potential to cause teratoma and induce carcinogenesis. Moreover, there is also legal and ethical controversies about using ESCs in clinical use. That's why induced pluripotent stem cells (IPSCs) are mentioned as an alternative promising type of stem cells that is used in wound healing and tissue regeneration. Studies indicated that iPSCs that have pluripotent stem qualities like ECSs by means of transfecting completely separated substantial cells with transcription factors known as c-Myc, Sox2. Klf4, Oct3 and Oct4. Despite the fact that iPSCs are acceptable choices for ECSs, their reprogramming limit is generally low. Besides, they cause cancer-causing nature and teratomas higher than ECSs paying not dependent to the transplantation place. Hence, adult tissue derived MSCs are the most secure and helpful application in tissue recovery applications without having any moral and legitimate contemplations. Due to the ease of their isolation and use, adipose derived stem cells (ADSCs) are very common within mesenchymal stem cells. Studies show that ADSCs induces wound healing and reduces scarring. Moreover, umbilical cord derived stem cells (UCSCs) are another alternative type of stem cells. Studies showed that UCSCs examined on dermal cells and have been shown to effectively differentiate into keratinocytes [73]. Several studies conducted with various stem cell types have shown that although certain stem cells such as ESCs are preferable for wound care, other types such as UCB stem cells are much more successful in the treatment of chronic wounds [73].

Motivating stem cell results contribute scientists to use them in dressing and scaffold formulations to facilitate adhesion, migration, and proliferation. In order to isolate, characterize, and deliver stem cells to the wound area, further studies are being conducted. Stem cells may solve the significant issue in wound management, regardless of the degree of their stemness. Although MSCs have a great deal to offer in this respect, their production, use and ethical issues must be taken into account, as is the case with ESCs and iPSCs and ESCs about their use in the clinic.

1.5. EXOSOMES

Cell to cell communication is compulsory in order to sustain tissue homeostasis and integrity of the organisms but most communication mechanisms between cells requires a coordination of the receptors and the signaling molecules [3]. Recently, nanovesicles mediated cell communication become prominence because of the ability to shuttle different molecules from producing cells to the target cells via extracellular vesicles called exosomes [4]. Initially 50 years ago, the presence of exosomes was described, but they were originally regarded as waste products that were released throughout the cell via shedding of the plasma membrane [4].

Exosomes are released from multiple cell types including stem cells, immune cells such as B & T cells, dendritic cells, endothelial and epithelial cells and cancer cells [11]–[16]. They are composed of multiple proteins, lipids, and 3'-5' exonucleases, that work together in a harmony through a functional unit [17]. In addition to proteins, various nucleic acids, such as functional microRNAs, mRNAs and other non-coding RNAs, have recently been found in the exosome. Aside from their ability to shuttle between cells due to their small Nano metric sizes (20-100 nm), they are also found in both nucleus and cytoplasm, and have function in the RNA processing of the cells [18], [19].

Free access to the any target cell makes exosomes a great companion for the drug delivery. According to the recent developments the exosome therapy is promising as a therapeutic strategy of diseases such as cancer and neurodegenerative disorders [20]–[23].

1.5.1. Biogenesis and the Structure of Exosomes

Exocytosis is an important and required for intracellular signaling and the growth of the plasma membrane. Exocytosis can be defined as the process of the vesicles fusing with plasma membrane and releasing their contents outside to the cell, intracellular trafficking.

Exosome secretion is controlled by the incorporation of plasma membrane-fused multivesicular bodies (MVBs) and continues with the release of their cargo. Multivesicular bodies (MVBs) produced through the inward budding into the endosomal lumen of the endosomal membrane have a role to play in mediating exosome secretion. MVBs carry endosome cargo molecules that later MVBs deliver for degradation to the lysosomes. MVBs also play a role in the secretion of their contents into the extracellular space through plasma membrane fusion, in addition to the delivery of cargo to lysosomes [49].

The mechanism for the development of MVBs and intraluminal vesicles (ILVs) is known to drive the transport-required endosomal sorting complex, also known as the ESCRT mechanism, which is categorized into four protein-associated complexes (ESCRT-0, -I, -II and -III) (VPS4, VTA1, ALIX, also known as PDCD6IP) [50]. The development of ILVs involves the complex of endosomal sequencing necessary for the ESCRT role [50As reported, the ESCRT Mechanism complex is a protein machine composed of four distinct ESCRT (0 to III) proteins that work together to form MVB formation, budding vesicles and classification or/and sorting of protein cargo [50]. The ESCRT process is primarily triggered through the identification and sequestration of ubiquitinized proteins through the binding subunits of the ESCRT-0 into unique areas of the endosomal membrane. Next, the ESCRT-I and -II interactions will be paired with ESCRT-III, a protein complex that plays a role in helping the overall budding process complex. Eventually, the ESCRT-III complex is isolated from the MVB membrane after splitting the buds to form ILVs, with the energy supplied by the Vps4 separation protein [50]. Various ESCRT components and ubiquitin proteins have been balanced in exosomes isolated from various cell types, regardless of whether exosome biogenesis is correlated with an ESCRT mechanism. Furthermore, the exosomal protein Alix, which is associated with a few proteins of the ESCRT system, such as TSG101 and CHMP4, participates in the exosome Cargo arrangement and layer sprouting through contact with sydnecan, was accounted for. [51]. These studies prompted a theory that infers ESCRT component work in exosomal biogenesis.

The exosomes found in MVBs are released into the extracellular space in mammalian cells when membrane fusion occurs and taken up by a recipient cell in three respective paths. The first way of the uptake is the fusion with plasma membrane of the recipient cell and the retrieval by release of its content into the cytosol; the second mechanism is the receptor-ligand binding which may occur between the exosome lipid membrane and recipient lipid membrane, and the last one is; endocytosis by phagocytosis. Exosomes have ability to alter molecular pathways for biological functions, when they enter the cell (Figure 2).

Briefly, multivesicular bodies, which are also known as late endosomes, produce exosomes. The generation occurs via the inward growing of the MVBs' membrane. Cytoplasmic proteins, tetraspannins, nucleic acids (such as DNA, mRNA and miRNA), some lipids such as cholesterol, ceramide, LBPA and flotiline are packed in a cell-dependent way into the exosomes during this generation process.

Exosomes are small vesicles with an endogenous origin which are homogeneous in size and shape. *In vitro*, exosomes are released from multiple cell types including stem cells, immune cells such as B & T cells, dendritic cells, endothelial and epithelial cells and cancer cells. *In vivo,* exosomes are found in body fluids known as, urine, plasma, saliva, blood, and serum. According to the literature, exosomes can be separated from other extracellular nanovesicles by their specific physicochemical property such as the density in a sucrose gradient (1.13 g / mL - 1.19 g / mL), their cup shaped morphology with a lipid bilayer, their size (which is between 20-100 nm viewed under transmission electron microscopy) and specific proteins that are related to exosome production and formation such as tetraspannins and heat shock protein family (HSP) [52].

The composition of exosomes may vary from cell to cell meaning that the composition may base on the cell that exosomes are originated from. Yet, exosomes contain various lipids, proteins and nucleic acids principally. Tetraspannin family members such as CD81, CD63 and CD9 are specially found in the plasma membrane of exosomes. Additionally, exosomes are enriched with heat shock protein family members such as HSP70 and HSP90. Besides especially exosomes derived from dendritic cells and B cells are also prospered with major histocompatibility complex (MHC) class I and class II proteins which have role in antigen presentation [53]. Integrin and immunoglobulin-like family members are located on the surfaces of the exosomes that are related with cell to cell communication (Figure 2) [53].

Additively, cytoskeleton associated proteins, known as actin and tubulin are also present in the exosomes. Specific members of Annexin family and Rab GTPase proteins have role in mediating the membrane fusion and exosome secretion via multivesicular bodies. All in all, to detect and characterize exosomes tetraspanin family members that are mentioned above are commonly used because these family are found all identified exosomes including plant exosomes and mammalian exosomes in the studies carried out until this time [54].

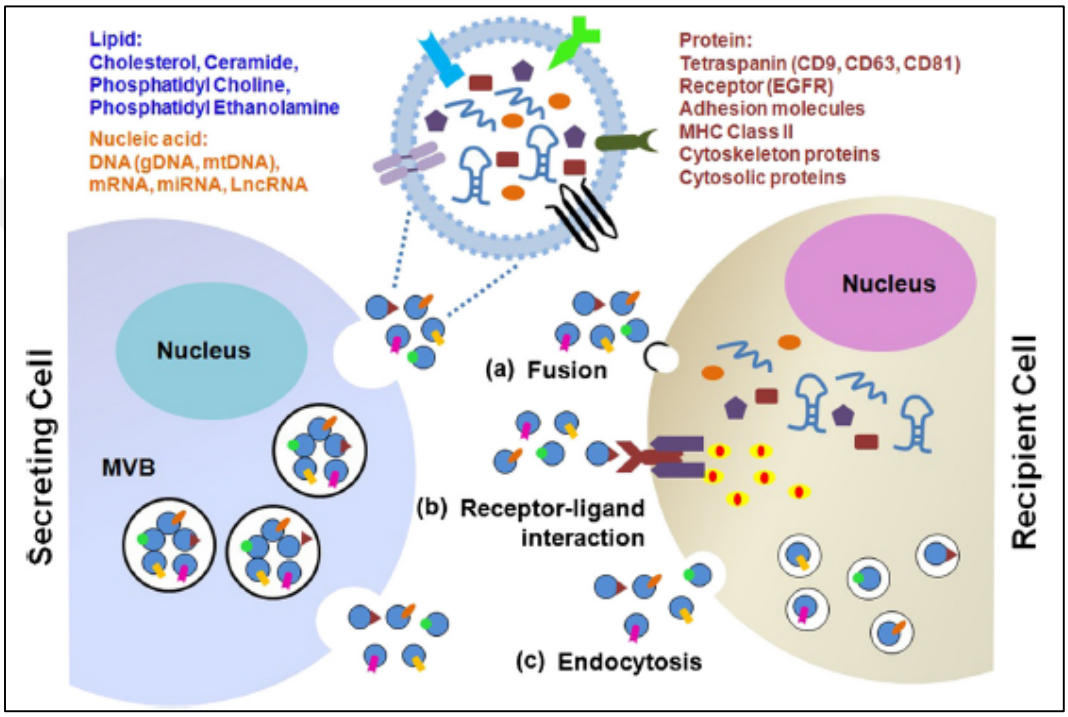


Figure 1.3. Biogenesis, release, structure, and uptake of exosomes [55].

Exosomes have a lipid composition meaning that they are rich in cholesterol and sphingolipids, which are in association with lipids rafts. Lysobisphosphatidic acid that is enriched in exosomes have an interaction with Alix protein which both induce the MVB formation [55]. According to the ExoCarta so far, (www.exocarta.org), discovered proteins and nucleic acids are collected in this database, 9769 proteins, 3408 mRNAs and 2838 miRNAs are identified in exosomes [56].

1.5.2. Cell to Cell Communication via Exosomes

Exosomes are known that they assume an important function in cell-to-cell interaction and communication. Moreover, exosomes are associated with typical physiological cycles as well as assume a significant function for the development and the progression of some diseases [56]. Studies demonstrated that cell to cell communication via exosomes can be mediated by exosomal protein, exosomal mRNA, exosomal miRNA and exosomal DNA. Initial research targeted on exosomal proteins and the function of these proteins which adjustments in protein expression would possibly play in physiological events.

The presence of molecules in the immune system which is involved in antigen presentation has given immune cell derived exosomes the role of potential immune response mediators. Exosomes have been shown to be published through both the apical and basolateral membranes in epithelial cell monolayers. The protein content of the exosomes apically released was indicative of clearance processes; proteins associated with antigen presentation were contained in exosomes released basolaterally. Exosomes have also been shown recently to regulate cell mobility by encouraging cell adhesion via their fibronectin-rich cargo [57]. However, the majority of research on exosomal proteins as intercellular communication mediators remains focused on pathophysiological states. Several studies in cancer have documented that tumor-derived exosomes can affect the extracellular matrix by release of matrix metalloproteinases (MMPs) or MMP activators, such as HSPs, with exosomes generally showing protumorigenicity [58]. It has also been shown that exosomal protein delivery is key to the spread of viral diseases such as hepatitis C [60] and human immunodeficiency virus type 1 (HIV), and neurodegenerative diseases such as Parkinson's and Alzheimer disease [61].

The first research to suggest a role for exosomes as intercellular messengers showed that targeted short interfering RNA could be transmitted to the mouse brain by systemically injected exosomes [62]. From this beginning stage, it is presently certain that exosomal mRNA can be conveyed into a cell where it very well may be transcribed into new, functional proteins, subsequently changing the functional capacity of the cell. Exosomes do not have the functional protein synthesis machinery itself and are only able to cause changes in functional protein once the RNA cargo is delivered into a recipient cell [51]. Indeed, functional delivery mediated by exosomes was demonstrated in a human mast cell line by

the use of murine cell line derived exosomes, thereby suggesting cross-species functional exosome delivery [63].

MiRNAs are among the most abundant regulatory molecules for genes and are known to control the expression of more than 60% of all genes coding for protein [63]. The contribution of the exosomal miRNA cargo to the intrinsic physiological role has been significant interest both *in vivo* and *in vitro* and studies [63]. Recent findings indicate that miRNAs in exosomes can be released via ceramide-dependent secreting machines controlled by a neutral sphingomyelinase 2 (nSMase2) enzyme encoded with a smpd3 gene that activates exosome secretion [64]. NSMase2 or ceramide were shown to assist exosome mediated miR-10b secretion in metastatic breast cancer cells, while ceramide antagonist inhibited that secretion. There are several studies based on exosomal miRNA mediating intercellular interaction, such as cancer , liver disease, cardiovascular disease, etc [65].

Lastly, very little research is available upon on function of exosomal DNA cargo in facilitating intercellular communication in physiological or pathophysiological conditions, indicating limited available information about the content of exosomal DNA. Undoubtedly, the discovery of both single-stranded mtDNA and double-stranded gDNA would result in greater scientific research on the potential roles exosomes in DNA regulation [66].

1.5.3. Plant Exosomes

Previous studies reported that, plant derived nanovesicles possess similar properties to mammalian exosomes [5]. In other words, there are evidences found that plant derived exosomes have similar characteristics with the mammalian exosomes. Additionally, the main secretion mechanism of plant exosomes is thought to be multivesicular body mediated release to the extracellular space [6].

According to the literature, once external or internal stimuli such as stress occur, plant derived nanovesicles are secreted via MVBs which have the same morphology with mammalian exosomes [6]. Besides their morphological mimicry, plant and mammalian exosomes have resemblance in their molecular composition. They all contain nucleic acids, mRNA, miRNA, solely the amount is different. To exemplify that, mammalian exosomes enriched with 100-300 miRNAs whereas plant exosomes have less [57]. As it mentioned

above, mammalian exosomes are enriched with cholesterol but mammalian exosomes contain relatively less phospholipids. On the other hand, researches showed that plant exosomes contain up to 98 percent phospholipids and some plant lipids such as galactolipids [57]. Besides that, lipid or protein content of plant exosomes is still need to be identified more.

Most notably, due to their effect of food absorption in homeostasis of the organs and the body, exosome like nanovesicles are isolated from edible plants. Communication between interspecies occurs when exosome-like nanoparticles generated from various forms of edible plants (grape, grapefruit, ginger and carrot) in inflammatory bowel diseases have anti-inflammatory properties [7], [8]. Although the underlying mechanisms of wound healing process of plant exosomes are still unknown whereas they are proven to have anti-proliferative and anticancer activities.

The first nanovesicles isolated from edible plants (from grape) was studied in 2013 [7]. According to the study, the size and the composition of grape exosome like nanovesicles (GELNs) were analyzed. Grape derived nanovesicles are shown to be 380nm in size, and contains phosphatidic acids (PA) and phosphatidylethanolamines (PE). In this study, GELNs were labeled with fluorescent dye which binds to the membrane and orally administrated to mice. Their results show that, intestinal epithelium cells are proliferated by WNT pathway, and components of the pathway are shown to be significantly upregulated after GELN administration. Additionally, once grape derived nanovesicles were administrated to mice with a having dextran sulfate sodium (DSS), which induces colitis, exosome treated group live longer by regulating the proliferation of intestinal stem cell while compared with control group [7].

In another study in respect of plant derived nanovesicles was about using Grapefruit-derived nanoparticles as a nano-carrier to use for the delivery of therapeutic agents [8]. Firstly, the size of the grapefruit derived nanoparticles were analyzed and found as an average of 250 nm in size. Cell viability analysis was performed both *in vivo* and *in vitro* which shows grapefruit nanovesicles treated cells resulted with no significant toxicity. Later on this study, chemotherapeutic drugs, nucleic acids, vectors and proteins are tried to load into grape derived nanovesicles and tested both *in vivo* and *in vitro*. Results implied that these nanovesicles can be used for the delivery of therapeutic agents efficiently to the site of disease and its biocompatibility gives an advantage in this process.

Further study conducted using grapefruit derived nanovesicles, results indicated that grapefruit derived nanovesicles have effect on increasing the resistance to DSS-induce colitis via upregulating E-cadherin. It is also revealed that grapefruit derived nanovesicles may regulate the expression of pro-inflammatory cytokines and chemokines withal minimizes the harm caused by DSS-induced colitis. To sum up, this study reveals that grapefruit derived nanovesicles can be used for preventing intestinal diseases through oral administration of grapefruit derived nanovesicles [59].

The effect of grapefruit derived nanovesicles as a drug carrier in brain tumor progression was also studied [67]. To inhibit brain tumor in mouse, grapefruit derived nanovector which carries miR17, regulated the natural killer cell activation, was administrated. Results showed that grapefruit derived nanovector which carries miR17 treated mice has a longer lifespan with brain tumor in compared with the control group. Additionally, this study reveals that modified plant exosome like nanovesicles can be used as a targeted therapy strategy in cancer [67].

One of the other plant derived nanoparticles used for the treatment of diseases is ginger. Ginger derived nanoparticles is used to investigate the effect of ginger derived nanoparticles (GDN) on alcohol induced liver damage [10]. Once, ginger nanoparticles are isolated and characterized, the localization of these nanovesicles is investigated *in vivo*. Results showed that GDNs were mostly localize in liver and lymph nodes in 12 hours. In alcohol-induced liver injury model, mice were treated with ethanol, the administration of GDNs in mice resulted with the decrease of the lipid droplets in the liver as well as decrease in triglyceride levels which represents that GDNs have ability to protect mice from alcohol-induced liver damage. In addition, in this study, endocytosis delivery of exosomes, uptake of exosomes by recipient cell is also studied by using endocytosis inhibitors which resulted with the uptake of GDNs is decreased, in other words, endocytosis mechanism is also regulated [10].

Another work that brings the most attention is *citrus lemon*-derived nanovesicles on different kinds of cancer cells proliferation and tumor growth [5]. The isolated nanovesicles from lemon was firstly identified with ranging size between 50-70 nm which in turn these vesicles can be exosomes and introduced into the different types of cancer. Cell viability results shows that lemon derived nano vesicles reduces the cancer cell viability whereas induces the healthy cell viability. In addition, gene expression analysis revealed that the lemon derived exosome treated cancer cells showed the increase of pro-apoptotic gene expression, BAD

and BAX while the decrease in anti-apoptotic gene expression of Bcl-xl. Additionally, in this study, *in vivo* effects of nanovesicles were studied and results indicated that tumor size was decreased after the treatment of lemon derived nanovesicles via TRAIL-mediated pathway.

Although presence of exosome-like nanoparticles in plants are supported by many evidences [6], [7], their role in mediating molecular interactions between species requires further detailed studies [8]. The influence of plant exosomes on cancer progression and inflammation was demonstrated previously [68]. The underlying mechanisms of wound healing process of plant derived exosomes has yet to be explored.

1.5.4. Exosome Isolation Methods

There are novel techniques as well as new kits came out to isolate exosomes and extracellular vesicles in addition to the traditional ultracentrifuge technique for the isolation of exosomes. Different techniques for the isolation of extracellular vesicles in their purity and quantity have been developed with the aid of development in science and technology. With respect to their density, form, scale, and surface proteins, each technique affects exosomes depending on the type of isolation [69].

As mentioned above ultracentrfugation is the oldest and most commonly used method in isolation of exosomes. The principle in this method involves segregation of extracellular particles based on their size, density, and shape in a sequential centrifugation scheme. The major advantage of ultracentrifugation is the ability to handle large sample quatities with high yields of exosomes. On the other hand, the potential disadvantage of this method is basically reliance on long periods of centrfugation at high speeds, which can incur damage on extracellular vesicles, thus impeding downstream analysis. Another isolation technique relies on size-based separation such as ultrafiltration that isolates exosomes with respect to their sizes. The major advantage of this technique is that it yields high exosome purity and relatively faster procedural times. On the other hand, it does introduce an important disadvantage of lowering the yield for intact exosomes due to its inherent shearing stress. The third alternative is called immunoaffinity capture where the isolation bases on interaction between exosomal receptor and its ligand anchored to the purification coloumn, and, hence, coining the name of the method as exosome fishing. Although this method is

very advantageous for the isolation of specific type of exosomes, its high cost and low yields are recognized as major disadvantages. Finally, a recently developed exosome precipitation method takes advantage of an aqueous-two-phase separation technique, where a water excluding-polymer interface captures exosomes through alteration of solubility or dispersibility. This method offers several advantages, including low cost, lack of need for expensive equipments, easiness in its application as well as ability to handle large sample capacity and high yields. The exosome precipitation method based on aqueous two phase, polymer based precipitation method is also used to isolate exosomes by altering the solubility or dispersibility or exosomes by the use of water-excluding polymers. This method is easy to use, low cost, no need for equipment and large sample capacity with a high yield [70].

1.5.5. Exosomes and Wound Healing

The effect of exosomes on cancer and inflammation has been demonstrated previously. In addition, due to the impact of food absorption on homeostasis of the organs and body, edible plants such as grape, lemon, ginger, and grapefruit are beginning to be used for exosome studies. In the intracellular interaction between cells, exosomes play a major role. Exosomes extracted from edible plant-use areas range from intestinal diseases to cancer, but their role in wound healing has yet to be explored.

Research has shown that topically applied MSCs contribute to both dermal regeneration and minimized scar formation promoting amelariaotion of chronic wound damages and rapid healing of cutaneous wounds [71]. As potential therapeutic agents exosomes offer a unique set of benefits for patients in terms lowering the risks for unwanted immune response or exerting antitumorigenic activity [72].

Recent studies showed that MSC exosomes can-in fact- trigger prominent signaling pathways of wound healing, including Akt, ERK, and STAT3 pathways and stimulate the expression levels of growth factors known as insulin-like growth factor-1 (IGF1), hepatocyte growth factor (HGF), stromal growth factor-1 (SDF1), nerve growth factor (NGF) and heat shock proteins such as Hsp70-90-72 and Wnt Signaling pathway [73] In addition to MSC-derived exosomes, plant-derived exosomes has produced promising therapeutic outcomes in treatment of various wounds [68]

The application of exosomes as a scaffold or wound dressing with various biomaterials has recently been investigated. To demonstrate, in a diabetic rat model, Chitosan wound dressings containing exosomes isolated from MicroRNA-126-overexpressing synovium M allow sustained exosome release and heal full-thickness skin deficits [74].

All in all, exosomes could be an important therapy strategy for wound healing due to their healing properties. However, more research is needed to explain the control of exosomes during the wound healing process and to identify new sources of exosomes or their contents for harvesting and to translate them for clinical use.

1.6. WHEAT

Wheat grass, '*Triticum aestivum* Lin.', belongs to the Gramineae family in Plantae kingdom which plays an important role in human diet, in the classification of vegetable foods around the world. Triticum species provide various types of wheat each year in Asian and Mediterranean regions [75]. *Triticum aestivum* seeds are germinated 6-10 days in order to obtain wheat grass. Studies conducted about wheat grass and its nutritional value demonstrated that wheat grass is enriched with high amounts of minerals such as iron, magnesium, phosphorus, essential amino acids, amino acids and also have high chlorophyll content [75]. Moreover, juice derived from wheat grass also contains rich amount of vitamins such as A, B, C, E, and K and minerals that is essential for human metabolism [76].

The utilization of grass juice as folk medicine for therapeutic purposes has been popular since 1930s. The epidemiological studies up to now have proven that wheat grass extract which is also known as natural medicine has a protective role in chronic thalassemia, high blood pressure, ulcer, diabetes and cancer [76].

Scientific research has shown that by implementing a diet rich in fruits and vegetables, cancer progression may be reduced around 7-31% [77]. Many plant-derived bioactive molecules inhibit oxidative stress [78] inhibit enzymes involved in carcinogenesis [79], prevent diabetes, Alzheimer and atherosclerosis [80] Moreover, plant-derived molecules have been shown to induce apoptosis in cancer cells, inhibit their proliferation while mitigate side effects of chemotherapeutics [81].

Wheat grass shows antioxidant characteristics due to its high content of biologically active compounds, bioflavonoids and minerals [76]. Many forms of wheat (wheatgrass or seed) have been developed for the treatment of diseases. Research findings has also shown that topical application of compounds containing antioxidants can be helpful for wound healing and tissue defense against oxidative disruption [82].

One of such study conducted with wheat has unraveled the detoxifying activity of the wheat grass.conducted about wheat is detoxifying activity of the wheat grass. In the study, detoxifying activity was studied and choline content in wheat grass was found to reduce fat deposition in the liver of animals which are fed a cholesterol rich diet [83].

The study that is in the matter of anti-inflammation activity of the wheat grass indicated that wheat grass have effect on wound healing via stimulating the re-epithelization process. Due to the wheat grass enriched in chlorophyll content, it can be used for the treatment of ulcers by enhance healing and induce anti-ulcer activity [84].

One of the most promising and barnburner studies about wheat is the anticancer activity of wheat grass extract based on its contents such as chlorophyll and sodium oxide dismutase. In the study, the cytotoxicity of the wheat extract (methanol extract of wheat grass) on Human laryngeal cancer (Hep2) was examined. Results revealed that after the treatment of wheat grass extract on Human laryngeal cancer (Hep2) cells, p53 gene expression increased and the expression of cyclin D1 decreased which resulted with the cell cycle arrest in G1 state. In other words, wheat grass extract has effect on halting the cancer cell progression [85].

To sum up, wheat (*Triticum aestivum* L.) is an important component of human nutrition. Cereal products have been mainly found to be protective against many chronic diseases and cancers, as well as antioxidant capacity due to free radical scavenging properties. During germination, mainly in wheat shoots, numerous primary and secondary metabolites and minerals are synthesized to protect newly formed cells from biotic and abiotic stresses from external sources [86]. It also contains high amount of chlorophyll, flavonoid, many vitamins, antioxidant enzymes and stress-relieving hormones [84]. The remarkable point in the wheat grass is to observe the decrease in the side effects caused by chemotherapy in patients suffered from cancer when the treatment is given simultaneously with both wheat grass and

chemotherapy drugs [85]. Some plants are used for the treatment of acute wounds, while others are used for the treatment of chronic wounds [86].

All in all, as a natural healer in traditional medicine, *T. aestivum* plant extracts are frequently used without any scientific evidence as to which particles play a major role in these processes. In other words, up to now, no attempts were made to examine the effects of wheat exosomes in technologies for regenerative medicine or drug discovery.

2. AIM OF THE STUDY

Wheat (*Triticum aestivum L.*)) is a vital component of Turkey's human diet. The protective effects of wheat extracts against cancer and neurodegenerative diseases have also been studied. Although number of studies on plant based compounds are escalating, exosomes derived from wheat as a therapeutic agent and their effects on wound healing has not been examined so far. In other words, up to now, no attempts were made to examine the effects of wheat exosomes in technologies for regenerative medicine or drug discovery. The aim of theis thesis was to demonstrate the wound healing property of Wheat (*Triticum aestivum L.*) derived exosomes using both *in vitro* and *in vivo* approaches.

3. MATERIALS AND METHODS

3.1. MATERIALS

3.1.1. Chemicals

The Chemicals which were used during this thesis was listed below:

- Dulbecco's Modified Eagle's Medium, high glucose, Gibco 41966, Thermo Fisher Scientific, USA
- Dulbecco's Modified Eagle's Medium, low glucose, Gibco 31885, Thermo Fisher Scientific, USA
- Fetal Bovine Serum (FBS) cell culture tested, Gibco 10500-056, Thermo Fisher Scientific, USA
- Penicillin-Streptomycin, Gibco 15140-122, Thermo Fisher Scientific, USA,
- Dimethyl Sulphoxide (DMSO), Santa Cruz Biotechnology sc-202581, USA
- Phosphate Buffered Saline (PBS), Lonza BE17-517Q, Switzerland
- Trypsin-EDTA Gibco 25200056, Thermo Fisher Scientific, USA
- Polyethylene glycol, Sigma81310, Sigma-Aldrich, USA
- Dextran, Sigma 9004-54-0, Sigma-Aldrich, USA
- TRIzol™ Reagent, 15596-018, ThermoFisher Scientific, USA
- RIPA Lysis and Extraction Buffer, 89900, ThermoFisher Scientific, USA
- Bovine Serum Albumin (BSA) $0332-100G, VWR LifeScience
- Mounting media F4680-25, Sigma, USA
- Protease Inhibitor (PI) Sigma, P8340, USA
- Matrigel, #354234, BD Biosciences, Bedford, MA

3.1.2. Instruments

The instruments which were used during this thesis was listed below:

- Laminar Flow Cabin, Heal Force Class II Biosafety Cabinet A2, China
- CO_2 incubator, InVitro Cell ES NU-5800, NuAire, USA
- Inverted Microscope, Nikon Eclipse, TS100,Netherlands
- Low-Speed Centrifuge, Gyrozen 416, Korea
- -80 Degrees freezer, New Brunswick, U410, Germany
- Microplate reader, Bio-tek ELx800, USA
- High-Speed Centrifuge, Sigma Aldrich 3-18KS, Germany
- Shaking Water Bath, Stuart SBS40, UK
- Nano-Drop 2000, Thermo Fisher Scientific, USA
- Thermal Cycler, Biorad Mycycler 1709703, USA
- CFX96 Real-Time PCR, Bio-Rad, C1000 Touch, USA
- Liquid Nitrogen Tank, Air Liquide Arpege 140, UK
- pH Meter, Milwaukee Mi151, USA
- Vortex, Dragon Lab, MX-F, China
- Heating Block, WiseTherm, HB-R, Switzerland
- Incubator Shaker, New Brunswick Innova 4330, USA
- Magnetic Stirrer, Heidolph MR3004, Germany
- Fluorescence Microscope, Axio Vert. Zeiss, Germany
- Confocal Laser Scanning Microscope, Zeiss LSM 700, Germany
- FACS Calibur, BD Biosciences, USA
- ChemiDOC XRS+Gel Imaging System, Bio-Rad Universal Hood 2, USA

3.1.3. Equipments

The lab equipments which were used during this thesis was listed below:

- Cell culture flasks, T-25, T-75, T-150, #90025, #90075, #90150, TPP, Switzerland
- Serological Pipettes 5,10,25,50 mL, SP-5-C,SP-10-C SP-25-C,SP-50-C, CAPP, Germany
- Micropipette, 2231300002, Eppendorf, Germany
- Micropipette tips, 301-03-051, Axygen, USA
- Microcentrifuge tubes 1.5, 2 mL, 078.03.002, 078.03.003, Isolab, Germany
- Falcon tubes 15 and 50 mL, LB.IS.078.02.003, Isolab, Germany
- Cryovials, 091.11.102, Isolab, Germany
- Graduated Cylinder, Isolab, Germany
- Filter 0.22 µm TPP, Switzerland
- Filter 0.45 µm Santorium Stedim_Biotech, Germany
- Bright-Line™ Hemacytometer, Sigma Aldrich, Z359629, USA
- Cover Slip, Sigma Aldrich, Z375357, USA

3.1.4. Kits and Assays

- Human Pro-Collagen I alpha 1 ELISA Kit ab210966, Abcam, USA
- RayBio. Human TGF-beta 1 ELISA Kit, #ELHTGFb1, RayBiotech, USA
- The Human Matrix MetalloProteinase 3 (Hu MMP3) ELISA kit, BMS2014-3, ThermoFisher Scientific, USA
- Human MMP-1 ELISA Kit (MMP1, ThermoFisher Scientific, USA
- Human VEGF ELISA Kit KHG0111, Invitrogen, USA
- The Muse Cell Cycle Kit, MCH100106, Merck Millipore, USA
- Annexin V Apoptosis detection kit, 640914,Biolegend, USA
- QuantiTect SYBR Green PCR Kit, 204145, Qiagen, USA
- iScript™ cDNA synthesis kit, #1708890, BioRad, USA
- Human Growth Factor Antibody Array C2000, #AAH-GF-1, RayBiotech, Inc., GA

3.1.5. Antibodies

- Anti-Collagen I antibody, ab34710, Abcam, USA
- Anti-Fibronectin antibody, ab2413, Abcam, USA
- Goat anti-rabbit IgGH&L, Alexa Fluor. 647) (ab150079, Abcam, USA
- DAPI, 4 6, 6-diamidino-2-fenilindol, DS1306, ThermoFischer Scientific, USA
- Anti-CD9 antibody, Anti-CD63 antibody, Anti-Hsp70 antibody, ab2215, ab8219, ab61907, Abcam, USA

3.1.6. Cell Lineages

- HDF, Primary human fibroblast, PCS 201-012
- HUVEC, Primary Umbilical Vein Endothelial Cells, CRL-1730
- HACAT, Human Keratinocyte, CLS 300493
- L929, Mouse Fibroblast, ATCC-CCL 1

3.2. METHODS

3.2.1. Exosome Isolation from *Triticum aestivum Lin.*

Exosomes are isolated from juice from *T. aestivum L.* **(**wheat grass). *T. aestivum* seeds from Ceyhan 99, Adana, was acquired. Wheat seeds were planted in moisturized perlite and grown at room temperature for 36 to 48 hours at dark conditions and after dark photoperiod, they were grown at light. Once wheat grass leaves length reaches around 10 cm (almost 5-7 days after the grass is formed) , lawn were collected, washed and squeezed in distilled water with a laboratory blender. After washing step, wheat grass juice was then centrifuged at 300 g for 10 minutes. In accordance with the aqueous two-phase system (ATPS) procedure mentioned in the literature [87], exosome isolation was conducted. In short, Dextran (DEX), Poly(ethylene glycol) (PEG) were prepared to obtain ATPS isolation solution via mixing all in distilled water with a 1:1 ratio. In other words, 20 mL of ATPS solution was mixed with 20 mL of plant lysate and centrifuged at 1000 x g for 10 minutes in order to separate aqueous two phase solution. 80 percent of the solution (volume) is discarded after the centrifugation

step and the same quantity of ATPS isolation solution has been applied and then centrifuged at the same speed again as a washing step. The washing step is conducted twice in order the increase the purity of exosomes. Lastly, after the washing steps, upper phase was discarded and lower phase which contains exosomes are filtered through 0.45 μm and 0.22 μm filter.

3.2.2. Exosome Characterization and Quantification

Isolated exosomes were then characterized using Nanoparticle tracking analysis (NanoSight, Malvern, UK), flow cytometry (FACS Calibur, BD,US), scanning electron microscope (ZEISS, Germany) and western blot (ProteinSimple, San Jose, USA). Exosome quantification was performed with Lowry Protein Assay.

Exosome quantification and size distribution nm were calculated using the NTA method with a laser of 488 nm. Samples were diluted with distilled water according to the suggested concentration range of 1:40, according to the manufacturers' protocol. The video captured nanoparticles at 60 second intervals for at least 5 captures and the test was carried via NTA software program 3.3.301.

For the characterization, 100 μl of isolated exosomes were incubated with 5 μl of CD9, CD63 and CD81 antibody for 30 minutes respectively. After the incubation 300 μl of PBS was added to the solution. CD surface markers of exosomes were detected using Becton Dickinson FACSCalibur flow cytometer.

WesTM Simple Western apparatus (ProteinSimple, San Jose, USA) was used to confirm the existence of CD81, CD63 and CD9 for the quantification of the exosome characterization from wheat juice. 0.5 gr exosomal protein was denatured in a DTT-based buffer according to the manufacturers' protocol and samples were then loaded onto WesTM multiwell immunoblotting plates. The seperation of exosomal proteins and the identificaiton of antibodies were detected with the capillary cartridge. Secondary antibody (anti-rabbit) was used and conjugated to horseradish peroxidase with respect to the manufacturers' protocol.

Isolated exosomes were analyzed with Scanning Electron Microscopy (SEM). On a glass microscope slide, samples were swapped and dried overnight at -20 °C. After that, the slide was covered with coverslip and examined via SEM.

Exosome quantification were examined by Lowry protein assay. 4 µl of exosomes is mixed with 16 µl of distilled water and 5 µl of the mixture was spread in three separate wells of 96-wellplate. 25 µl of reagent A solution and 220 µl of reagent B solution was added into each well and incubated for 30 minutes in dark at room temperature. After the incubation, absorbance of wells were measured with Multiscan Spectrophotometer at 490 nm.

3.2.3. Cell Culture

Human dermal fibroblast (HDF), human vascular endothelial cell (HUVEC), human keratinocytes (HACAT) cells were cultured in DMEM High media, both supplemented with 100 units/mL Penicillin and 100 µg/mL Streptomycin (1% PS) and 10% (v/v) FBS (Fetal Bovine Serum). In addition, primary mouse dermal fibroblast (L929) was cultured in DMEM low media with the same conditions.

All cell lines mentioned above were cultured at 37 ^{o}C, 5% CO2 (v/v and 95% air (v/v).

3.2.4. Cell Passaging

When cells achieved 70-80 percent confluency, cell passing was required to execute. The monolayer of the cell was rinsed with 1X PBS pH 7.4 (Phosphate-Buffered Saline). After the washing step, cells were detached from the cell culture flask with 0.05% (w/v) trypsin at 37 ^{o}C for 2-3 minutes. After trypsinization, 10% (v/v) fetal bovine serum (FBS) containing media was added onto the cell suspension in order to neutralize trypsin activity and cells were collected. Then, cell suspension was centrifuged at 300 x g for 5 minutes, supernatant was discarded and the cell pellet was suspended in fresh growth media.

3.2.5. Cell Counting

After the cell passaging protocol described above, 10 µl of cell suspension was taken and placed into hemocytometer. Under the inverted light microscope, the middle square of the hemocytometer was counted using an 4x objective.

Total cell number/mililiter was counted with the formula below:

Cell number/ml= ‘counted cell number x dilution factor, factor/ mm2 x chamber depth’.

3.2.6. Cell Cryopreservation

Freezing mix solution was prepared with 10% (v/v) Dimethyl sulfoxide (DMSO) and 90% (v/v) FBS. After the centrifugation step mentioned as section 3.2.4., cell pellet was resuspended in 1mL of freezing mix and put into a new labeled cryovial. Cryovials were placed at -80 ^{o}C firstly, and then put into liquid nitrogen for long term storage.

3.2.7. Cell Thawing

Cells were obtained from liquid nitrogen and warmed to 37 ^{o}C immediately. 5 mL of warmed growth media was poured into the freezing drop by drop in order to protect cells from the increase of the osmotic pressure. Cell suspension was centrifuged at 300 x g for 5 minutes. Supernatant was discarded and the cell pellet was resuspended in complete growth medium. The day after, cell medium was changed with fresh medium to remove all DMSO.

3.2.8. Cell Viability

Cellular viability of HDF, HACAT, HUVEC and L929 were analyzed using WST1 assay. HDF, HACAT, HUVEC and L929 cells were seeded into 96 well plates with a density of 3000 cells/well and incubated 24 hours in humidified conditions, at 37 °C, 5% carbon dioxide. The following day, wheat exosomes were added at different concentrations, 25 µg/mL, 50 µg/mL, 75 µg/mL, 100 µg/mL and 200 µg/mL onto cells. Cellular viaility was then analyzed with WST1 assay, according to the manufacturer’s instructions. By assigning the absorbance value obtained from untreated cells to 100%, the percentage of cell viability was determined.

3.2.9. Scratch Assay

In order to determine the effect of wheat exosomes on wound closure scratch assay was performed. $3x10^5$ cells were seeded onto 6 well plates and incubated overnight at humidifed

conditions. The following day, cells were scratched with using 200 µl tip, By using 1000-µL sterile tip, and cell culture media were changed with fresh media containing containing 2% exosome-depleted FBS and 200 µg/mL wheat exosome. Cells were incubated for 24, 48 and 72h. Wound closure was determined every 24h and measured with an inverted microscope. Wound closure is measured using Image J software.

3.2.10. Matrigel Tube Formation Assay

To analyze the angiogenic effect of wheat exosome on HUVEC cells, tube formation assay is conducted. Matrigel Membrane Matrix is left to thaw at 4°C overnight. 24-well plate is coated with 200 µl/well of Matrix and incubated at 37 °C for 1 hour to allow polymerization. HUVECs are seeded with the density of $2x10^5$ cells/well, then incubated with 200 µg/mL wheat exosome and incubated at humidified incubator. After 16 hours, images of tube formation are obtained by inverted light microscope. Analysis of tube formation is calculated by Wimasis program.

3.2.11. Anti-Inflammation Assay

H_2O_2 is used to investigate the response of wheat exosomes under the inflammation. For this purpose, HDF, HACAT, HUVEC and L929 cells were seeded onto 96 well plates with the concentration of $5x10^3$ cell/well, and treated with 1.0 mM H_2O_2, 100 µg/mL WE and 1.0 mM H_2O_2 and 100 µg/mL WE together for 24 hours in 37 °C. After 24 hours, cell viability assay with WST-1 reagent is conducted as previously discussed at section 3.2.8.

3.2.12. RNA Isolation

HDF, HACAT, HUVEC cells were seeded into 6-well plate with the density of $3x10^5$ cell/well. Following day, 200 µg/mL wheat exosome was added onto the cells. After 2 days of incubation of cells with exosome, conditioned medium was taken, each well was washed once with 1X PBS pH7.4 (37 °C). According to the manufacturers' protocol, the Trizol solution was applied to each well. In a new 1.5mL tube, cell suspension in trizol was transferred and chloroform was applied to each cell suspension. Each sample was inverted

multiple times and then cultivated for 2-3 minutes at 37 °C. Samples were then centrifuged at 12000 x g for 15 minutes at 4 °C after incubation. Three phases were observed after the centrifugation stage. The upper step is defined as the stage in which the RNA is present. The second phase is identical to the yellowish color known as the protein phase, and DNA was present in the third phase. The RNA phase was then taken and moved into a new tube. In order to clean the samples, pre-cooled isopropanol was added as well as the amount taken from the clear phase and the eppendorf tubes were inverted several times. Samples were incubated for 10 minutes at room temperature and then centrifuged for 10 minutes at 4 °C at 12000 x g. Supernatant was then discarded and 75 percent ethanol was applied to each sample in pre-cooled form. At 7500 rpm for 10 minutes at 4 °C, the samples were centrifuged. After centrifugation, ethanol was discarded and pellet was dried as a air dry. After all, RNase/DNase free water was added into each sample and the RNA concentration was calculated with nano drop.

3.2.13. Reverse Transcriptase Polymerase Chain Reaction

Qiagen Senscript Reverse Transcriptase is used for the translation of RNA into cDNA. According to the following table, the master mix was prepared. The PCR reaction was conducted for 60 minutes at 37 °C. After reaction the concentration of cDNA samples were measured using Nano Drop.

Table 3.1. RT-PCR reaction master mix

Reagents	**Volume per reaction**
10x Buffer	2 µl
dNTP mix (5mM)	2 µl
10 µM Oligo dT primer (from 10X to 1X)	2 µl
Senscript Reverse transcriptase enzyme	1 µl
Rnase-Dnase free water	variable
Template	variable
Total reaction volume	20 µl

3.2.14. Quantitative Polymerase Chain Reaction

The mRNA expression levels of gene list that is given below at table 3.2. were measured via using Syber-green Quanti-Tech (Qiagen) kit. Including Syber green PCR mix, universal primer, Rnase-Dnase free water and 500 ng for each sample, the reaction mixture was prepared, and reactions were conducted according to the conditions shown in table 3.3.

During the analysis, the 18S rRNA reference gene was used to produce relative quantification and absolute quantification was calculated using the standard curve.

Table 3.2. Quantitative PCR conditions

Cycle	Temperature	Time	Phase
1	94°C	15 min	
2	95°C	5 min	Initial denaturation
3 (39 repeat)	95°C	60 sec	Denaturation
	55°C	60 sec	Annealing
	72°C	60 sec	Extension
4	72°C	10 min	Final extension
5 (80 repeat)	50-80°C 0.5 °C increase /12 sec		Melt curve
6	4°C	∞	Cooling

Table 3.3. Gene and primer list used in RT PCR assay

Gene	Species	Sequence
Akt	Human	F 5’ GGGACCTGAAGCTGGAGAA 3’ R 5’ CCTGGTTGTAGAAGGGCAGG 3’
Col1A1	Human	F 5’ -CCACGCATGAGCGGACCCTAA-3’ R 5’-ATTGGTGGGATGTCTTCGTCTTGG-3’
Col3A1	Human	F 5’ 5’-TGGTCTGCAAGGAATGCCTGGA-3’’ R 5’ TCTTTCCCTGGGACACCATCAG’
Vimentin	Human	F 5'-TGCAGGAGGCAGAAGAATGGTACA-3' R 5'-TTCCATTTCACGCATCTGGCGTTC-3'
Fibronectin	Human	F 5’ AGCCTGGGAGCTCTATTCCA 3’ R 5’ CTTGGTCGTACACCCAGCTT 3’
Laminin	Human	F 5’ CACATGTCCGTCACAGTGGA 3’ R 5’ TAGAGGCTGACCACCTCCTC 3’
MMP1	Human	F 5’ ACATTGCAGTGCAGGATGTGCAGGC3’ R 5’ CTTGGGTACTGGTGACCGGT3’
MMP2	Human	F 5’ TTTCCATTCCGCTTCCAGGGCAC 3’ R 5’ TCGCACACCACATCTTTCCGTCACT 3’
MMP9	Human	F 5’ GACGCAGACATCGTCATCCAGTTT 3’ R 5’ GCCGCGCCATCTGCGTTT 3’

3.2.15. Cell Cycle Analysis

HDF, HUVEC, HACAT and L929 cells were seeded with the concentration of of 3×10^5 cell/well onto 6-well plates and incubated overnight. Cells were treated with a serum-free medium for 24 h after incubation to synchronize the cell cycle, accompanied by incubation with wheat exosomes of 200 µg / mL. After the incubation, cells were detached with trypsin and washed once with PBS. Then, cells were fixed with 70% EtOH (30% PBS, 70% EtOH) and incubated at – 20 °C for 2 h for the fixation process. The cells were then washed with PBS and resuspended with cold PBS containing 0.1% RNAse A and 1% Noridot to remove

the existing ethanol, then incubated for 30 min at 37 ° C. After all, 5 µL of Propidium iodide solution was added onto cells and the cell cycle distrubution was analyzed via flow cytometry.

3.2.16. Annexin V Apoptosis Assay

To observe the apoptotic effect of wheat exosome on HDF, HACAT, HUVEC and L929 cells, Annexin V Apoptosis Assay was performed. Cells were seeded into a 6-well plate ($3x10^4$ cells/well), after 24h 200 µg/mL of wheat exosome was added onto cells. Cells were incubated 48h at cell culture conditions. After the incubation, conditioned media was collected and cells were washed with PBS. Cells were then trypsinized and collected. Cell pellets were washed with 1 mL PBS for 5 minutes after centrifugation. Following this step, 250 µl of the Annexin V binding buffer was added to the sample pellet and 5 µl of the Annexin V PE antibody were applied to the cell pellet and incubated on ice for 20 minutes. 1 µl of PI antibody was administered only prior to conducting flow cytometry. Samples were then analyzed via BD FACS Calibur Flow Cytometry.

3.2.17. Enzyme-Linked Immunosorbent Assays

Human MMP1, human MMP 3, human VEGF, human pro-collagen 1 alpha 1, human TGF beta 1 Enzyme-Linked Immunosorbent Assays (ELISA kits) were performed individually. Each kit has its own 96 well plate coated with related antibodies. Into each well, 100 µl of each samples were added and incubated for 2.5 hours. The medium was discarded after incubation and 100 µl of biotin antibody (1X) was applied to each well and incubated for 1 hour at room temperature. Wells were decanted and washed three times with a 1X wash buffer after incubation. 100 µl of 1X HRP Avidin solution was applied to each well following the washing phase and incubated at room temperature for 1 hour. Wells were aspirated and washed four times with a 1X wash buffer after incubation. On each well, 90 µl of TMB Substrate Solution was added and incubated at dark and room temperature for 30 min. Finally, in each well, 100 µl of the stop solution has been applied. With an ELISA plate reader at 450 nm with 620 nm as reference wave length, the absorbance values of the wells were evaluated.

3.2.18. Growth Factor Array Assay

Human Growth Factor Antibody Array C2000 (RayBiotech, Inc., GA) was used to assess growth factors secreted by HDF, HUVEC and HACAT cells cultured in the presence of wheat exosomes, according to the manufacturer's instructions. In a humidified incubator with 5% CO2, these cells were treated with 200 µg/ml wheat exosome for 24 hours at 37 °C. The antibody bound membranes were incubated with blocking buffer for 30 minutes. The membranes were incubated overnight at 4 °C with samples diluted in 1ml of blocking buffer. Membranes were incubated with 1ml of Biotinylated Antibody Cocktail overnight at 4 °C upon being washed three times. Membranes were incubated with HRP-Streptavidin solution overnight at 4 °C after the purification steps. Membranes were visualized with luminometer device after washing samples. Fiji ImageJ software was used to measure average spot levels of intensity for each growth factor, which were then correlated to their respective control samples.

3.2.19. Immunocytochemistry

HDF, HUVEC, HACAT cells were seeded onto 8 well chamber with the cell concentration of 3000 cells per chamber and incubated overnight at cell culture conditions. The control group was treated with complete DMEM medium , and sample group was treated with 200 µg/ml of wheat exosomes for 24 h prior to immunocytochemistry analysis. Cells were fixed with 2% (w/v) of paraformaldehyde solution for 30 minutes at room temperature and washed for 5 min in PBS-T solution, PBS with 0.1% Tween 20 for three times. 0.1% (v/v) Triton X-100 solution was then added for 5 min for the permeabilization of cells at room temperature. To inhibit non-specific binding of antibodies, cells were washed with PBS-T; cells were then incubated with 2 percent goat serum and incubated at 4 C overnight with the 0.2 percent primary antibody against collagen type I. Cells were washed using the same procedure after incubation and incubated with 0.4 percent goat anti-mouse AlexaFluor-647 antibody at 4 °C for 1 h after incubation. Cells were washed gently and nuclei were stained at 4 C with 0.1 percent DAPI solution (4', 6-di-amidino-2-phenyl-indole) for 20 min. Finally, the cells were washed with PBS-T and observed under a confocal microscopy.

3.2.20. Cellular Uptake

According to the manufacturer's protocol, the cell nucleus was dyed with Hoechst dye at a final concentration of 1 g/ml. Then, attached cells were dyed with CellTrackerTM Green CMFDA Dye according to the protocol. Wheat exosomes were dyed with PKH26 dye. 1ml of diluent C is mixed with 4 µl of PKH26 antibody and 1.1 ratio of exosomes were then added into solution and incubated for 5 minutes. After the incubation, in order to get rid of the excess dye, solution poured into spin coloumns and centrifuged at 750 x g for 2 minutes. After this, dyed exosomes were given to the cells and their photos were taken at 24h under fluroscent microscope.

3.2.21. *In vivo* Animal Study

Female Wistar rats (n=35) weighing 200±50 g were used in wound healing experiments. The animals were housed at a constant temperature (21±1 °C) and humidity (60±10%), and subjected to an artificial 12-h light/dark cycle and fed with food and water ad libitum as normal. Animal handling and surgical procedures were approved with an ethical permission obtained from the Elazığ University Ethics Committee of Experimental Animal Use and the Research Scientific Committee at the same institution.

3.2.21.1. Anaesthesia

Intramuscular injections of 85 mg/kg ketamine hydrochloride (Ketalar, Parke-Davis, 50 mg/ml) and 6 mg/kg xylazine hydrochloride (Rompun, Bayer, 23.32 mg/ml) were anaesthetized in the animals.

3.2.21.2. Wound model and Treatment Protocol

The rats were divided into 5 main groups, and 7 rats were randomly selected for each group. A disposable 5 mm diameter skin punch biopsy tool (Kai Medical, Japan) was shaved and punched on the dorsal surface of the rat skin to create a full-thickness excisional wound in the 2nd, 3rd, 4th and 5th groups.

Right away after infliction of the wound, placebo cream (Control), 200 µg/ml, 500 µg /ml exosome, 1000 µg/ml exosome and 2000 µg/ml exosome treatment twice daily were topically applied to the wounded areas in the second, third, fourth and fifth classes. These applications were repeated twice a day until, after completion of the procedure, the animals

were sacrificed, that is, on day 15. The wounds were examined each day in each group. For further study, animals that completed the postoperative follow-up duration of 15 days were sacrificed.

3.2.21.3. Qualitative Examination of the Wound and Wound Area Measurements

On a clear plastic film with a fine-tipped marker, the wound borders were numbered each day. With the assistance of the millimetric paper and planimeter, the wound areas were assessed. The day wound contraction began during the evaluation, the wound contraction rate, the wound fraction healed, the expansion rate, the day epithelialization was first observed, the wound fraction healed with epithelialization, and the number of days in which healing was completely completed were measured during the examination.

3.2.21.4. Histopathological Examinations

At day 15, the animals were decapitated under light ether anesthesia, and skin specimens were taken and stored in 10% neutral formaldehyde at room temp. The samples were washed in a graded sequence of alcohols (70, 90, 96, and 100%) upon being treated with toluene. Tissues were stored in microscope slides.

3.2.21.5. Histological Grading

The general morphology, epithelization, fibroblast, and inflammatory cell number of tissue samples which were stained with H&E were examined.

3.3. STATISTICAL ANALYSIS

For multiple data comparisons using GraphPad Prism statistical software 5.0 (GraphPad Software, La Jolla, CA, USA), one-way variance analysis (ANOVA) followed by Tukey's post hoc test was carried out.

4. RESULTS

4.1. EXOSOME CHARACTERIZATION BY FLOW CYTOMETRY AND SCANNING ELECTRON MICROSCOPY

Wheat derived exosomes were isolated with aqueous two phase solution method as described previously at section 3.2.1. Exosomes were firstly characterized by clustred of differentiation (CD) surface markers such as CD9, CD63 and HSP70 markers which are known as tetraspanin proteins and mostly found at exosomal membrane. In addition, the size of the wheat derived exosomes were analyzed by scanning electron microscopy.

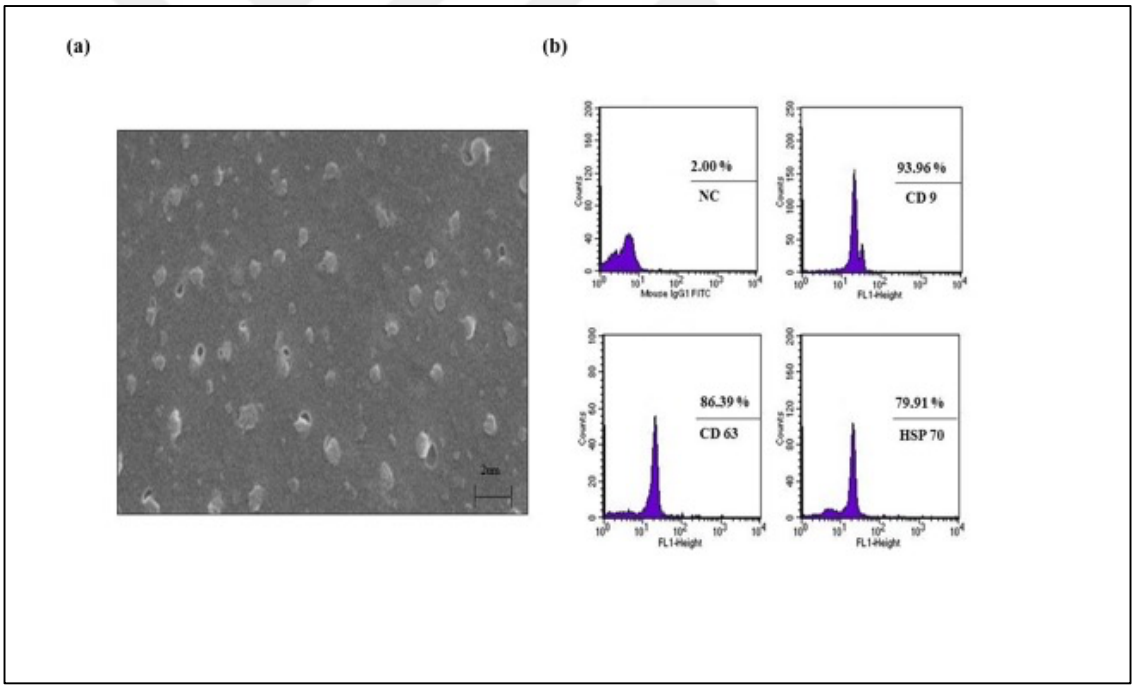


Figure 4.1. Characterization of wheat derived exosomes via (a) Scanning Electron Microscopy (SEM) and (b) Flow Cytometry. The SEM image showed irregularly shaped multiple vesicles with a scale bar suggesting 2 μm (a) and flow cytometry analysis showed high percentage of binding between exosome-specific surface antigens CD9, CD63 and HSP 70.

The percentage of these CD markers were analyzed by flow cytometry (Figure 4.1). Negative control was used exosomes without any marker and CD9, CD63, and HSP70 CD surface markers were tested and calculated as 93.96 percent, 86.39 percent and 79.91 percent respectively.

In addition, the size of the wheat derived exosomes were analyzed by scanning electron microscopy. Results demonstrated that, exosomes were ranging between 40 and 100 nm (Figure 4.1A, B), was indicated by scanning electron microscope analysis.

4.2. EXOSOME CHARACTERIZATION BY NANOPARTICLE TRACKING ANALYSIS (NTA), ZETA SIZER AND IMMUNOBLOTTING

Wheat derived exosomes were then characterized by NTA and Zeta Sizer. NTA was used to analyze the nanoparticle size and the concentration of exosomes with respect of nanoparticles' Brownian motion.

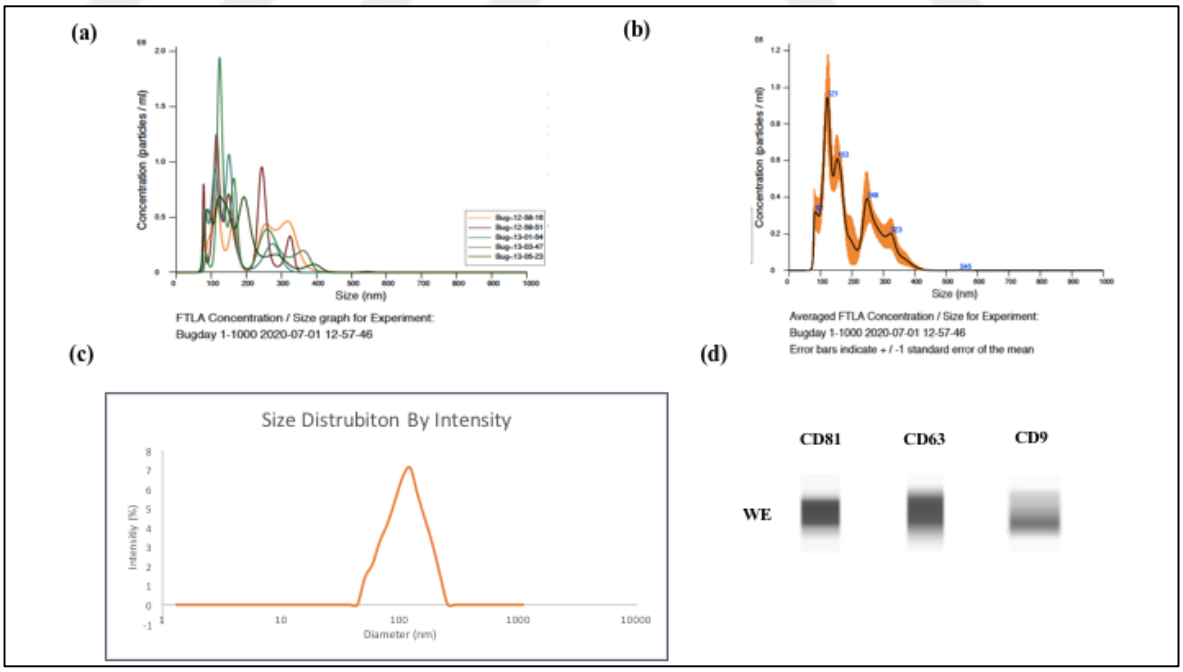


Figure 4.2. Nanoparticle tracking analysis (NTA) data demonstrated the size and the concentration of exosomes (a) The finite track length adjustment (FTLA) concentration / size graph , (b) The average of FTLA concentration /size, (c) Dynamic light scattering analysis. Size distribution of exosomes derived from wheat derived exosomes, (d) WES

Simple Western™ Analysis with exosome spesific CD Surface Markers named as CD81, CD63 and CD9

According to the Figure 3.2 A and B, the size of exosomes were found as an average with a mean of 188.4 +/- 11.4 nm and the mode at 126.7 +/- 6.4 nm. Additionally, exosome size nanoparticle concentration were found as $9.35e^{+07}$ +/- $3.54e^{+06}$ particles/ml and a particle number by frame was found as 16.5 +/- 0.8 particles/frame (Figure 3.2 A, B). Additionally, Zeta sizer analysis were also confirmed the average size of exosomes around 100 nm (Figure 3.2 C). Moreover, as it shown in Figure 4.2. D, results demonstrated that wheat derived exosomes showed positive intensity signal for CD81, CD63 and CD9 cell surface markers.

4.3. CELLULAR VIABILITY AND PROLIFERATION

For the detection of the cytotoxic effect of wheat exosomes on HDF, HUVEC, HACAT,and L929 cells cell viability was measured via WST1 assay, performed according to the manufacturer's protocol as described Section 3.2.8.

The cell proliferation percentage was determined by assigning 100 percent of the absorbance value obtained from the untreated cells.

The toxicity of different concentrations (30–200 μg/mL) of wheat exosome on HDF, HUVEC, HACAT and L929 cells was determined within the 3 days of the incubation (24, 48, and 72 h). There were no cytotoxic effects at concentrations of up to 200 μg / mL of wheat exosomes on these types of cells. Furthermore, in all cell types, cell proliferation was increased significantly in a dose dependent manner.

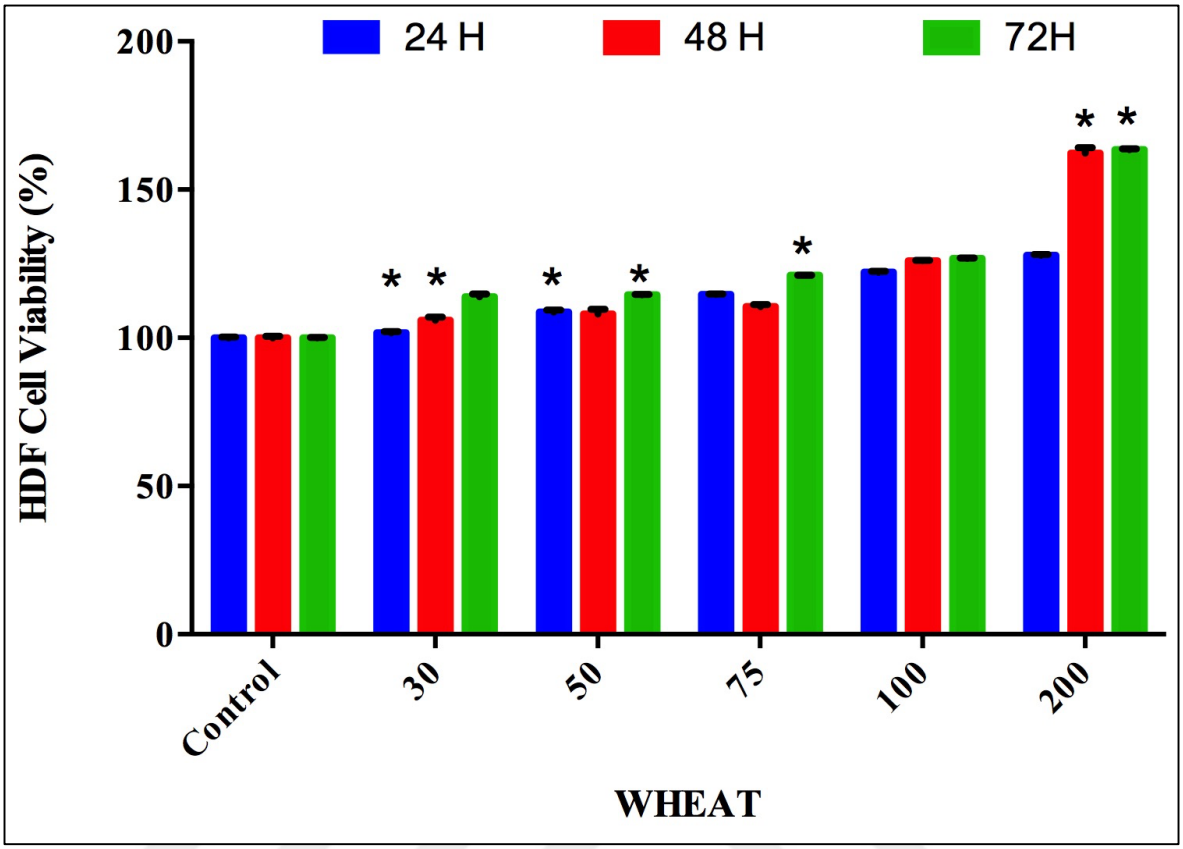


Figure 4.3. The cell viability of HDF cells after the treatment of different concentrations of wheat exosome.Mean absorption values derived from WST-1 cell viability assay were normalized to post 24 hours of untreatd cell data. Results represents the mean ± SD of three independent experiments. Asterisks are used to indicate statistically significant values with respect to control (0 µg/ml) (*p ≤ 0.05; **p ≤ 0.01).

24 hours after the treatment of different concentrations of wheat exosome (30-200), HDF cells showed approximately 20 percent increase in the cell number for lower doses (30 to 100 µg/ml), however at 200 µg/ml of the exosome treated HDF cells resulted with a significant cell proliferation rate which is around 50 percent 48th hour ($P<0.05$).

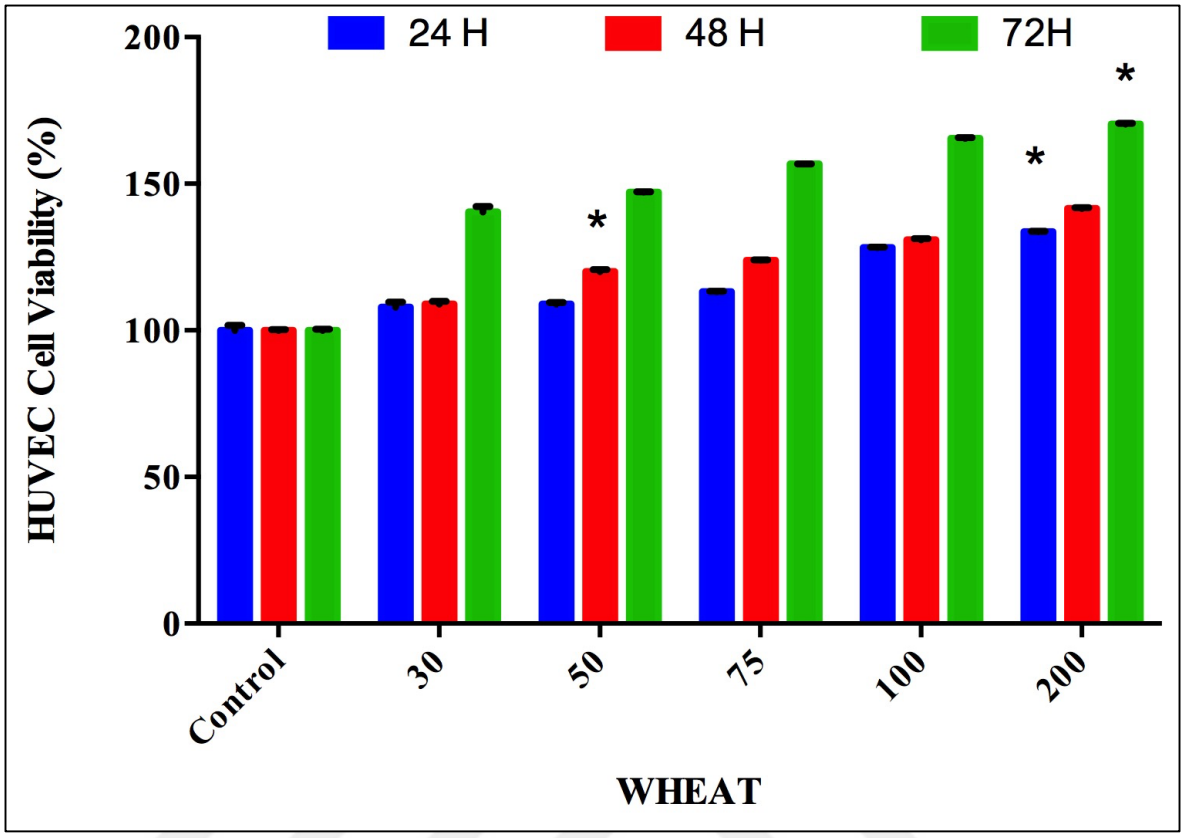


Figure 4.4. The cell viability of HUVEC cells after the treatment of different concentrations of wheat exosome.Mean absorption values derived from WST-1 cell viability assay were normalized to post 24 hours of untreated cell data. Results represents the mean ± SD of three independent experiments. Asterisks are used to indicate statistically significant values with respect to control (0 µg/ml) (*$p \leq 0.05$; **$p \leq 0.01$).

Similarly, wheat exosome treated HUVEC cells showed approximately 20 percent increase in the cell number for lower doses (30 to 100 µg/ml) at 24 and 48 hours, whereas at 72hour wheat exosome treated HDF cells resulted with 30-50 percent increase in cell proliferation ($P<0.05$).

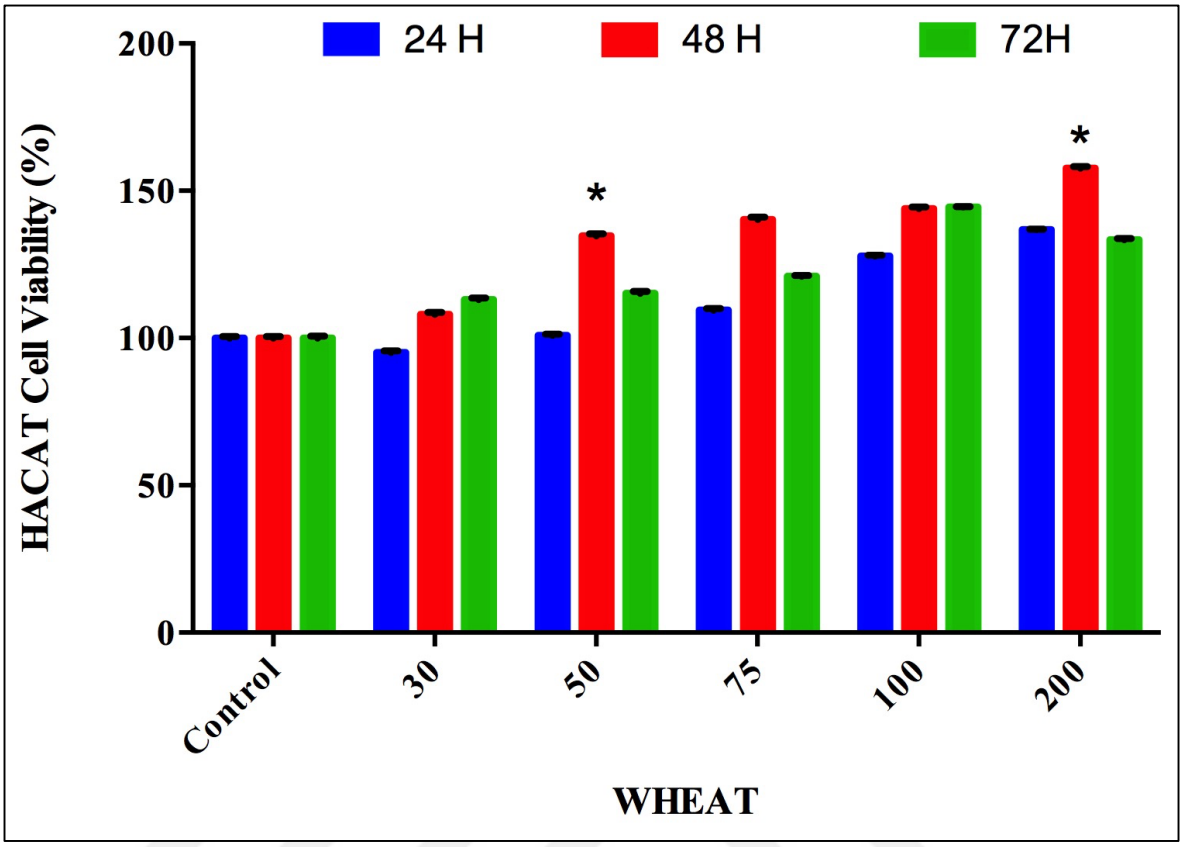


Figure 4.5. The cell viability of HACAT cells after the treatment of different concentrations of wheat exosome. Mean absorption values derived from WST-1 cell viability assay were normalized to post 24 hours of untreated cell data. Results represents the mean ± SD of three independent experiments. Asterisks are used to indicate statistically significant values with respect to control (0 µg/ml) (*$p \leq 0.05$; **$p \leq 0.01$).

The toxicity of different concentrations (30–200 µg/mL) of wheat exosome on HACAT cells was resulted as there was no cytotoxic effects at concentrations of up to 200 µg / mL of wheat exosomes. Furthermore, cell proliferation was increased significantly in a dose dependent manner.

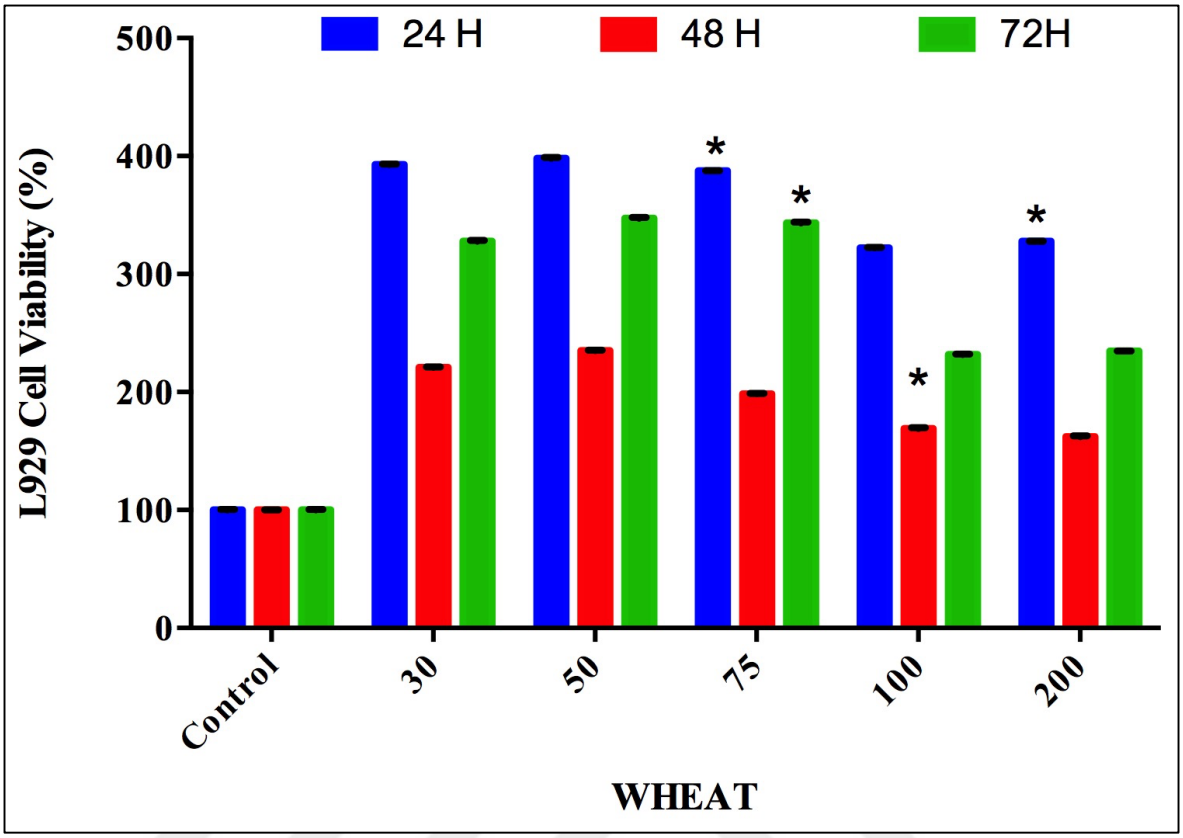


Figure 4.6. Relative quantification of L929 cell viability. Mean absorption values derived from WST-1 cell viability assay were normalized to post 24 hours of untreated cell data. Results represents the mean ± SD of three independent experiments. Asterisks are used to indicate statistically significant values with respect to control (0 µg/ml) (*$p \leq 0.05$; **$p \leq 0.01$).

For three days (24h, 48h, 72h), mouse fibroblast cell line (L929) was treated with 30 µg/ml, 50 µg/ml, 100 µg/ml, and 200 µg/ml of wheat exosomes. Based on the results, relative cell viability was given in Figure 4.3.3. Results demonstrated that each concentration significantly increases the cell viability in respect to the control (0 µg/ml). No cytotoxic effect is observed for each concentration. It is seen that wheat exosomes induce cell proliferation in time and concentration dependent manner.

4.4. SCRATCH ASSAY

After the treatment of 200 µg / mL of wheat exosome, the proliferation of HDF, HUVEC, HACAT and l929 cells was increased as discussed as section 4.3. That's why for further experiments, 200 µg / mL of wheat exosome was chosen as a optimal dose. In order to investigate the effect of wheat exosomes on cell migration functions, the activity of the wheat exosome on cell migration was evaluated by scratch assay method.

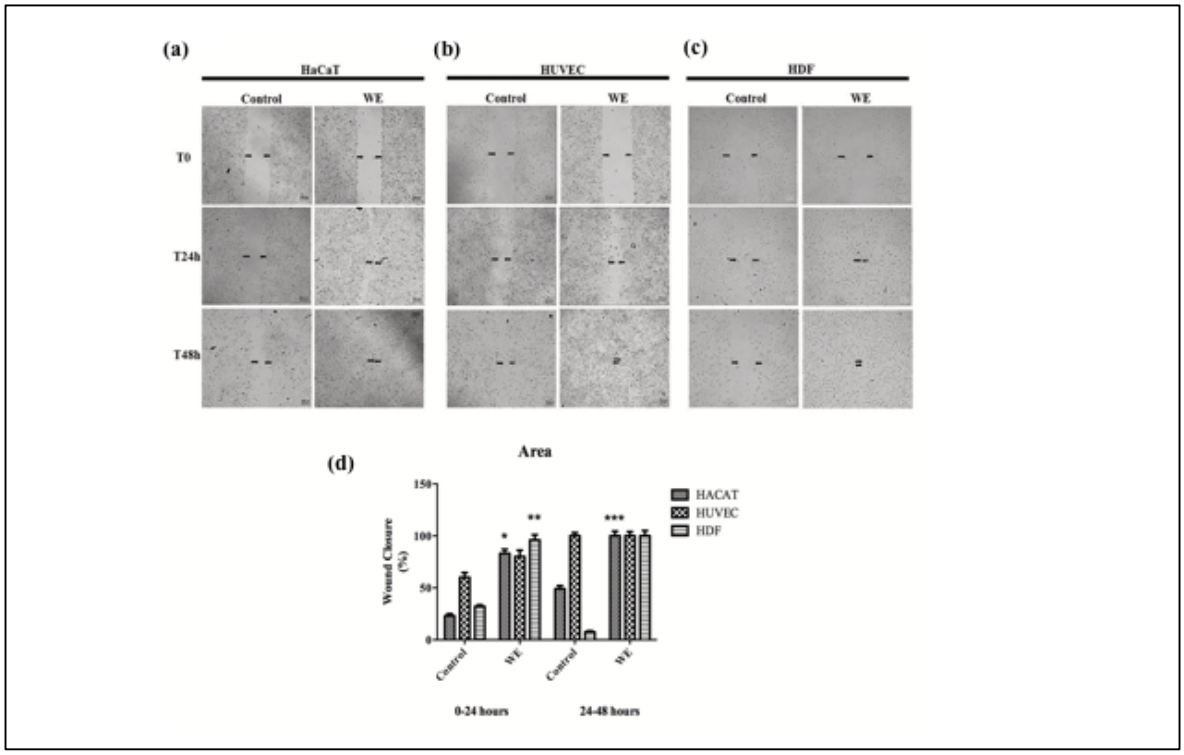


Figure 4.7. Microscope images (Å~ 40 magnifications, 400 µm scale bar) to test in vitro wound healing throughout the scratch assay using (a) HACAT, (b) HUVEC, and (c) HDF cells. Immediately following the wound, and after 24 and 48 h of incubation for each cell line, cell migration was recorded. In DMEM medium, all control groups and treated wheat exosome (WE) groups were cultured (37 °C, 5 percent CO2 with 10 percent FBS), exosome-treated groups contained 200 µg / mL exosomes D The wound closure rate among cells of HACAT, HUVEC, and HDF. *P < 0.05

As a consequence of the scratch assay, while control group cells moderately closed the scratched area after 48 h, HDF, HUVEC, and HACAT cells treated with wheat exosome

were reported to migrate faster, which was around 50 percent higher wound closure rate after 24 hours of incubation.

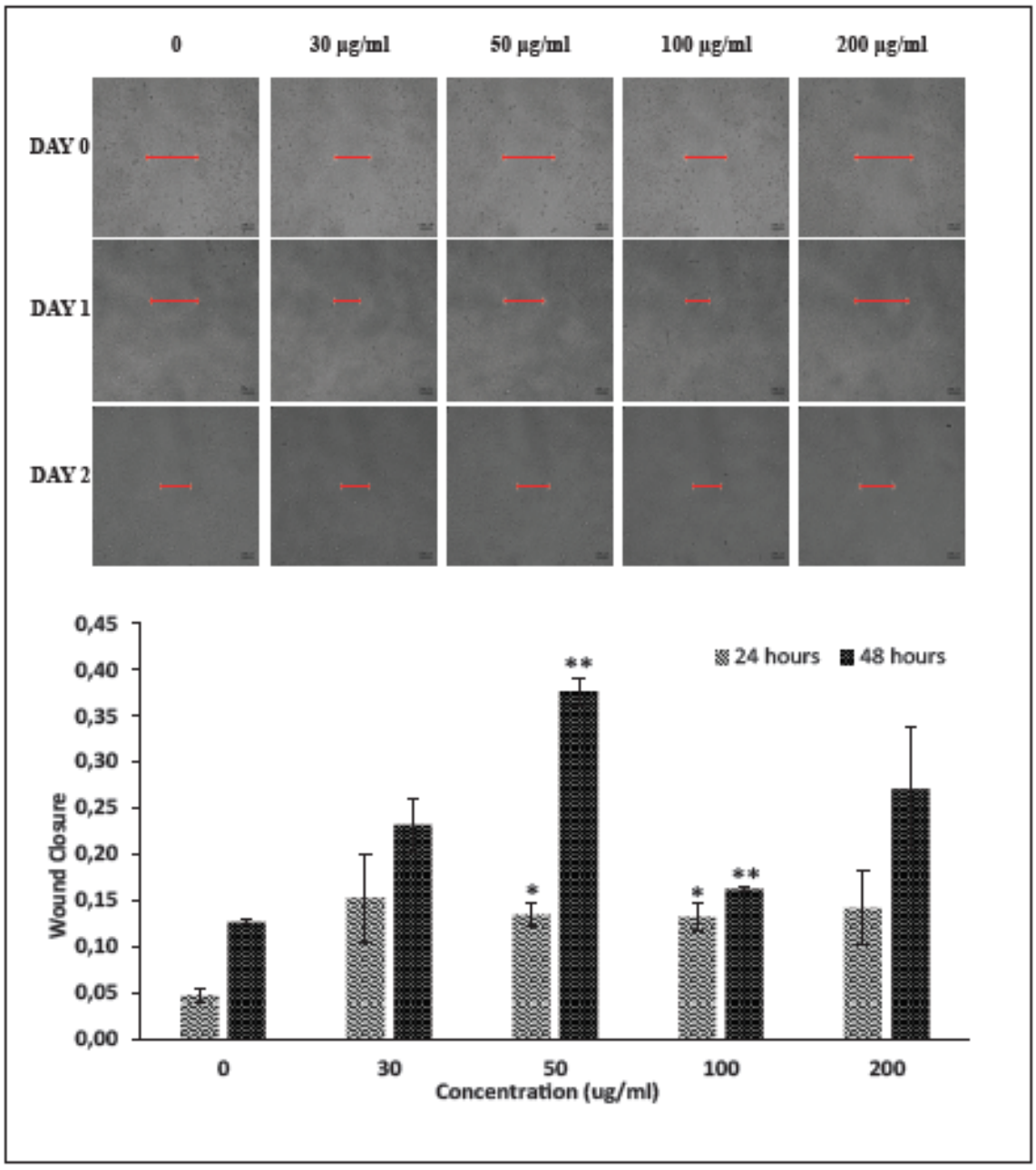


Figure 4.8. Scratch assay results. Images obtained at day0, day1 and day2, showing the effect of WE at different concentration. Graph shows the wound closure. Results represents the mean ± SD of three independent experiments. Asterisks are used to indicate statistically significant values with respect to control (0 µg/ml) (*$p \leq 0.05$; **$p \leq 0.01$).

Cell migration of L929 was assessed with scratch assay using 30 µg/ml, 50 µg/ml, 100 µg/ml, and 200 µg/ml wheat exosomes. Images were obtained at day0, day1 and day2.

Wound closers were indicated with red arrows in Figure 4.4.2. Day0 images are obtained after the plate was scratched and provides a control for further days. Results indicate that after 24h, wound closure have started for each concentration. Wound closure was measured using ImageJ program and graph was plotted by the results. It is seen that concentrations 50 µg/ml and 100 µg/ml of WE have shown significant closure after both 24h and 48h treatment relative to the control (0 µg/ml)

4.5. MATRIGEL TUBE FORMATION ASSAY

To test the angiogenic properties of the wheat exosomes, human umbilical vein endothelial cells (HUVECs) are used to conduct tube formation assay. Cells are seeded over Matrigel and treated with 100 µg/ml (Figure 4.5.1) and 200 µg/ml (Figure 4.5.2) wheat exosome for 16 hours respectively. Images are analysed by Wimasis program and results are obtained as number of branching points. All parameters have shown an increase in cells treated with 100 and 200 µg/ml WE with respect to control. Significant difference occurred in number of branches with respect to control.

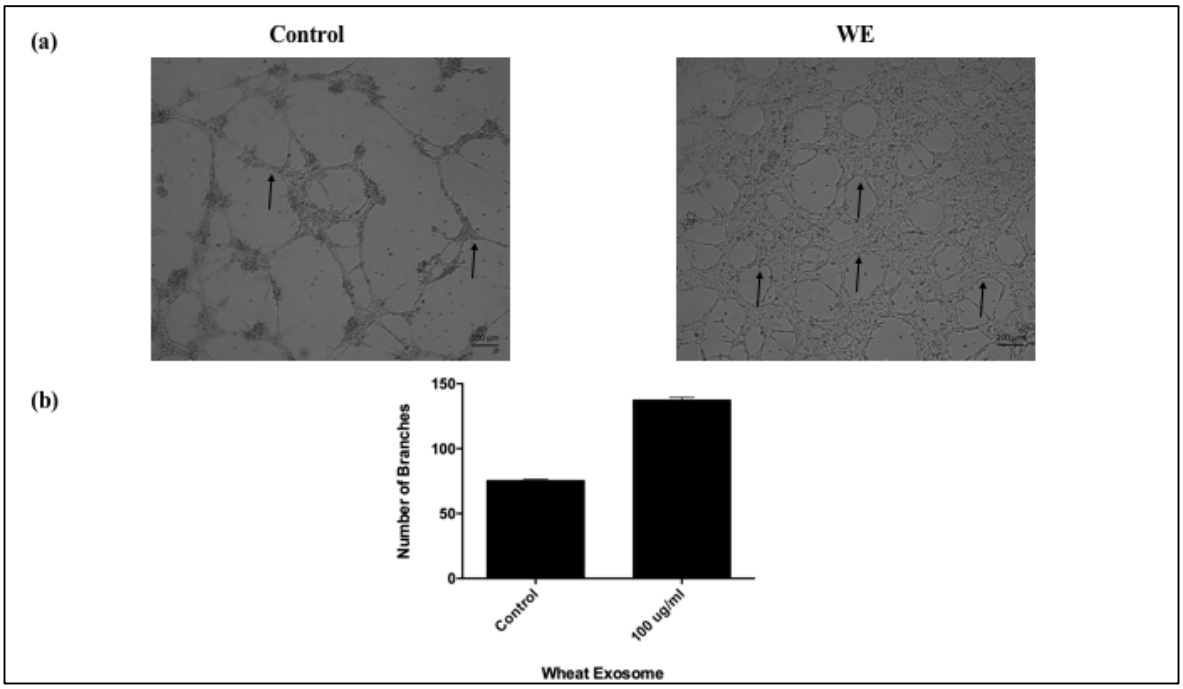


Figure 4.9. Tube formation analysis (100 µm bar scale) to test angiogenesis stimulation in HUVEC cells through 100 µg/ml wheat exosome treatment. (a) Control, HUVEC cells with any treatment and 100 µg/ml wheat exosome treated HUVEC cells. (b) Average

number of branching are analyzed by Wimasis program and given as graphs. Results represents the mean ± SD of three independent experiments (*p ≤ 0.05; **p ≤ 0.01).

Images are obtained after 16 hours and show a 2 folds increase in branching in 100 µg/ml wheat exosome treated HUVECs (Figure 4.5.1) and compared to the control group, the number of branches in the 200 µg/ml wheat exosome treated group was about 2.5 to 3 times higher, suggesting a functional link between wheat exosomes and angiogenesis. (Figure 4.5.2).

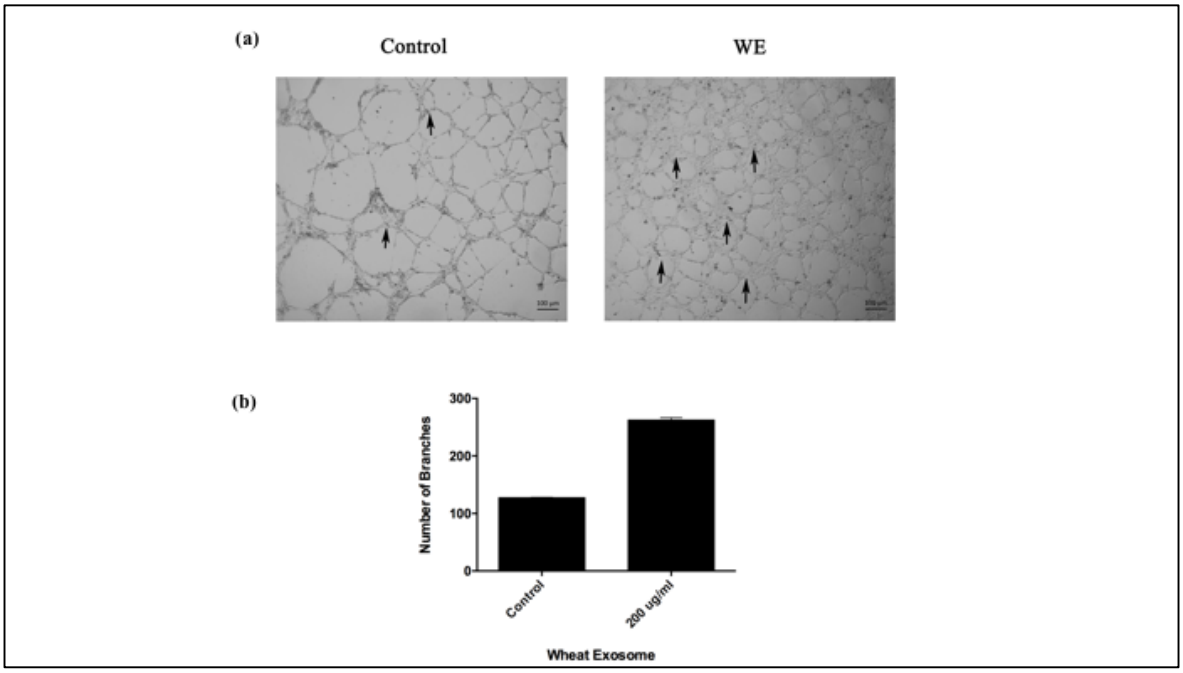


Figure 4.10. Tube formation analysis (100 µm bar scale) to test angiogenesis stimulation in HUVEC cells through 200 µg/ml wheat exosome treatment. (a) Control, HUVEC cells with any treatment and 200 µg/ml wheat exosome treated HUVEC cells. (b) Average number of branching are analyzed by Wimasis program and given as graphs. Results represents the mean ± SD of three independent experiments (*p ≤ 0.05; **p ≤ 0.01).

All in all tube formation analysis showed that, wheat exosomes are capable of increasing the development of tube-like structures by significantly increasing the number of branches, suggesting that wheat exosomes play a significant role in vascularization, angiogenesis during wound healing.

4.6. ANTI INFLAMMATION ASSAY

Anti-inflammation effect of wheat exosomes is measured using H2O2. Cells are treated with 100 µg/ml of wheat exosomes or 1.0 mM hydrogen peroxide or both of them together. 24h after treatment, WST-1 assay is conducted, as explained previously as section 3.2.8. By taking 100 µg/ml WE treated data as positive control, % cell viability graph is produced.

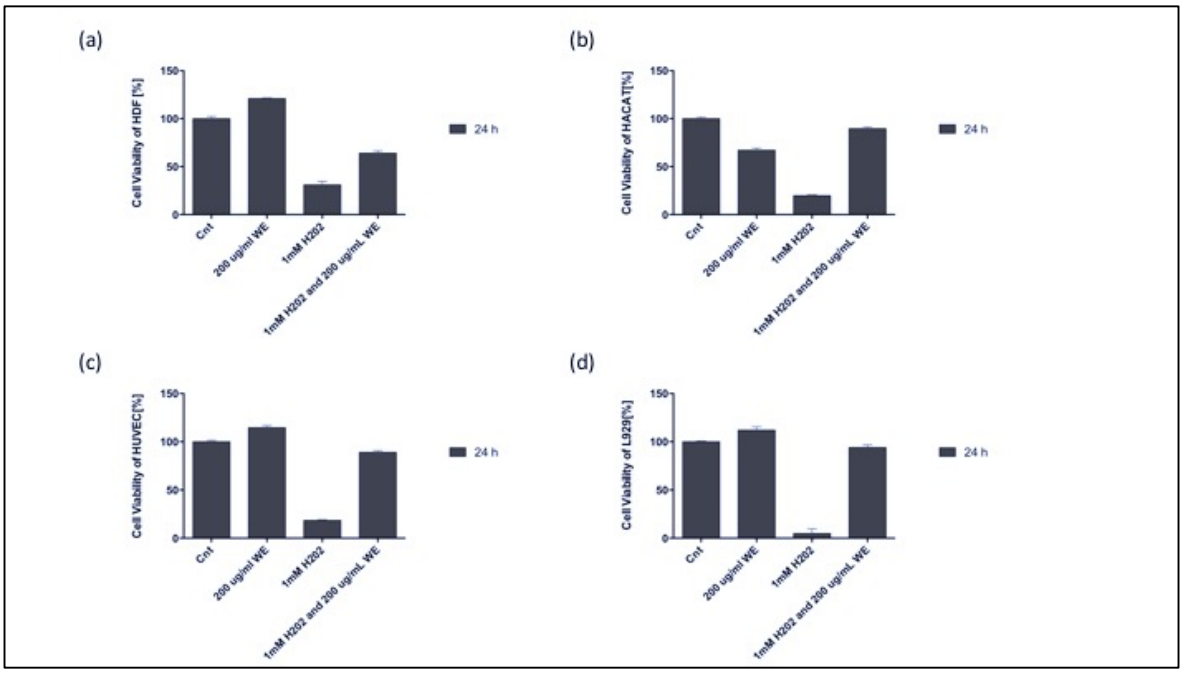


Figure 4.11. Anti-inflammation effect of wheat exosomes (WE) using H2O2 on (a) HACAT, (b) HUVEC, (c) HDF and (d) L929 % cell viability is obtained by taking 100 µg/ml WE data as positive control. Results represents the mean ± SD of three independent experiments.

Results demonstrated that 1 mM H_2O_2 has shown inflammatory effect decreased the cell viability around 3 fold on all healthy cell lines, causing cytotoxicity. On the other hand, cells treated with both wheat exosome and hydrogen peroxide show similar result as the positive control, pulled back the cell viability rate to the control level which means, wheat exosome could block the inflammatory effect of H_2O_2. In other words, wheat exosomes have shown to be anti-inflammatory in HDF, HUVEC, HACAT and L929 cell lines.

4.7. APOPTOSIS

To assess whether wheat exosome resulted with apoptosis in HDF, HUVEC, HACAT and L929 cells, cells treated with wheat exosome with the concentration of 200 µg/mL exosome for each cell for 48 hours. After the treatment, HDF, HUVEC, HACAT and L929 cells were stained with Annexin V PE conjugated as described in Section 3.2.16. Annexin V is a protein that has a higher affinity for the phospholipid phosphatidylserine found in cells which undergoes apoptosis.

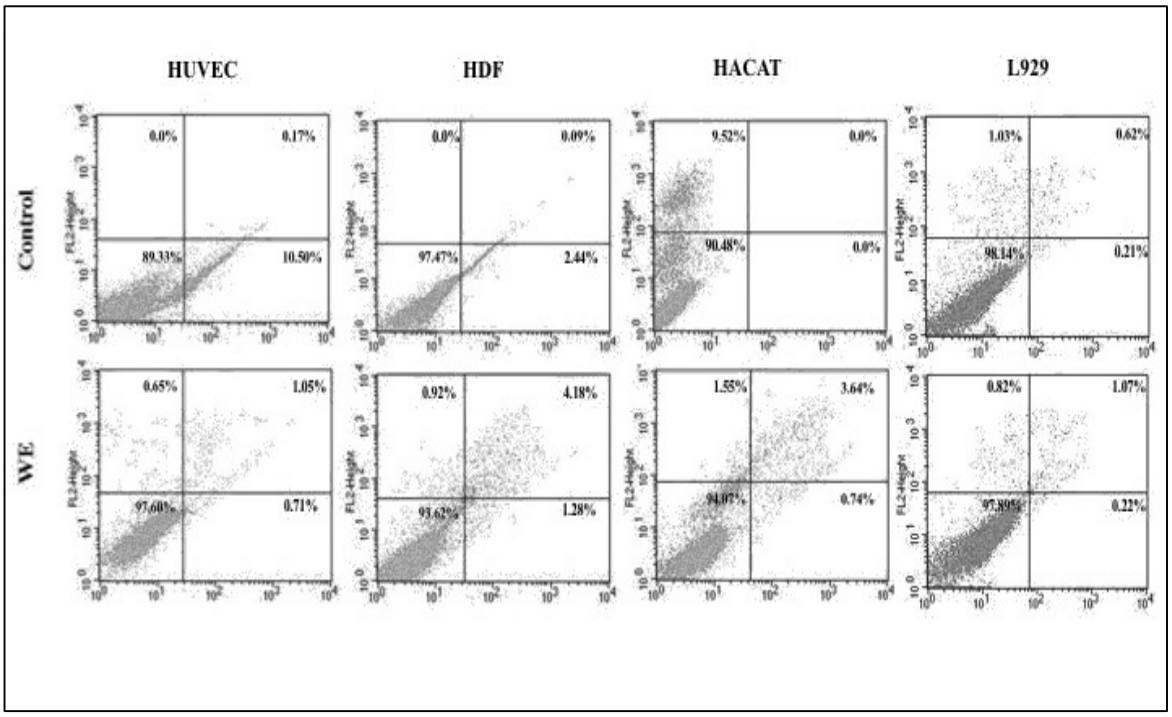


Figure 4.12. The Annexin V staining on 200 µg/mL wheat exosome treated HUVEC, HDF, HACAT and L929 cells. Apoptotic cells that are positively stained with PE conjugated Annexin V are marked in the upper left quadrant of representative histograms. The apoptotic rate of control and the apoptotic rate of cells treated with 200 µg/mL wheat exosome are represented respectively.

In order to confirm the apoptotic effect of wheat exosomes on HUVEC, HDF, and HaCaT cells, annexin V staining was assessed. The results demonstrated that 200 µg/mL of wheat exosome treated HUVEC cells resulted with the number of 1.76 % apoptotic cells, HDF cells resulted with the number of 5.1 % percent apoptotis, HACAT cells resulted with the number

of 5.19 % apoptotic cells and lastly, L929 cells resulted with the number of 1.89 % apoptotic cells with respect to the control group (Figure 4.7). In brief, there was no noticeable apoptosis detected after the treatment of wheat exosomes on these healthy cell lines.

4.8. CELL CYCLE

Besides the apoptotis, we also investigated the effect of the wheat exosome treatment on the cell cycle distribution.

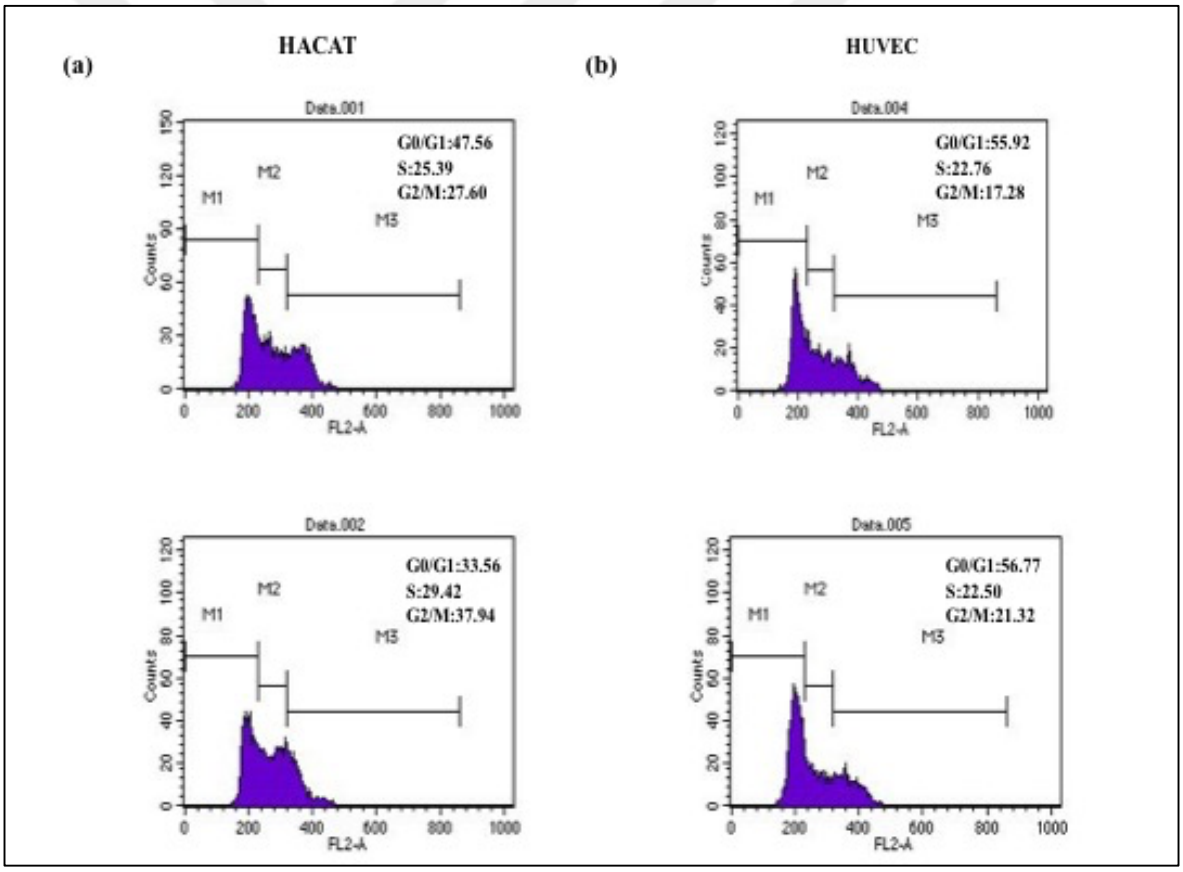


Figure 4.13. Cell cycle Analysis of (a).HACAT and (b).HUVEC cells upon 200 µg/mL wheat exosome treatment. Both the control (above) and wheat exosome (below) cell cycle distributions have exosomes that do not induce a remarkable alteration in the cell cycle The data were demonstrated and analyzed with ± SEM, n=4, *P<0.05

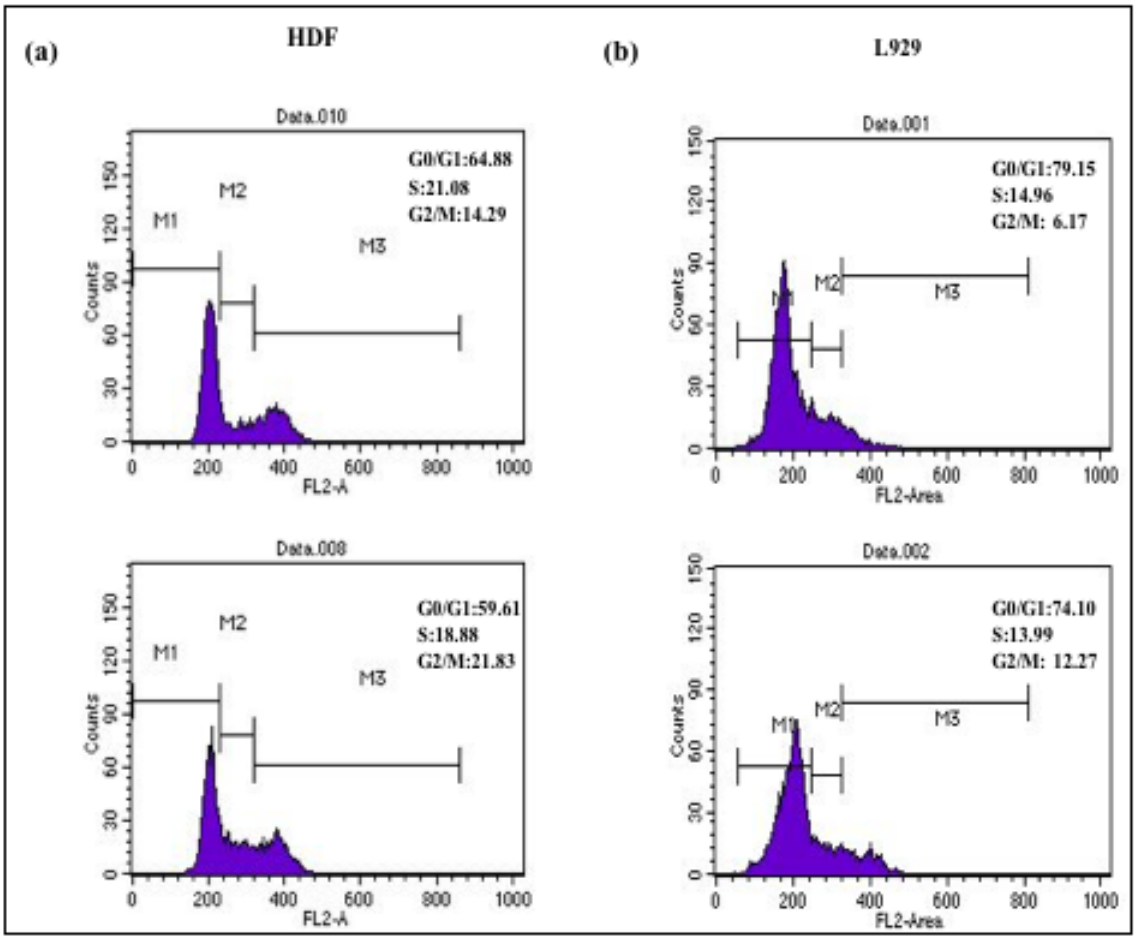


Figure 4.14. Cell cycle Analysis of (a) HDF and (b). L929 cells upon 200 µg/mL wheat exosome treatment. Both the control (above) and wheat exosome (below) cell cycle distributions have exosomes that do not induce a remarkable alteration in the cell cycle The data were demonstrated and analyzed with ± SEM, n=4, *P<0.05

As a consequence of the cell cycle study, in compare to control suggesting the anti-apoptotic activity of wheat exosome, this did not lead to a blockage in phases of the cell cycle. (Fig. 4.13 and 4.14.). In the cell cycle phase, HUVEC and HDF cell lines showed minor differences in the distribution profile; only a decrease of HaCaT cells in the G0 / G1 phase was observed with the increase of HACAT and HUVEC cells in the and G2 / M phase. For the HACATs, after the treatment of wheat exosome, number of cells in the S phase increased by 16 percent significantly with respect to control cells which were treated with growth medium. On the other hand, L929 cells, on the other hand, responded to 200 µg/ml exosome

treatment with the increase in the G2-M phase which is 50 percent with respect to control cells.

4.9. ELISA

Upon exosome application, Pro-COL1A1 ELISA was performed to test procollagen 1 levels of HDF, HACAT, HUVEC and L929 cells.

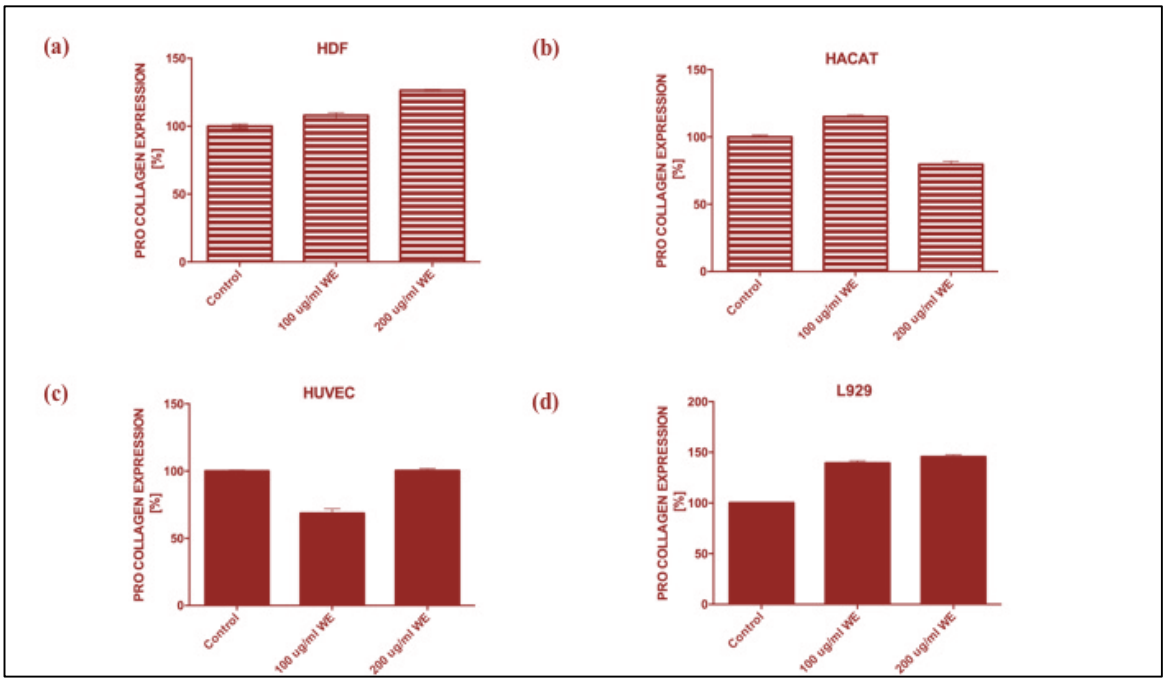


Figure 4.15. Pro Collagen expression levels of (a) HDF, (b) HACAT, (c) HUVEC and (d) L929 cells. Control was treated with only complete growth media. Datas were then analysed by two-tailed multiple t tests and demonstrated with Å} SEM, n=3, *P<0.05.

Results demonstrated that 100 µg/mL of wheat exosome treated HDF cells resulted with the 10 percent induction in procollagen expresion, moreover 200 µg/mL wheat exosome treated HDF cells resulted with almost 30 percent increase in procollagen expression. Besides, after the treatment of 100 µg/mL of wheat exosome on HACAT cells, procollagen expression was increased about 10-15 percent. On the other hand, 200 µg/mL of wheat exosome treated HACAT cells resulted with the 20 percent decrease in procollagen expresion. Moreover, for

the HUVEC cells, procollagen expression was slightly decrease after the treatment of 100 µg/mL of wheat exosome. On the other hand, pro collagen expression of 200 µg/mL of wheat exosome treated HUVEC cells pulled back to the control levels. Lastly, after the treatment of wheat exosomes, procollagen expression level of L929 cells were increase in a dose dependent manner.

Upon exosome application, TGF B ELISA was performed to test TGFB levels of HDF, HACAT, HUVEC and L929 cells.

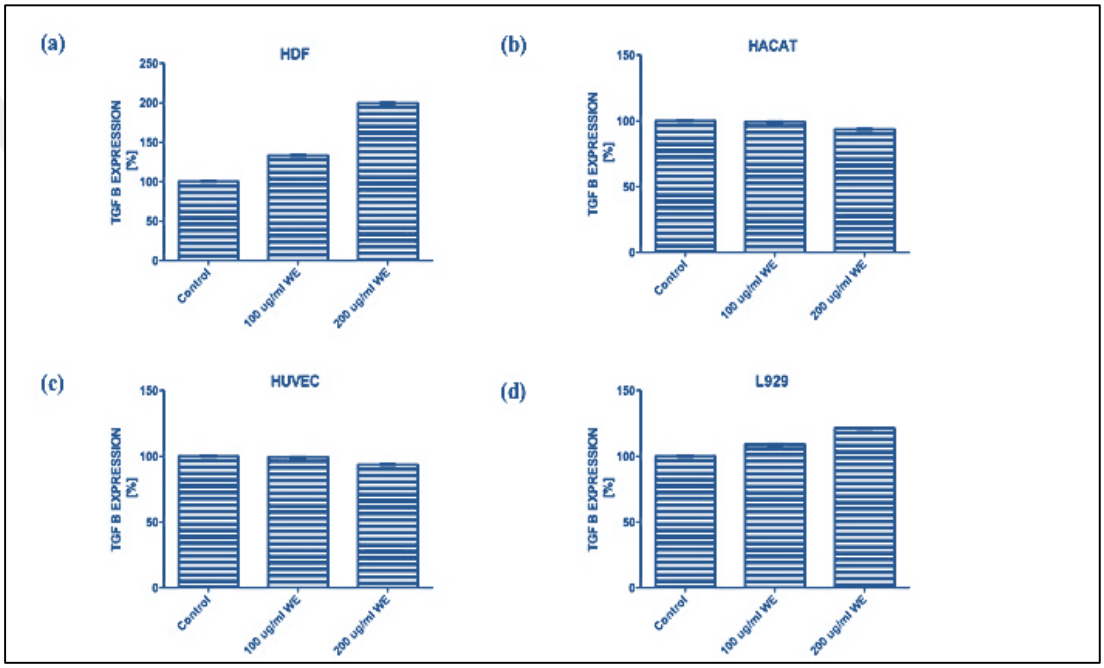


Figure 4.16. TGF B expression levels of (a) HDF, (b) HACAT, (c) HUVEC and (d) L929 cells. Control was treated with only complete growth media. Datas were then analysed by two-tailed multiple t tests and demonstrated with Å} SEM, n=3, *P<0.05.

TGFB ELISA results showed that, the treatment of wheat exosomes with the different doses did not significantly affect the TGFB expression on HACAT and HUVEC cells. On the other hand, after the treatment of 100 and 200 µg/mL of wheat exosome, the TGFB expression level of HDF and L929 cells were increase in a dose dependent manner.

After the treatment of wheat exosomes with the different doses, VEGF ELISA was performed to test VEGF levels of HDF, HACAT, HUVEC and L929 cells.

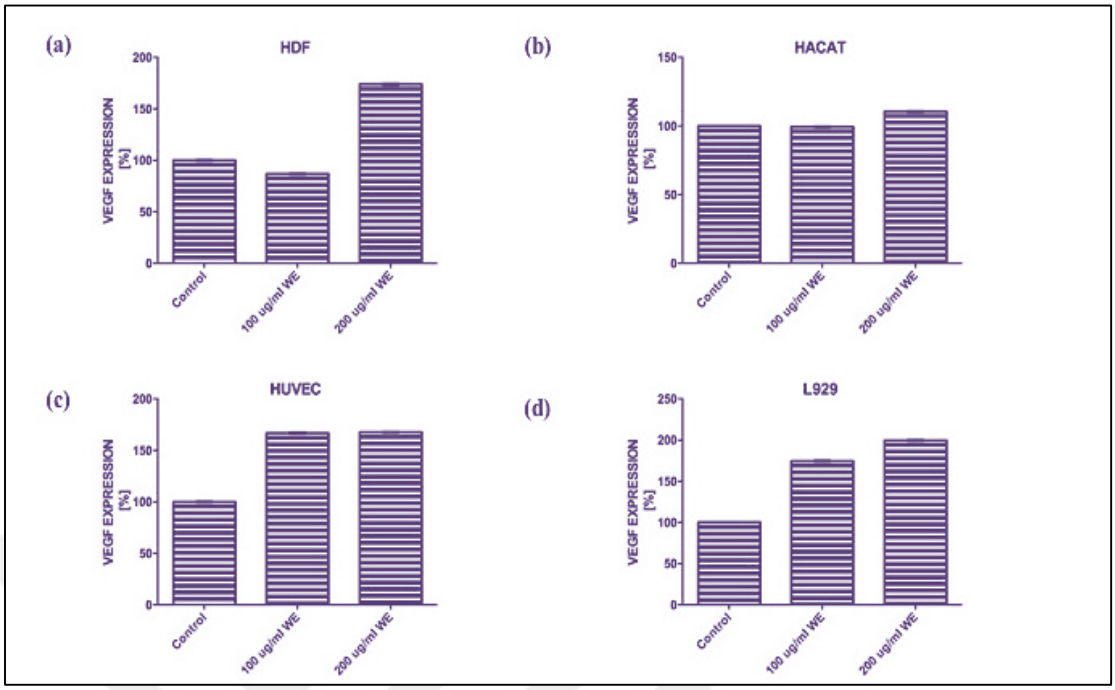


Figure 4.17. VEGF expression levels of (a) HDF, (b) HACAT, (c) HUVEC and (d) L929 cells. Control was treated with only complete growth media. Datas were then analysed by two-tailed multiple t tests and demonstrated with Å} SEM, n=3, *P<0.05.

The treatment of 100 and 200 µg/mL of wheat exosome on HACAT, HUVEC and L929 cells, VEGF expression levels were slightly increase in a dose dependent manner. On the other hand, 200 µg/mL of wheat exosome treated HDF cells showed significantly, 50 percent, increase in VEGF expression.

MMP1 and MMP3 expression was analyzed after 100 and 200 µg/mL of wheat exosome treatment on these cells to determine the effect of matrix metalloproteinase activity.

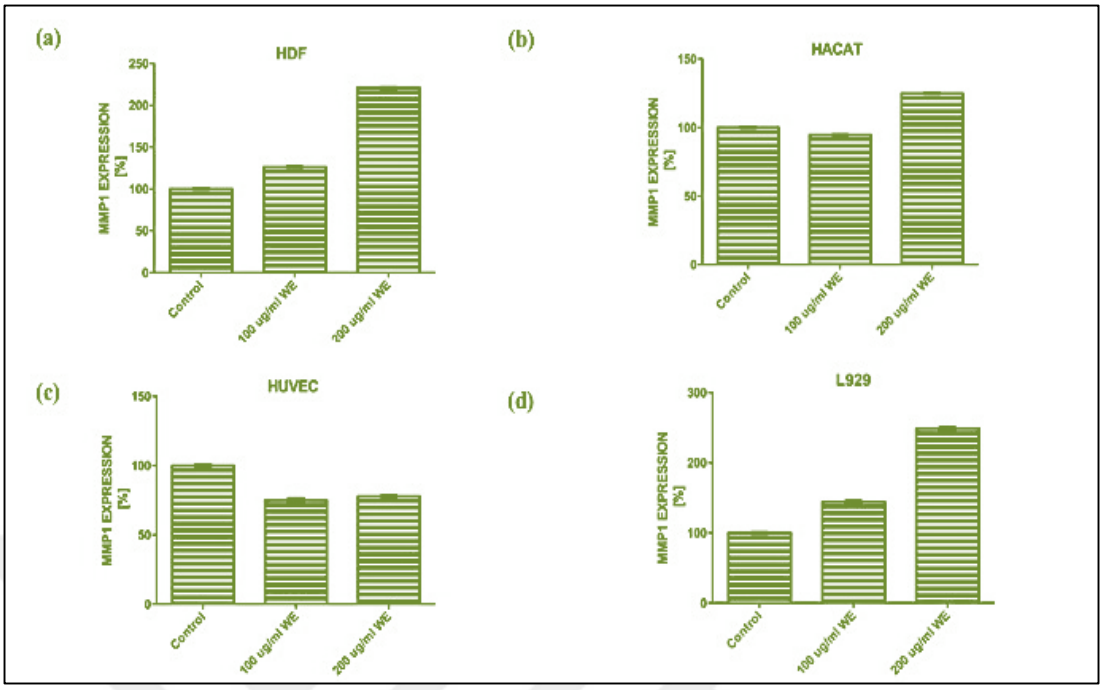


Figure 4.18. MMP1 expression levels of (a) HDF, (b) HACAT, (c) HUVEC and (d) L929 cells. Control was treated with only complete growth media. Datas were then analysed by two-tailed multiple t tests and demonstrated with Å} SEM, n=3, *P<0.05.

Upon exosome application on HDF and L929 cells, MMP1 expression was found 2 fold higher with respect to control in dose dependent manner. On the other hand, exosome treated HUVEC cells at every doses, resulted with almost 20 percent decrease in MMP1 expression level.

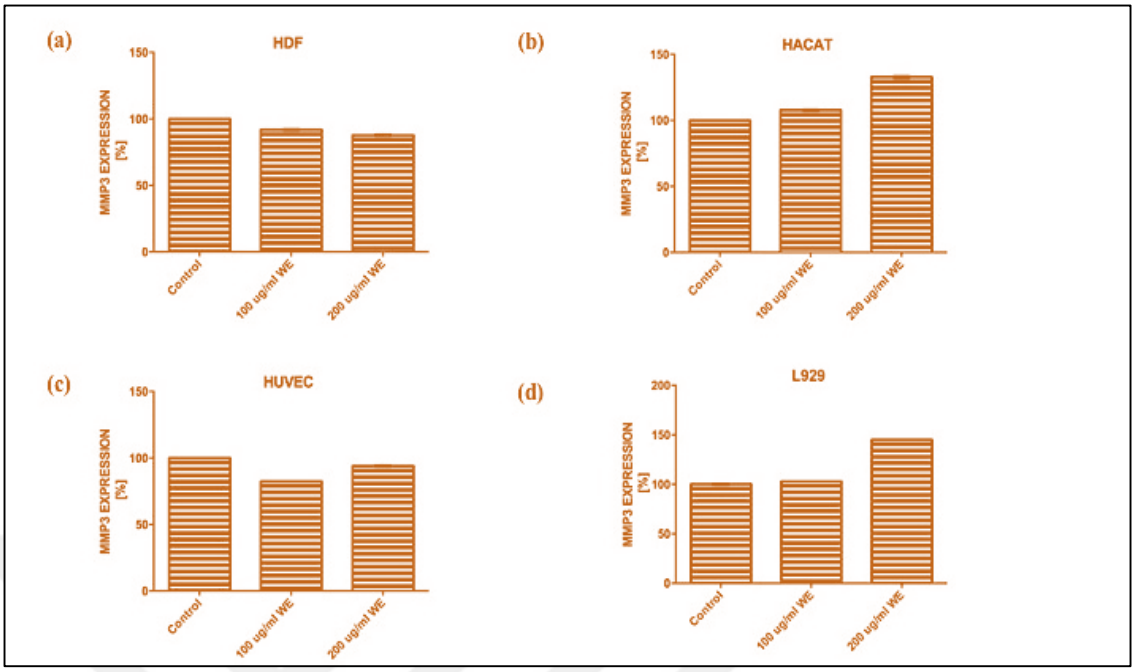


Figure 4.19. MMP3 expression levels of (a) HDF, (b) HACAT, (c) HUVEC and (d) L929 cells. Control was treated with only complete growth media. Datas were then analysed by two-tailed multiple t tests and demonstrated with Å} SEM, n=3, *P<0.05.

Upon exosome application on HACAT and L929 cells, MMP3 expression was found almost 1.4 fold higher with respect to control. On the other hand, exosome treated HUVEC and HDF cells displayed no significant change in MMP1 expression level.

4.10. RT-PCR GENE EXPRESSION ANALYSIS

To examine the potential effect on the mechanism for proliferative and migratory activity of wheat exosomes, HDF, HUVEC, HACAT and L929 cells were treated with 200 µg/ml of wheat exosomes for 24h and with RT-PCR method, wound healing related gene expression levels were assessed as shown below (Figure 4.10.1, Figure 4.10.2 and Figure 4.10.3).

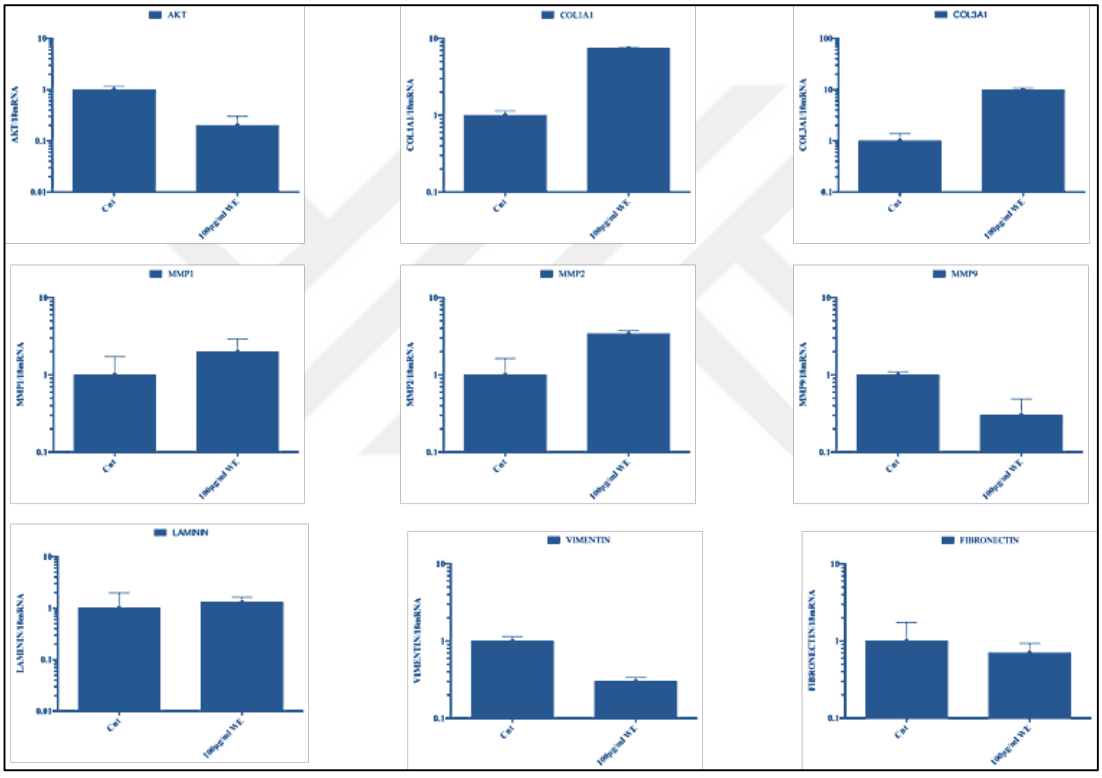


Figure 4.20. The mRNA expression levels of HDF cells after the treatment of 200 µg/ml of wheat exosomes for 24h, HDF cell mRNA levels were evaluated using qPCR, wound healing related gene mRNA levels were normalized against 18s RNA expression levels. Abbreviations: COL1A1: Collagen type I alpha 1, COL3A1: Collagen type III alpha 1, MMP2: Matrix metalloproteinase-2, MMP9: Matrix metalloproteinase-9

Results demonstrated that HDF cells after the treatment of 200 µg/ml of wheat exosomes resulted with around 7 fold increase in ColA1 and10 fold increase in Col3A1 mRNA expression levels respectively. On the other hand, Fibronectin and Vimentin, gene

expression levels were down regulated 3 and 7 fold respectively. On the other hand, Laminin gene expression levels were found to be 1.2 fold upregulated. Moreover, after the treatment of wheat exosome on HDF cells, matrix metalloproteinases, MMP1, and MMP2 gene expressions were resulted with 1.9, and 3.4 increase respectively. Lastly, MMP9 and AKT mRNA level was found to be downregulated 8 folds interestingly upon wheat exosome treatment.

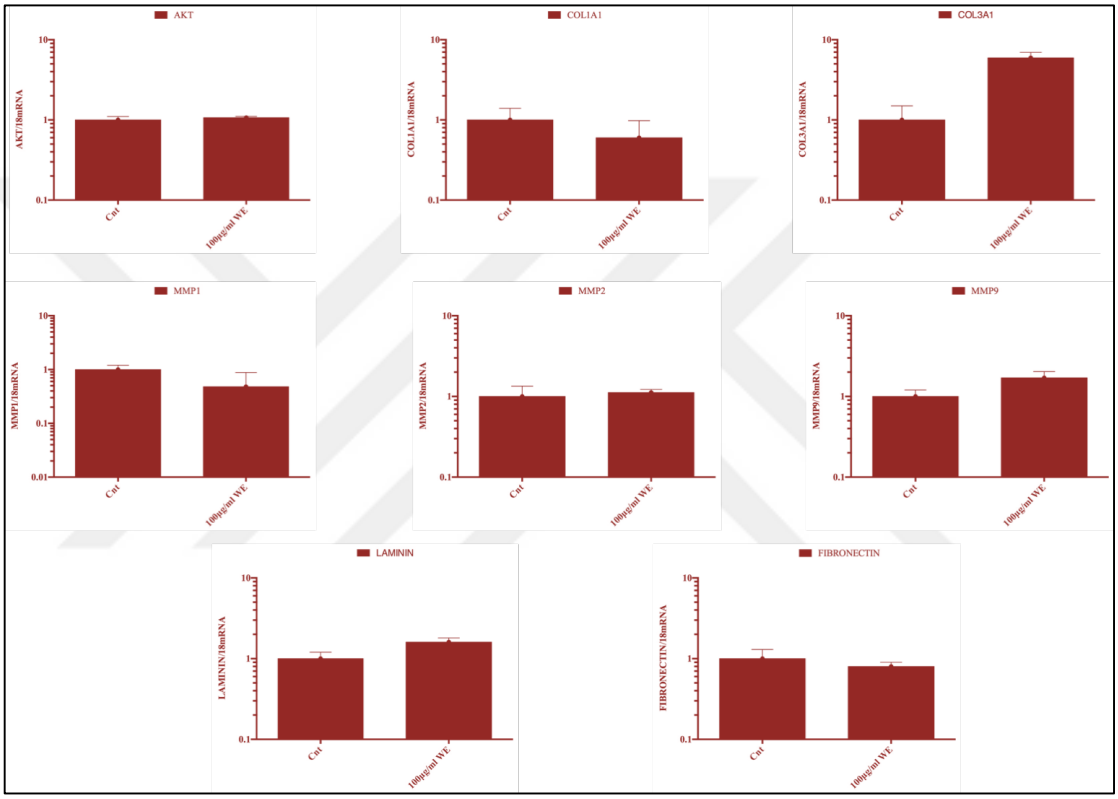


Figure 4.21. The mRNA expression levels of HACAT cells after the treatment of 200 µg/ml of wheat exosomes for 24h, HACAT cell mRNA levels were evaluated using qPCR, wound healing related gene mRNA levels were normalized against 18s RNA expression levels. Abbreviations: COL1A1: Collagen type I alpha 1, COL3A1: Collagen type III alpha 1, MMP2: Matrix metalloproteinase-2, MMP9: Matrix metalloproteinase-9

Results demonstrated that HACAT cells after the treatment of 200 µg/ml of wheat exosomes resulted with 6 fold increase in Col3A1 gene expression level whereas, ColA1 gene expression levels did not show any significant change. Moreover, there is no significant change in Fibronectin and Laminin mRNA level after the treatment of wheat exosomes. Moreover, MMP1 mRNA expression downregulated by 5 fold while MMP2 and MMP9 and AKT mRNA expression levels are upregulated by 2 folds upon wheat exosome treatment.

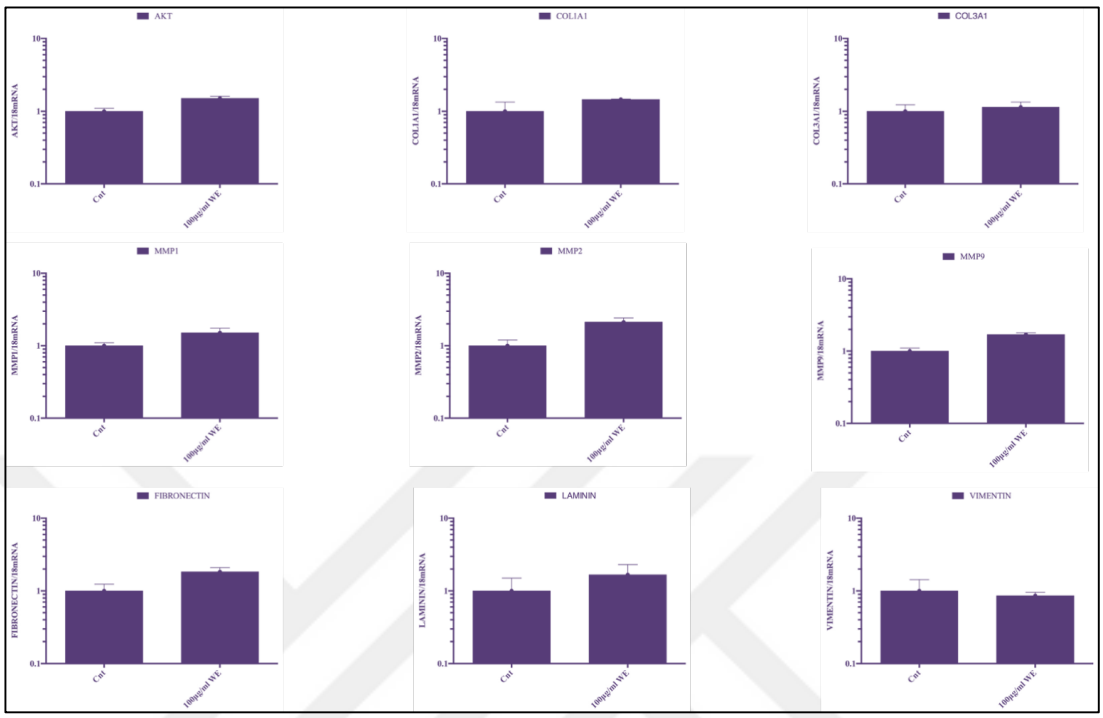


Figure 4.22. The mRNA expression levels of HUVEC cells after the treatment of 200 µg/ml of wheat exosomes for 24h, HUVEC cell mRNA levels were evaluated using qPCR, wound healing related gene mRNA levels were normalized against 18s RNA expression levels. Abbreviations: COL1A1: Collagen type I alpha 1, COL3A1: Collagen type III alpha 1, MMP2: Matrix metalloproteinase-2, MMP9: Matrix metalloproteinase-9

Results demonstrated that HUVEC cells after the treatment of 200 µg/ml of wheat exosomes resulted with 1-2 fold increase in ColA1 and Col3A1 gene expression levels. Similarly, Fibronectin and Laminin, which are known as extracellular matrix proteins, gene expression levels were upregulated 1.8 and 1.6 fold respectively. On the other hand, Vimentin gene expression levels were found to be not significantly downregulated. Moreover, after the treatment of wheat exosome on HUVEC cells, matrix metalloproteinases, MMP1,MMP2 and MMP9 gene expressions were resulted with 1.5, 2, and 1.7 fold increase respectively. Lastly, AKT mRNA level was upregulated 1.5 folds upon wheat exosome treatment.

4.11. GROWTH FACTOR ARRAY

Wound healing is regulated with the expression of series of cytokines and growth factors. That's why, growth factor array analysis was performed to check the effect of wheat exosomes on the growth factor and cytokine stimulation.

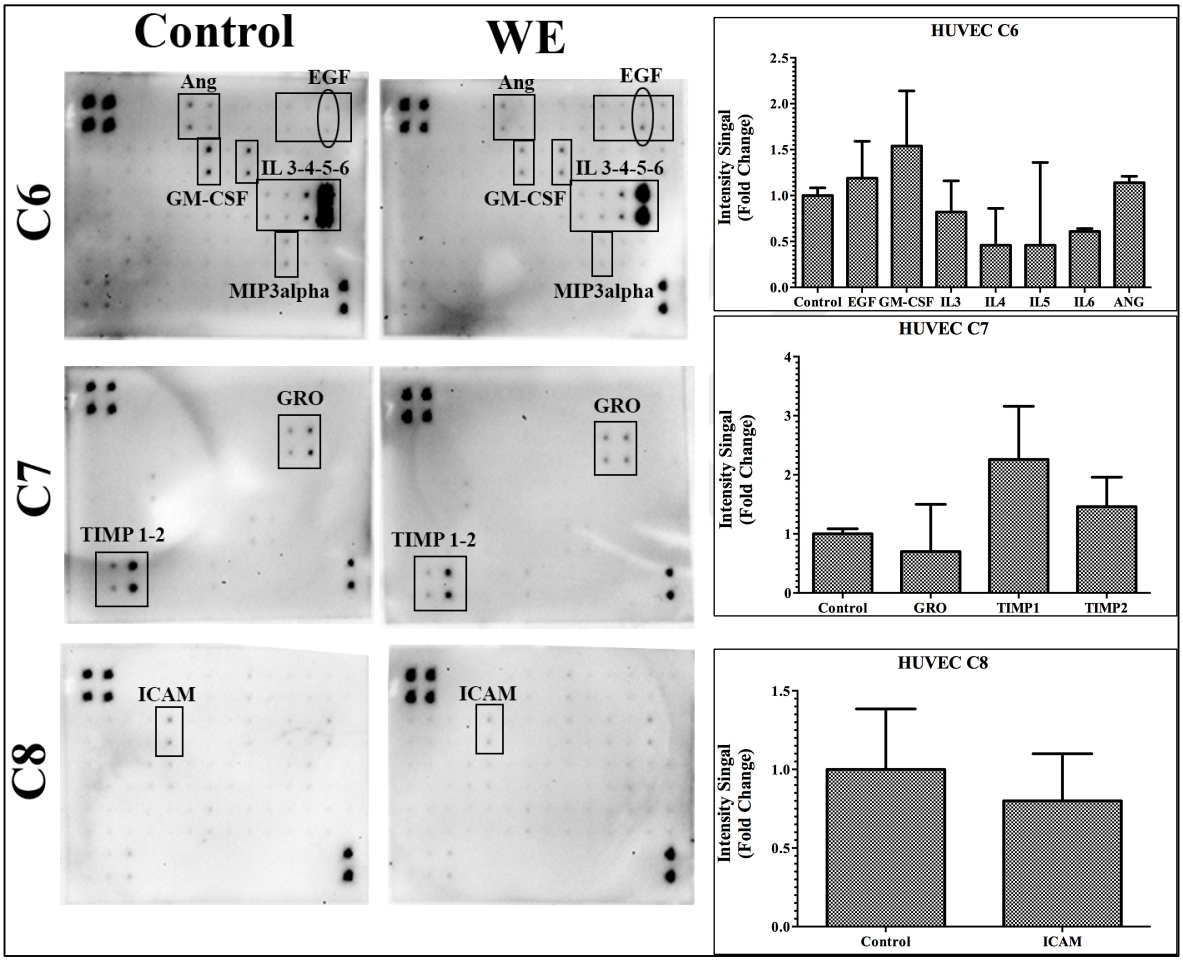


Figure 4.23. The Growth Factor analysis of HUVEC cells treated with 100 µg/ml of wheat exosomes, C6, C7 and C8 represents the array names

Exosome treated HUVEC cells resulted with the overexpression of EGF (Endothelial growth factor), VEGFR (Vascular endothelial growth factor), and IGFBP-1 (insulin-like growth factor-binding protein), GM-CSF (Granulocyte macrophage colony stimulating factor) and Angiogenin in HUVEC cells. Besides downregulation of pro inflammatory cytokines, IL3,4,5,6 were downregulated

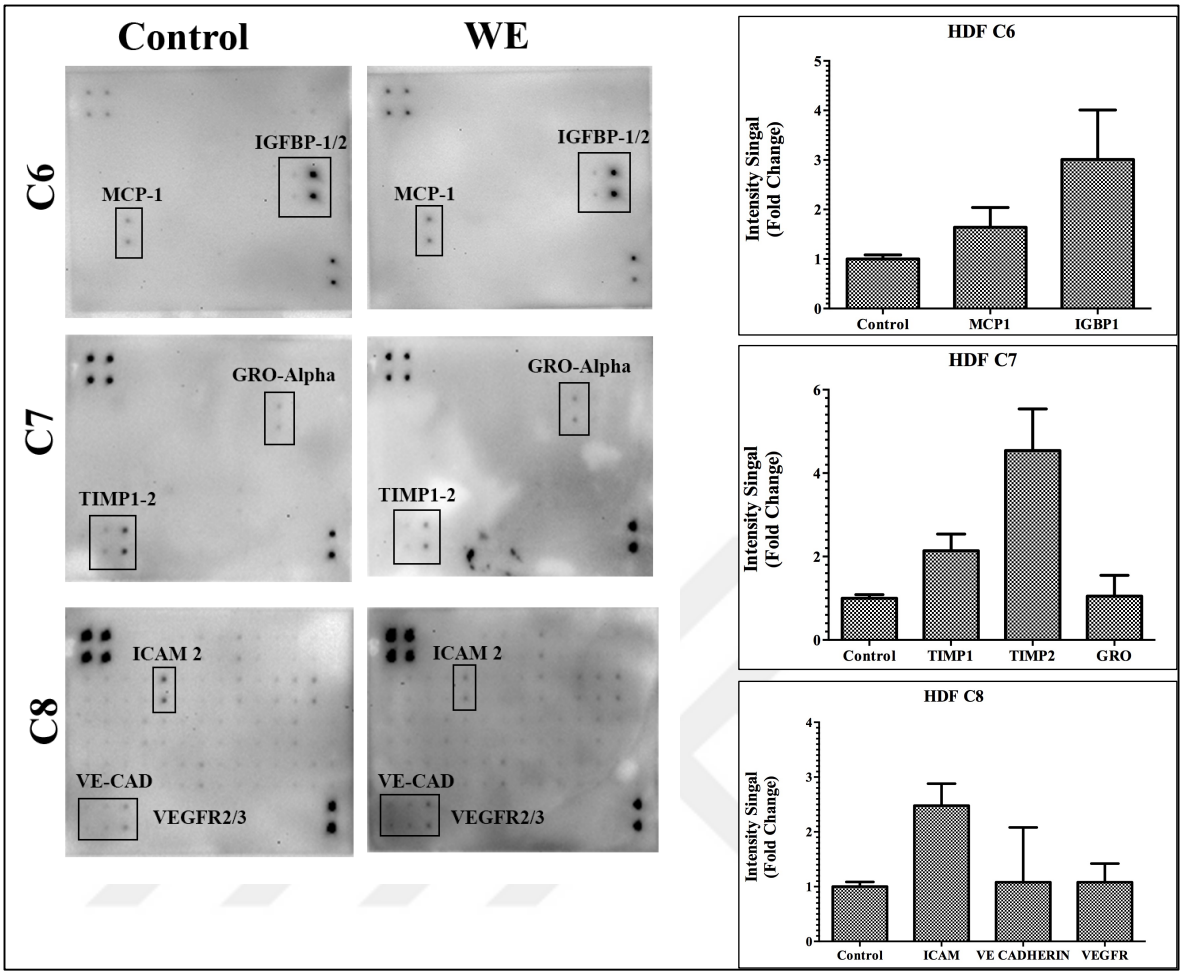


Figure 4.24. The Growth Factor analysis of HDF cells treated with 100 µg/ml of wheat exosomes, C6, C7 and C8 represents the array names

Wheat exoosme treated HDF cells resulted with the over expression of insulin-like growth factor-binding protein (IGFBP)-1, MCP1 (Monocyte chemoattractant), TIMP1&2, ICAM, VE Cadherin and VEGFR

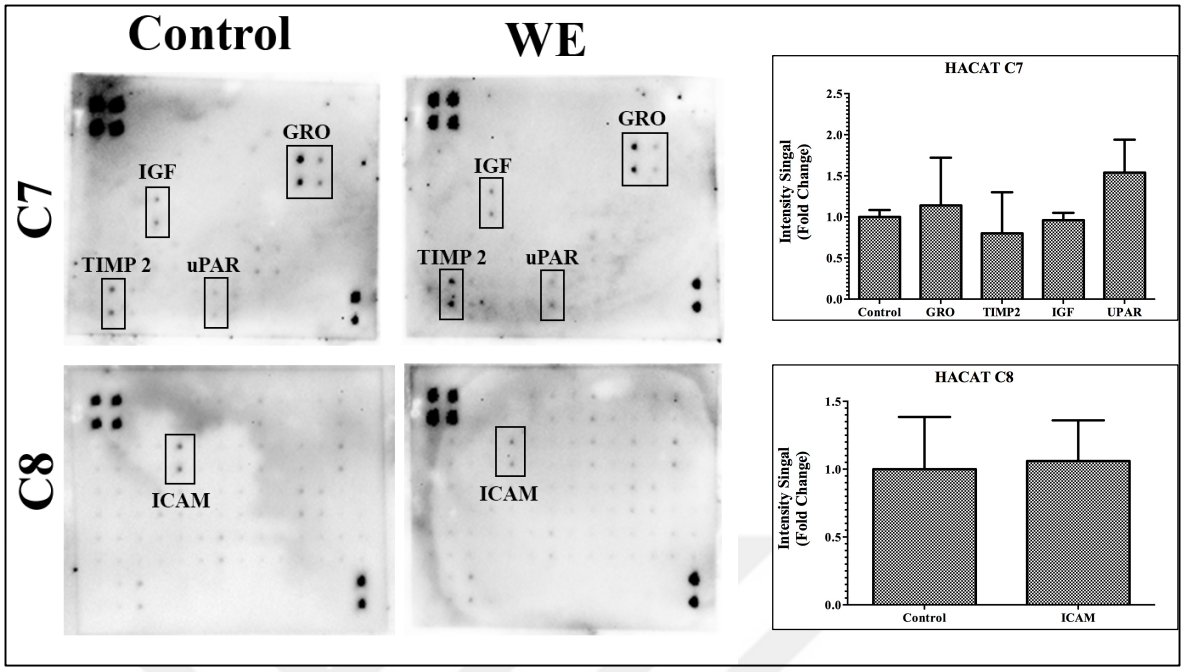


Figure 4.25. The Growth Factor analysis of HACAT cells treated with 100 µg/ml of wheat exosomes, C7 and C8 represents the array names

HDF, HUVEC and HACAT cells were treated with 200 µg/ml of wheat exosome for 24h and growth factors were evaluated with the growth factor array assay (Figure 4.23, 4.24 and 4.25). Results showed that wheat exosome treatment resulted with the overexpression of GRO (Growth factor oncogene), UPAR (Urakinase plasminogen), and ICAM (Intercellular adhesion molecule) in HACAT cells whereas downregulation of TIMP2 (Mmps inhibitor) were noted.

4.12. IMMUNOCYTOCHEMISTRY

In order to demonstrate the expression of collagen type 1 in HDF, HUVEC and HACAT cells treated with wheat exosomes, collagen type I was investigated through immunocytochemistry study. Specific antibodies against collagen type I were labeled for the cells. According to the immunocytochemistry results, in contrast with the control group following treatment of wheat exosomes, HDF, HUVEC and HACAT cells were moderately stained with collagen type 1 antibody (Figure 4.11.)

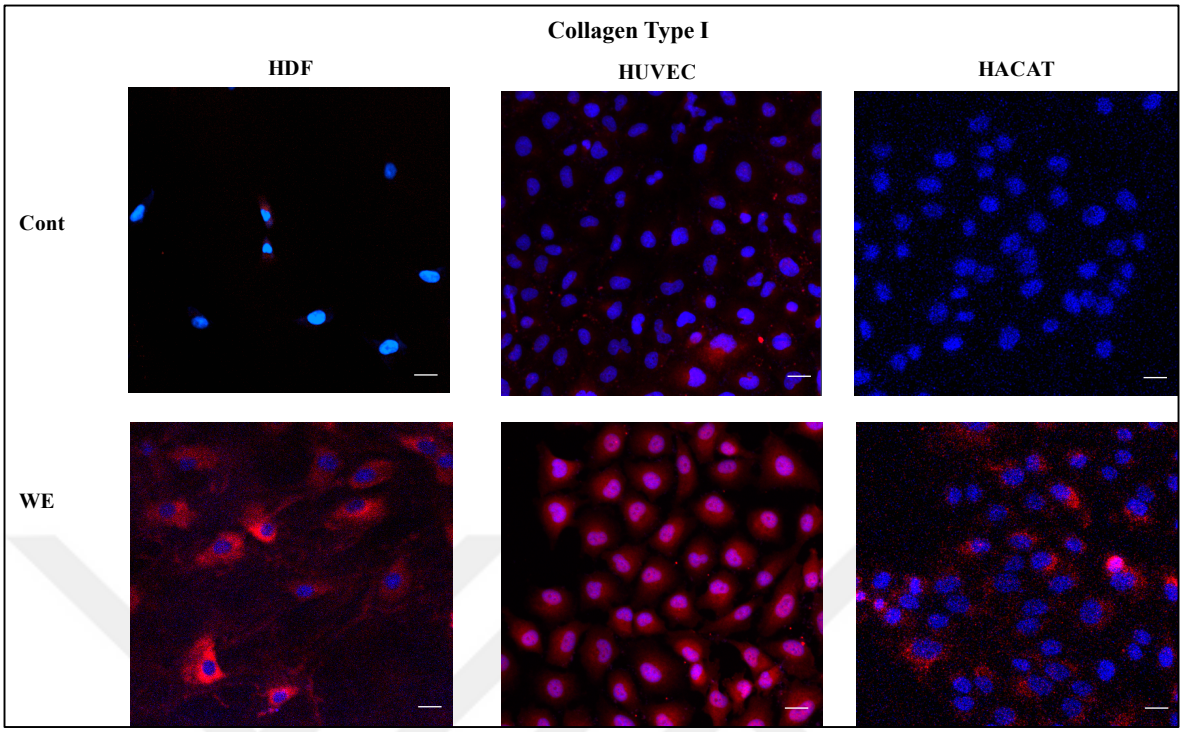


Figure 4.26. Immunocytochemistry location and quantification of Collagen Type 1 protein expression of HDF, HUVEC and HACAT cells analyzed via confocal microscopy. Control represents cells without any treatment, WE represents, cells treated with of 200 µg/ml of wheat exosomes for 24h.

4.13. CELLULAR UPTAKE

To check the cellular uptake of exosomes, cell nucleus, cell membrane and exosomes were dyed. Exosome uptake began about an hour after the application of exosomes. Exosomal uptake is high in HDF, HUVEC, and HACAT cells at 24 hours after the treatment (Figure 4.27).

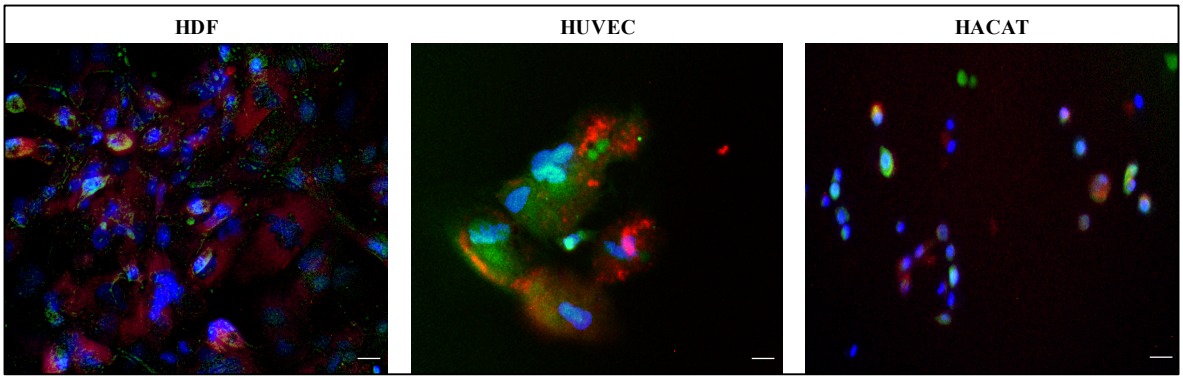


Figure 4.27. Wheat exosome uptake of HDF, HUVEC and HACAT cells. (20X magnification, scale bar at 20μm.

4.14. *IN VIVO* ANIMAL STUDY

4.14.1. Wound Contraction

In this study, we create a full-thickness excisional wound in animals then wounds were treated with different concentrations of exosomes such as in the 2nd group with a placebo cream, 3rd group with 500 μg/ml WE exosomes containing hydrogel, 4th group with 1 mg/ml of WE exosome containing hydrogel and 5th group with 2 mg/ml of WE exosome containing hydrogel for 15 days. After the completion of the treatments, photographs of wounds were taken every 5 days and the wound closure rate was shown as a as percentage of original wound area

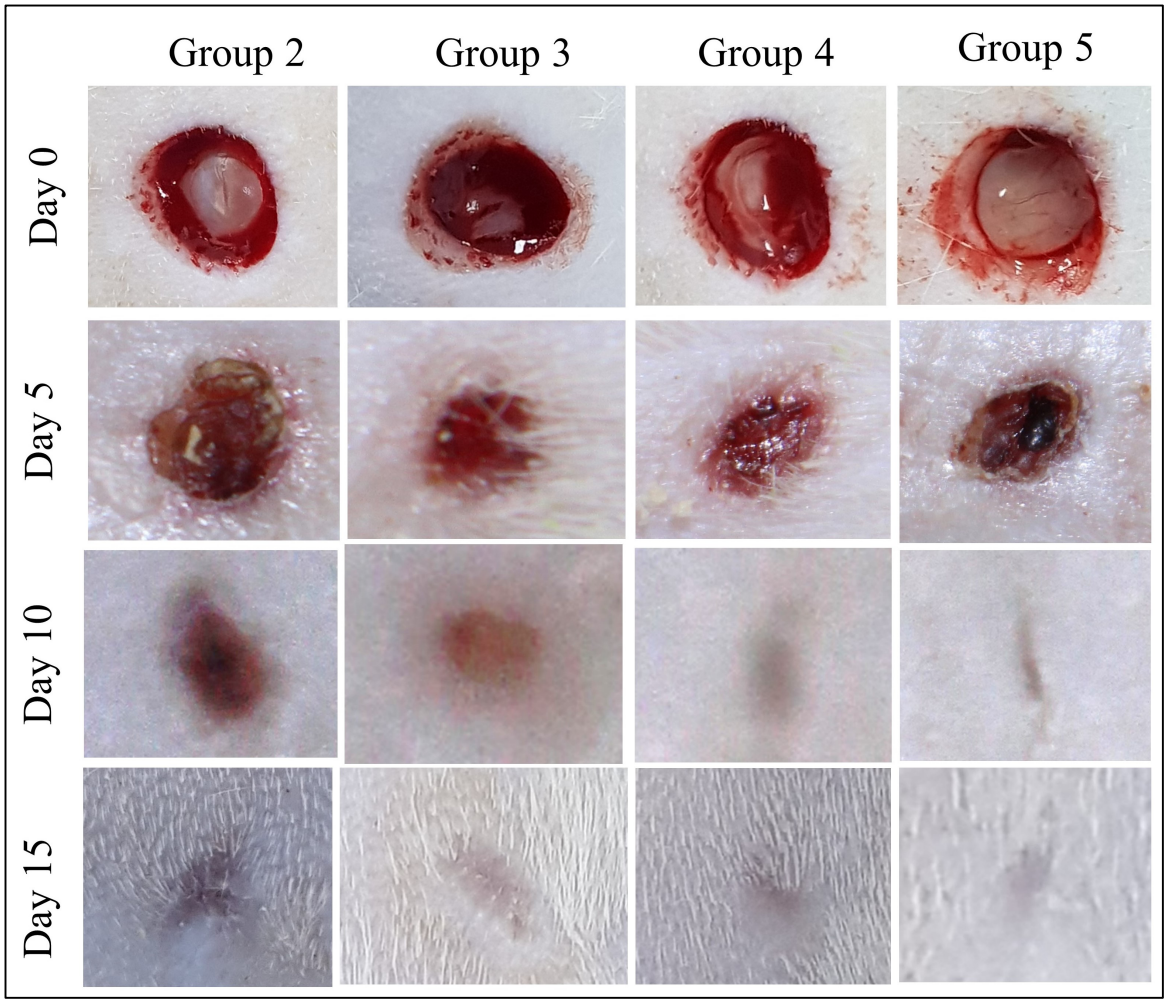


Figure 4.28. Macroscopic wound contraction measurement for 15 days in all the experimental groups. Group 2, Control rats represents a placebo cream, Group 3 represents 500 µg/ml exosome containing gel treated rats, Group 4 represents 1000 µg/ml exosome containing gel treated rats and Group 5 represents 2000 µg/ml exosome containing gel treated rats.

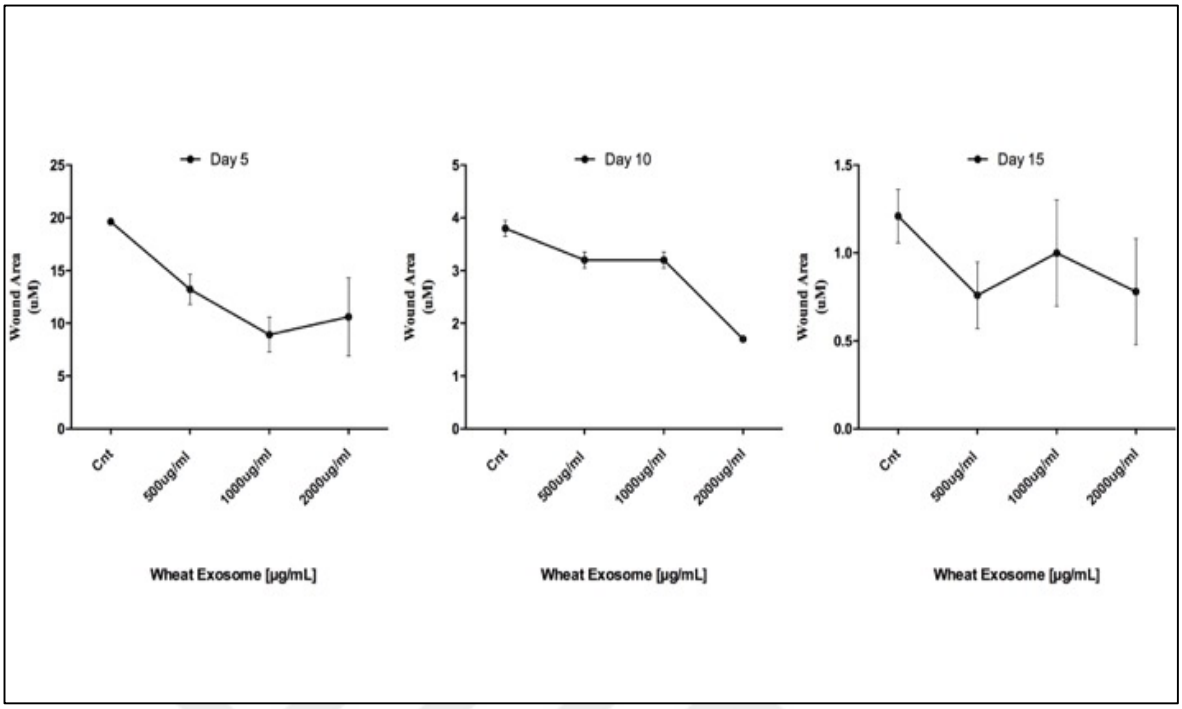


Figure 4.29. The measurement of the wound area at day 5, day 10 and day 15 post-wounding for each experimental group. Group 2, Control rats represents a placebo cream, Group 3 represents 500 µg/ml exosome containing gel treated rats, Group 4 represents 1000 µg/ml exosome containing gel treated rats and Group 5 represents 2000 µg/ml exosome containing gel treated rats.

The results revealed that the wound closure and contraction rate was maximum in group 5 which is 2000 µg/ml exosome treated rats (Figure 4.28.). The mean unhealed wound area was considerably lower and the total average wound area in exosome-treated wounds was considerably higher than in control wounds in dose-dependent manner ($p<0.05$).

4.14.2. Macroscopic and Microscopic Evaluation

During this study, no mortality was observed and all the rats survived until the end of the study. Compared with exosome groups, the wounds in the control group showed a greater degree of inflammation. In exosome-treated rats, the wound sites developed less exudate than the control wounds.

In the control group, filling the wound with granulation tissue to the skin level was substantially slower than in the exosome groups (Figure 4.28.).

4.14.3. Histopathological Examinations

For histopathological analysis, specimens of skin tissue were collected from each animal and fixed in formaldehyde. Histopathological outcomes of multiple variable cross sections from each experimental group (Control, Placebo group 2, 500 µg/ml gel containing exosome group 3, 1000 µg/ml gel containing exosome group 4 and 2000 µg/ml gel containing exosome which is group 5 were analyzed using routine H&E stains.

The sections were analyzed in terms of morphology, intensity of the fibroblast, granulation tissue formation and inflammatory cell infiltration.The histopathology of the skin tissue corroborated with the macroscopic findings. The healed wound area is covered with a dense epithelial layer in all rats. Cellular infiltration of usually mononuclear leukocyte form was seen (Figure 4.30.). Dose-related reduction in Fibroblast and inflammatory cell count was also detected.

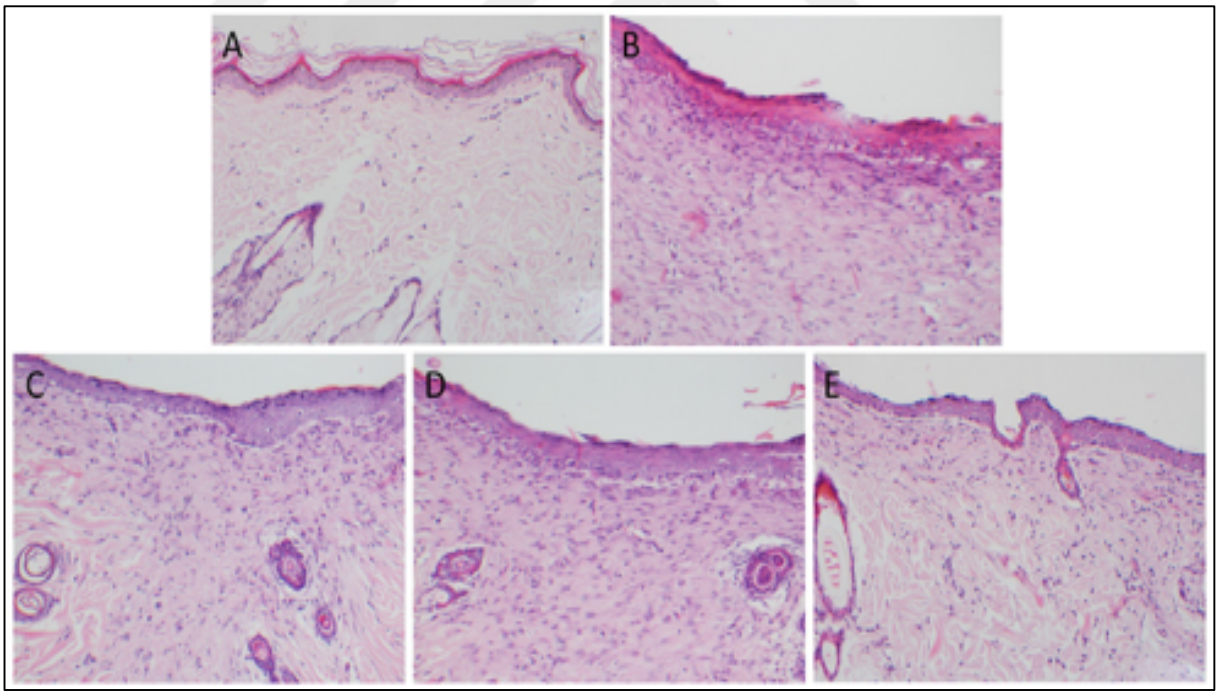


Figure 4.30. Histopathological analyses of H&E (Magnification=200X) stainings of wound tissue sections. (a).Normal appearance of the skin in the epidermis and dermis.; (b) significant increase in the number of ulcers, fibroblasts and inflammatory cells.; (c-e): dose-related reduction in Fibroblast and inflammatory cell count.

5. DISCUSSION

Several intrinsic and extrinsic factors as well as stimuli from cytokines and participating growth factors provide skin integrity in order to maintain the longevity of the skin in the event of any tissue damage [7]. Although there is much evidence supporting the existence of exosome-like nanoparticles in plants their function in interspecies communication still needs much detailed study.

The effect of plant exosomes on the oncogenic transformation and inflammation has been shown previously. In fact, due to the impact of food absorption on homeostasis of the organs and the body, edible plants such as grape, grapefruit, ginger, and lemon are have been the major focus of exosome studies. Exosomes play a key role in the maintenance of intercellular communication. Despite increasing number of studies demonstrating therapeutic effect of plant exosomes in various intestinal diseaases and cancer, their potential impact on wound healing is yet to be examined. [17]. In the meantime, a guideline (Minimal Information for Studies of Extracellular Vesicles-MISEV) to distinguish distinct types of extrcellular vesicles, including microvesicles, apoptotic bodies, and exosomes is established with the aim of defining purer populations isolated nanovsicles as exosomes [88].

The purpose of this study was to investigate the effect of nanoparticles called exosomes from wheat (grass) on wound healing using both *in vitro* and *in vivo* approaches.

Experimental procedure includes isolation and characterization of wheat exosomes from wheat grass by using centrifugation and ATPS method with Polyethylene Glycol (PEG)-Dextran (DEX) two phase gradient. According to the results, the isolation of relatively homogenous and stable exosome-like extracellular vesicles from wheat grass juice was isolated succesfully as shown in Fig. 4.1. and Fig 4.2. Exosomes should be evaluated, at least 3 positive exosomal surface markers should be identified, and either electron microscopy or NTA should be observed, according to the MISEV guidelines.Therefore, wheat derived exosomes were isolated and characterized by nanoparticle tracking analysis, scanning electron microscopy (SEM), dynamic light scattering based on their size and morphology, and CD surface markers, CD9, CD63 and HSP70 were investigated by flow cytometry. Since their contents are susceptible to modification, one of the indicatives of their carriers structures is known as evident size and morphology of the exosomes. As shown in Fig 4.1,

exosomes are shown to have round, spherical shapes with sizes varying from about 100 nm with a negatively charged; this is also consistent with the literature. Positive surface expression of tetraspanins (CD9, CD63) and heat-shock proteins (HSP 70) was demonstrated by wheat exosomes, which are calculated as exosome CD surface antigens. Here, polyethylene glycol was used to enrich the concentration and isolation efficiency [89]. Dextran has surface properties that allows selective separation by specifically attarcting exosomes and repelling/excluding remainder extracellular vesicles. the exosomes and separate them from other residues [90]. Two phase separation allows exosomes to remain at the bottom DEX phase while contaminant and proteins remain at the top PEG phase [90]. This method provides cheaper and easier way for isolation of extracellular vesicles such as exosomes for various purposes [69]. As per this protocol, following the removal of the PEG phase, the DEX phase was filtered through 0.22 micron pore size filters to remove any contaminant that might remained. Concentration of the freshly isolated exosomes were measured by Lowry Assay. Furthermore, uptake analysis revealed that exosomes and dermal cells communicate, as they become increasingly visible within the cells 24 hours after application.

In acute wounds, after the inflammatory process, the proliferative process is initiated. To close the gap, this stage involves both the migration and proliferation of cells. This step is essential for proper healing and is known as a rate-limiting healing factor. Based on our findings from the WST1 studies application of wheat exosomed to the cell lines analyzed in this study resulted in a significant increase in cell proliferation (40-50 %) in a dose dependent manner. In order to evaluate the cytotoxicity of wheat exosomes and their influence on cell proliferation cell viability assay WST-1 was conducted for 3 days manitoring cell viability at 24h, 48h and 72h timepoints at 4 different concentrations ranging in 30-200 µg/ml in the HDF, HACAT, HUVEC, and L929 cell lines. The working principle of WST-1 depends on the cleavage of tetrazolium salt by cellular mitochondrial dehydrogenases to form formazan [91]. An increase in cell viability increases the number of mitochondrial dehydrogenases therefore an intense color forms. By this method, cell viability is measured by colorimetric means. 6 different concentrations were used as 30 µg/ml, 50 µg/ml, 75 µg/ml 100 µg/ml, and 200 µg/ml and tested for 24h, 48h and 72h. Results are given in Figure 4.3.1 to 4.3.4, as seen, no cellular toxicity was detected in any of the concentrations after treatment to the HDF, HACAT, HUVEC and L929 cells. All dosages have shown to be significantly

increasing the cell viability, therefore the cell number as well. In conclusion, wheat exosomes displpay no cytotoxic effects and presemts a potential to aument cell viability.

Migration is also known as another rate-limiting factor of the wound healing process and, therefore, it is essential for the growth of all living cells (organisims) as well as prevention of inflammation, and triggering of immune response [92].

Scratch assay is recognized as a gold standard in assessing wound closure rate. To demonstrate the potential effect of wheat exosomes on cell migration scratch assay was conducted. In this assay a gap created by scratching the plate is monitored over a timecourse whereby efficiency of gap closure in terms of cell migratory patterns is considered as a representation of wound closure. The fact that this is an easy and low-cost assay which mimics *in vivo* wound closure makes it highly used [93]. In this assay, optimal concentration of wheat exosomes (200 µg/ml of wheat exosomes) which was found at WST-1 assay is chosen and used under 2% serum supplemented conditions, and images were obtained at the day of the wound as day0, day1 and day2. To exclude any contributory effect due to the presence of growth factors introduced by the serum, potential effects of wheat exosomes in wound closure were measured under either low or zero serum supplied conditions. As a consequence of the scratch assay, while control group cells moderately closed the scratched area after 48 h, HDF, HUVEC, HACAT and L929 cells treated with wheat exosome were reported to migrate faster, which was around 50 percent higher wound closure rate after 24 hours of incubation.

Angiogenesis is the main component of the wound healing process, and when vascularization and endothelial cell activation take place, nutrients and waste materials can be properly transferred between the cells. Tube formation assay includes coating the surface with basement membrane to mimic *in vivo* conditions, then seed the cells over and treat the cells. Formation of branches of endothelial cells start to form within an hour, if the treatment induces angiogenesis [94]. Single dose of 200 µg/ml of wheat exosomes were used on HUVECs and images are obtained 16 hours post treatment. All in all to observe angiogenesis of the endothelial cell line, we conducted a matrigel tube formation assay. Strikingly, results of the tube formation assay conducted in presence or absence of wheat exosomes indicate that these nanovesicles exert a strong stimulatory effect on cellular proliferation as well as tube formation on human endothelial cells.

To evaluate the anti-inflammatory abilities of wheat exosomes, hydrogen peroxide (H2O2) was used to induce oxidative stress on HDF, HUVEC HACAT and L929 cells. Hydrogen peroxide generates reactive oxygen species (ROS) in fibroblast cells therefore causing cell viability to decrease [93]. Cells were either treated with 1 mM of H2O2, or with 100 µg/ml wheat exosome or with both 1 mM H2O2 and 100 µg/ml wheat exosome for 24 hours. WST-1 assay was conducted after 24h. Results are given in Figure 4.6. Here, only cell culture media used sample were taken as positive control and taken as 100% viable. It is seen that when hydrogen peroxide only was used, high cellular toxicity was deected whereas when hydrogen peroxide and wheat exosome were administrated at the same time onto healthy cell lines to start a inflmmation reaction, results showed similarity with positive control, meaning that wheat exosomes had effect on pulling inflammation back to the control level. Here it is concluded that wheat exosomes have the ability protect the fibroblast cells against inflammation made by reaktive oxygen species (ROS).

It has been shown to have anti-cancer, anti-ulcer, and anti-oxidant activities consisting of high chlorophyll content, essential vitamins, nutrients, essential enzymes, amino acids, and dietary wheat fibers[75]. Similarly, we suggested that the active compounds of wheat grass juice may be linked to their anti-apoptotic activity, as verified by the staining of HDF, HACAT, HUVEC and L929 cells with Annexin V following the treatment of wheat exosomes which was resulted with a decrease of apoptotic cell number.

Because of the common phenomenon of cell cycle arrest right before apoptosis, the impact of wheat grass-derived nanoparticles on cell cycle distribution was also investigated. After wheat exosome treatment, the cell cycle distribution of HDF, HACAT, HUVEC, L929 cells was changed as more cells were in the G2-M phase than the control cells as a result of increased proliferation. In addition, HACAT and HUVEC cells were prepared to divide in the S phase after wheat exosome treatment, but HDF and HUVEC cell numbers were higher than control cells in the G2-M phase. Exosome application, as predicted, resulted in changes in cell cycle distributions of each cell line, as well as an increase in proliferation rate. As a result of increased proliferation, the cell cycle distribution of all dermal cells changes after exosome therapy, with more cells in the G2-M cycle than control cells. These cells be in either the S phase, where they intend to synthesize DNA, or the G2-M phase, where they divide. However among all cells, exosomes have also been found not to induce so significant

change in the distribution of the cell cycle. The proliferative effect of the exosomes of wheat must therefore be held responsible for their anti-apoptotic properties.

Components of the extracellular matrix such as collagen, fibronectin, laminin and vimentin play a significant role in wound healing [95]. The healing process involves the replacement of the type I collagen matrix with the previously formed fibrin clot. Additionally, during skin healing, collagen provided by fibroblasts regulates both cell adhesion and migration [48]. Interaction between cell surface proteins with ECM proteins such as laminin, vimentin, collagen and fibronectin is the key factor causing cellular migration [96]. Furthermore, actin and microtubules collaborate directly with vimentin for invasive cell elongation [97]. These ECM proteins control cellular behaviour directly or indirectly and are the main factors for wound repair. As such, our data generally validate that wheat exosome increases type I and type 3 collagen mRNA level results in collagen synthesis, and increases fibroblast cell proliferation and migration during the wound healing phase. Moreover, immunocytochemistry and ELISA results are also validated that wheat exosome has effect on collagen synthesis significantly. Fibronectin, Vimentin and Laminin gene expression levels did not change significantly after the treatment of wheat exosomes on four different cell lines. In order to provide a suitable environment for cell migration, metalloproteinases like MMP-1, MMP-2, MMP-3, and MMP9 degrade temporary wound matrix and extracellular matrix proteins [98]. The migratory activity may have been caused by an increase in MMP-1 and MMP-3 in HDF and HACAT cells treated with wheat exosomes. In other words, wheat exosome treated cells showed upregulation of MMPs which have role in angiogenesis, one of the important stage of wound healing. MMP-1 is also strongly expressed in ECM remodeling on fibroblasts [99]. Furthermore while MMP-1 stimulates keratinocytes to migrate to fibrillary collagen, MMP-3 influences wound contraction and both are synthesized at the edge of the wound by keratinocytes. Increased protein levels of MMP-1 at the ELISA study in both HACAT and HDF could therefore also be induced by these cells' migratory activity. Moreover, VEGF, which is also known as vascular endothelial growth factor, important in vascularization in wound healing, was significantly upregulated after the treatment of wheat exosomes on HDF, HUVEC and L929 cells. Moreover, wheat exosome treated HACAT, HUVEC and L929 cells resulted with slightly increase in dose dependent manner VEGF expression levels whereas 200 μg/mL of wheat exosome treated HDF cells showed significantly, 50 percent, increase in VEGF expression

due to the ELISA results. Another explanation for enhanced cell proliferation and levels of migration may be due to higher levels of expression of AKT mRNA levels as well as levels of AKT protein expression. AKT is indeed one of the core PI3K pathway mediators that regulates cell proliferation, migration, invasion, and angiogenesis through MMPs and VEGF. AKT mRNA levels caused a 2-fold increase in HACAT and HUVEC cells, while in HDF cells, a serious decrease in AKT mRNA levels was observed. Wheat exosomes has been found to reduce AKT levels in human fibroblasts, suggesting a species-specific wheat exosome response. This may not be a drawback for ulcer management since elevated levels of MMPs and degraded matrix proteins are usually accompanied by chronic wounds [98]. The exosome treatment of wheat resulted in an inarguable impact on the gene expressions of extracellular matrix proteins, Akt and MMPs, but to determine their protein levels and functions, further work is required with multiple cell lines and animal models.

Even though exact function of TGF proteins in wound healing is not properly known and there are some questionable reports in the literature, it has been reported that TGF-β1 and TGF-β3 increase fibroblast and keratinocyte cell migration and proliferation, granulation tissue formation, extracellular matrix development, production of growth factor secretion, angiogenesis and tissue remodeling [100-104]. In line with these studies, TGFB1 ELISA results showed that, the treatment of wheat exosomes with the different doses did not significantly affect the TGFB expression on HACAT and HUVEC cells. On the other hand, after the treatment of 100 and 200 µg/mL of wheat exosome, TGFB expression levels were increased in response to the treatment with wheat exosomes at 100 and 200 µg/ml concentration in HDF and L929 cells.

A range of cytokines and growth factors secreted by different cell types have role in wound healing stages such as inflammation, proliferation and remodelling. Studies reported that deficiency of one or more cytokine could resulted with the delay in chronic wounds. The analysis of the growth factor profiles of wheat exosome-treated fibroblasts, endothelial cells, and keratinocytes can provide insight into the possible mechanisms and pathways by which wheat exosome aids wound healing. Results showed that wheat exosome treatment on HDF cells resulted with the overexpression of IGFBP1 which is known to trigger IGF1 induced cell migration and proliferation. Moreover, the intensity signal of MCP1 was higher in WE treated cells which is a chemokine have role in fibroblast migration and also regulate the macrophages to the wound area to clear the wound as it shown in literature [122]. The

increase of intercellular adhesion molecules, ICAM and VE-Cadherin was also upregulated [122]. TIMP1&2 which are known as Tissue inhibitor of metalloproteinases, anti-inflammatory cytokine, *In vitro,* TIMP-2 has been shown to both slow and speed up cell migration during healing. TIMP2 is also expressed in wound fibroblast cells, which are mostly adjacent to blood vessels [121] was also found to be higher intensity signal in WE treated HDF. Additionally, exosome treated HUVEC cells resulted with the overexpression of EGF (Endothelial growth factor) which triggers signaling pathways about cell proliferation and migration, VEGFR (Vascular endothelial growth factor) which have role in cell motility and angiogenesis, and IGFBP-1 (insulin-like growth factor-binding protein), GM-CSF (Granulocyte macrophage colony stimulating factor) which have role in angiogenesis in ischemic diseases and Angiogenin which have role in angiogenesis in HUVEC cells. Besides downregulation of pro inflammatory cytokines, IL3,4,5,6 were downregulated. Wheat exosome treated HACAT cells resulted with the over expression of GRO (Growth factor oncogene) which plays a signficant role in the activation of neutrophils after a tissue damage or pathogen entry to the wound and UPAR (Urakinase plasminogen) which have an important role in promoting cell migration in cutaneous wounds.

For an *in vivo* model, we decided to use full thickness skin wounds in rats. (We chose to use a full thickness skin model where through biopsy punch, we could generate standardized wounds more easily and have controls and samples in the same animal, thus minimizing inter-animal variability). Interestingly, our findings demonstrated that when compared with exosome groups, the wounds in the control group showed a higher degree of inflammation and the wound sites developed less exudate than the control wounds in exosome treated rats. Furthermore, the filling of the wound to skin level with granulation tissue was substantially slower in the control group than in the exosome groups. (Figure 4.12.1.). Moreover, the wound closure and contraction was also maximum in the gel containing the highest concentration exosome which is 2 mg/ml exosome containing gel. Macroscopic assessments revealed that as predicted, the closure rate of full thickness wounds was increased upon treatment with gel formulation containing the highest concentration of wheat exosomes. The macroscopic point made the histopathology of the skin tissue. Histopathological examinations were also validated the presence of inflammatory cells, the healed wound surfaces were covered with a thick epithelial layer, high amount of fibroblasts, mononuclear leukocyte type cellular infiltration, well-organized and abundant collagen secretion in wheat

exosome containing gel treated groups in comparison with control rats, which shows increasing and promoting wound healing promoting properties of wheat exosomes. These findings are directly consistent with *in vitro* results, supporting the anti-inflammatory activity of wheat exosomes, and also stimulating effects on the proliferation and migration of fibroblasts, keratinocytes, endothelial cells and supporting the development of the extracellular matrix.

In order to increase the absorption rate of wheat exosoms through the cell membrane, the formulation base could be modified from hydrogel to cream polymers such as, liposome or polymersome could be used in the formulation. Biodegradable wound dressings which continuously release exosomes for a long time, apart from everyday applied products, may be a future therapeutic option to minimize psychological stress and facilitate healing. The next milestone of this project will be clinical trials conducted with different acute and chronic wounds to validate the study results, after deciding optimum concentration, formulation, treatment amount and intensity.

Although plant-based studies have recently escalated wheat exosomes and their effect on wound healing have not been investigated to date. Therefore, this was the first study shown that wheat exosome has the wound healing properties using both *in vitro* and *in vivo* approaches.

6. CONCLUSIONS

Lastly, wheat-derived exosomes improve wound healing by boosting collagen formation, matrix metalloproteinases, and different growth factors that play a role in the wound healing process. These exosomes, in particular, trigger the proliferative and migratory capabilities of dermal cells and boost the angiogenic characteristics of endothelial cells via transitory overexpression of MMPs and VEGF. Enhanced amounts of ECM proteins after exosome therapy can also be ascribed to increased levels of migratory characteristics of keratinocytes and fibroblasts. According to the results, wheat exosomes stimulate cutaneous cell proliferation, speed up wound healing, improve angiogenesis, and exhibit anti-scar capabilities. These findings suggest that wheat exosomes might be useful in wound healing.

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APPENDIX A: ETHICAL APPROVAL FORM

Evrak Tarih ve Sayısı: 16/03/2020-385045

T.C.

FIRAT ÜNİVERSİTESİ REKTÖRLÜĞÜ

Hayvan Deneyleri Yerel Etik Kurulu

Sayı :97132852/604.01.02/
Konu :Prof. Dr. Kazim ŞAHİN' nin projesi hk

Sayın: Prof. Dr. Kazim ŞAHİN

İlgi :06/03/2020 tarih ve 383202 sayılı yazı

"Buğday eksozomlarının yara iyileşmesi üzerine etkisi" başlıklı araştırma projeniz Etik Kurulumuzda görüşülmüş olup Etik Kurulu Kararı örneği ekte gönderilmiştir.

Bilgilerinizi rica ederim.

e-imzalıdır.
Prof. Dr. Mustafa İSSİ
Kurul Başkanı

EK :
Etik Kurul kararı

Evrak Tarih ve Sayısı: 11/03/2020-384971

T.C.
FIRAT ÜNİVERSİTESİ
HAYVAN DENEYLERİ YEREL ETİK KURULU
KARARLARI

Oturum Tarihi	Oturum Saati	Oturum Sayısı
11/03/2020	14:00	2020/04

Hayvan Deneyleri Yerel Etik Kurulu 11/03/2020 tarihinde saat 14:00'da Kurul Başkanı Prof. Dr. Mustafa İSSİ başkanlığında, aşağıda imzaları bulunan kurul üyelerinin katılımlarıyla toplanarak gündemdeki konuları görüşmüş ve aşağıdaki kararları almıştır.

"Buğday eksozomlarının yara iyileşmesi üzerine etkisi" başlıklı araştırma projenizde ***35 Adet Wistar Albino veya Spraque-Dawley Sıçan*** kullanılacağı ve hayvanlar üzerinde yapılacak girişimlerde hayvan kullanım etiği ilkelerine uyulacağı tarafınızdan beyan edilmiştir. Bu çerçevede aşağıda ismi bulunan araştırmacılara ait projenin "Fırat Üniversitesi Hayvan Deneyleri Yerel Etik Kurul Yönergesi" gereği altı ay aralıklarla Hayvan Deneyleri Yerel Etik Kuruluna bilgi verilmesi şartıyla uygun olduğuna oybirliği ile karar verilmiştir.

1. Prof. Dr. Kazım ŞAHİN
2. Polen KOÇAK
3. Prof. Dr. Ali Said DURMUŞ
4. Doç. Dr. Cemal ORHAN
5. Prof. Dr. Fikrettin ŞAHİN
6. Prof. Dr. İbrahim H. ÖZERCAN

Kurul Üyeleri:
Prof. Dr. Mustafa İSSİ
Prof. Dr. Gülsüm ÖKSÜZTEPE
Prof. Dr. Sinan CANPOLAT
Prof. Dr. Asiye BAŞUSTA (Bulunmadı)
Doç. Dr. Serkan DÜNDAR
Doç. Dr. Burcu GÜL BAYKALIR
Dr. Öğr. Üyesi Esef BOLAT
Dr. Öğr. Üyesi Özgür BULMUŞ (Bulunmadı)
Vet. Hek. Ebru GÖKDERE
Onur UYGUR
Murat DAĞHAN (Bulunmadı)

Başkan
E-İmzalıdır
Prof. Dr. Mustafa İSSİ
Kurul Başkanı

Prof. Dr. Gülsüm ÖKSÜZTEPE

Prof. Dr. Sinan CANPOLAT

T.C.
FIRAT ÜNİVERSİTESİ
HAYVAN DENEYLERİ YEREL ETİK KURULU
KARARLARI

Oturum Tarihi	Oturum Saati	Oturum Sayısı
11/03/2020	14:00	2020/04

Onur UYGUR