UNIVERSITI PUTRA MALAYSIA

ROS STATUS OF NORMAL AND DIABETIC MURINE FIBROBLAST-TREATED WITH GALLIC ACID LOADEDGRAPHENE OXIDE (GAGO) NANO FORMULATION

NUREEN ALIA BINTI ABDUL RAHMAN

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NUREEN ALIA BINTI ABDUL RAHMAN

A PROJECT PAPER SUBMITTED AS PARTIAL REQUIREMENT FOR THE DEGREE OF BACHELOR SCIENCES (BIOMEDICAL SCIENCE)

DEPARTMENT OF BIOMEDICAL SCIENCES
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UNIVERSITI PUTRA MALAYSIA
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ABSTRACT

ROS Status of Normal and Diabetic Murine Fibroblast-Treated with Gallic Acid Loaded Graphene Oxide (GAGO) Nano Formulation

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Introduction: Diabetes Mellitus (DM), a state of chronic hyperglycaemia, affecting over 124 million individuals worldwide. One of the major complications of DM is impaired wound healing. Wound healing is a normal physiological process that proceeds through a series of co-ordinated cellular and cytokine-mediated events, culminating in the restoration of functional integrity of tissues. It has been reported that low levels of anti-oxidant accompanied by raised levels of markers of free radical damage may have contributory role in delaying the healing process in diabetic rats. Gallic acid (GA) is a phenolic compound found in almost all plants and has been reported to possess powerful health benefits such as anti-oxidant, anti-inflammatory, anti-cancer, and anti-diabetic properties. However, GA suffers a short half-life, in which affects its anti-oxidant property when administrated *in vivo*. Recent investigations have employed graphene oxide (GO), a biocompatible and cost-effective graphene derivative, as a nanocarrier for GA. There is a significant advantage of using nanomaterial in medicine for the diagnosis and treatment of diseases, especially in drug delivery. However, the effect of this formulated nano-compound has not been fully studied. **Objective:** Thus, this study aimed to evaluate the anti-oxidant level of gallic acid-loaded graphene oxide (GAGO) in normal and diabetic fibroblast. **Hypothesis:** It was hypothesized that better anti-oxidant level measured in 3T3 (normal fibroblast) and 3T3-L1 (diabetic fibroblast) treated with GAGO nano formulation compared to control groups. **Methodology:** Briefly, 3T3 normal fibroblast and 3T3-L1 diabetic fibroblast cell lines were treated with nine (9) different concentrations of GAGO nano formulation, ranged between 0-500 µM, for up to 24 hours. Reactive oxygen species (ROS) level was measured at 12 and 24 hours of post-treatment. As comparisons, pure GO and pure GA were used as controls in this study. **Results:** Our data showed a bell-shaped curve of ROS productions when 3T3 and 3T3-L1 cells were treated with pure GA, pure GO or GAGO nano formulation. All results showed time-dependent pattern. As compared to 12hr post-treatment, lower ROS productions were observed at 24hr post treatment of 3T3-treated with GAGO. Interestingly, the same observation was seen earlier in 3T3-L1 treated with GAGO, at 12hr post-treatment. **Conclusion:** Our results suggest that anti-oxidant effect of GAGO-treated cells is improved, both in 3T3-L1 and 3T3 cells. Altogether, this study indicates that GAGO nano formulation is worth to explore for its therapeutic properties. Therefore, further analysis is still required to establish the effects of ROS production and drug-release of this GAGO nano formulation, both in normal dan diabetic cells.

Keyword: Diabetes mellitus; gallic acid; graphene oxide; nanoparticles; anti-oxidant

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ABSTRAK

Status ROS Terhadap Sel Fibroblas Normal dan Diabetes Mencik Dirawat dengan Formulasi Nano Asid Galik-Grafena Oksida (GAGO)

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Pengenalan: Diabetes Mellitus (DM) merupakan penyakit hiperglisemia kronik, yang mempengaruhi lebih daripada 124 juta individu di seluruh dunia. Salah satu komplikasi utama DM adalah menyebabkan terjejasnya penyembuhan luka. Penyembuhan luka adalah proses fisiologi normal yang berlangsung melalui satu rangkaian kejadian selular dan sitokin yang diselaraskan, yang memuncak pada pemulihan integriti fungsi tisu. Telah dilaporkan bahawa tahap anti-oksidan yang rendah disertai dengan peningkatan tahap penanda kerosakan radikal bebas mungkin berperanan dalam melambatkan proses penyembuhan pada tikus diabetes. Asid Gallic (GA) adalah sebatian fenolik yang terdapat di hampir semua tanaman dan telah dilaporkan mempunyai manfaat kesihatan seperti sifat anti-oksidan, anti-radang, anti-barah, dan anti-diabetes. Walau bagaimanapun, GA mengalami separuh hayat yang pendek, yang mempengaruhi sifat anti-oksidannya apabila diberikan secara *in vivo*. Kajian baru-baru ini menggunakan grafena oksida (GO), derivatif grafena yang bersifat biokompatibel dan kos efektif, sebagai pembawa nano untuk GA. Terdapat kelebihan ketara menggunakan nanomaterial dalam perubatan untuk diagnosis dan rawatan penyakit, terutama dalam penyampaian ubat. Namun begitu, kesan sebatian nano yang dirumuskan ini belum dikaji sepenuhnya. **Objektif:** Oleh itu, kajian ini bertujuan untuk menilai tahap anti-oksidan GAGO pada fibroblas normal dan diabetes. **Metodologi:** Secara ringkas, sel fibroblas normal (3T3) dan fibroblas diabetes (3T3-L1) dirawat dengan sembilan (9) kepekatan formulasi nano GAGO yang berbeza, berkisar antara 0-500 μM, sehingga 24 jam. Tahap spesies oksigen reaktif (ROS) diukur pada 12 dan 24 jam selepas rawatan. Sebagai perbandingan, GO tulen dan GA tulen digunakan sebagai kontrol dalam kajian ini. **Keputusan:** Data kami menunjukkan lengkung produksi ROS berbentuk loceng ketika sel 3T3 dan 3T3-L1 dirawat dengan formulasi nano GA, GO tulen atau GAGO. Semua hasil menunjukkan corak bacaan bergantung pada masa. Berbanding dengan 12 jam selepas rawatan, pengeluaran ROS yang lebih rendah diperhatikan pada 24 jam selepas rawatan terhadap 3T3 yang dirawat dengan GAGO. Menariknya, pemerhatian yang sama dilihat sebelumnya pada 3T3-L1 yang dirawat dengan GAGO, pada 12 jam selepas rawatan. **Kesimpulan:** Hasil kajian kami menunjukkan bahawa kesan anti-oksidan sel yang dirawat GAGO bertambah baik, sama ada dalam sel 3T3 dan 3T3-L1. Secara keseluruhan, kajian ini menunjukkan bahawa formulasi nano GAGO bernilai untuk diterokai untuk sifat terapeutiknya. Oleh itu, analisis lebih lanjut masih diperlukan untuk menentukan kesan pengeluaran ROS dan pelepasan ubat dari formulasi nano GAGO ini, baik dalam sel normal atau diabetes.

Kata kunci: Diabetes mellitus; asid galik; grafena oksida; nanopartikel; anti-oksidan

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LIST OF ABBREVIATIONS

4-HNE	4-hydroxy-2-nonenal
ANOVA	Analysis of variance
ARE	Antioxidant responsive element
CD4+	Cluster of diffentiation 4
CD8+	Cluster of differentiation 8
CDC	Center for Disease Control and Prevention
DFU	Diabetic foot ulcer
DM	Diabtes Mellitus
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EGCG	Epigallocatechin gallate
EGF	Epidermal growth factor
FGF	Fibroblast growth factor
GA	Gallic acid
GAGO	Gallic acid-loaded grapheme oxide
GO	Graphene oxide
H_2O_2	Hydrogen peroxide
MDA	Malondialdehyde
MMP	Matrix metalloproteinase
NRF2	Nuclear factor E2-related factor 2
O_2	Oxygen
PDGF	Platelet derived growth factor
PI3K	Phosphoinositide-3 kinase
ROS	Reactive oxygen species
TGF-β	Transforming growth factor

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CHAPTER 1

INTRODUCTION

1.1 Background

According to Centers for Disease Control and Prevention's (CDC) National Diabetes Report, 2017, throughout the United States, 90-95% of individuals suffer Type 2 diabetes while just 5% of individuals have Type 1. Over 100 known physiological causes contribute to deficits in the wound care of diabetes patients (Brem & Tomic-Canic, 2007). Many studies focus on oxygen as the factor that affect the impairment of wound healing in both normal and diabetic condition (Abiko & Selimovic, 2010; Guo & DiPietro, 2010). However, the mechanism is different in normal and diabetic healing of wound. In normal wound healing, reactive oxygen species (ROS) play a vital role. They act as act as secondary messengers to many immunocytes and non-lymphoid cells, which are involved in the repair process, and appear to be important in coordinating the recruitment of lymphoid cells to the wound site (Dunnill et al., 2017). This original paper by Christopher Dunnill and his friends stated that low levels of ROS are essential in stimulating effective wound healing. ROS will burst into pathogen presents in wounds, leading to their destruction. Nevertheless, during this point, excess ROS that leak into surrounding, contribute to bacteriostatic environment. ROS can eventually cause cellular damage and delay wound healing. Additionally , those cellular defects are responsible for inadequate bacterial clearance and delayed or impaired repair in people with diabetes as well (Loots et al., 1998; Sibbald & Woo, 2008). Therefore, ROS are needed at low levels in cells to prevent oxidative damage and to ensure that these ROS signals do not reach damaging levels, it is needed to encounter it with antioxidant strategy. In order to solve this issue, together with today's evolving nanotechnology, it is also important to look at a new alternative that has been encapsulated with a carrier that can enhance skin to

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effectively speed up healing. This study is done to compare the antioxidant properties of Gallic Acid (GA), its carrier Graphene Oxide (GO) and the hero, which is the gallic acid-loaded graphene oxide (GAGO). Finding showed in an observation-based study that GA accelerates wound closure in both normal and diabetic environments (Yang et al., 2016). Thus, our results provide new insight for improvisation of current drug delivery system.

1.2 Hypothesis

GAGO will express high anti-oxidant level in normal fibroblast and diabetic fibroblast-treated cells compared to its pure GA- and GO- compounds.

1.3 Objectives

1.3.1 General Objective

To measure anti-oxidant properties of GA, GO and GAGO in normal and diabetic fibroblast.

1.3.2 Specific Objective

To measure anti-oxidant level of GAGO on ROS in normal and diabetic fibroblast, and comparing to its pure GA-and GO-.

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CHAPTER 2

LITERATURE REVIEW

2.1 Skin as the Largest Organ

2.1.1 Definition and Structure

Skin is the largest outer covering of the body, providing physical barrier, which protects from heat and light, injury and infection. Skin serves as a waterproof, insulating shell, shielding the body from temperature spikes, damaging radiation and toxic chemicals. Its composition is made up of two main strata: the epidermis and the dermis. The epidermis includes keratinocytes, melanocytes, dendritic cells, Langerhans and other immune cells, sensory axons, and the basement membrane epidermal-dermal (Pasparakis, Haase, & Nestle, 2014; Rousselle, Braye, & Dayan, 2019). Meanwhile, the dermis includes the skin appendages, mast cells, fibroblasts, dermal cell receptor, resident and circulating immune cells (Kupper & Fuhlbrigge, 2004). In addition, the dermis regulates the functions of cytokine, growth factors and contains complex of extracellular matrixes that sustain intercellular connections and cellular movement.

Extracellular matrix (ECM), components that gives the skin its unique properties of elasticity, tensile strength and compressibility, is responsible for giving the skin cells structural and biochemical support. Schultz et al. (2005) explained the proteins of normal skin found in the ECM are critical in healing acute and chronic wounds. ECM, a gel-like matrix formed by the surrounding cells, has two main groups of fibrous structural proteins and proteoglycans combined. Fibrous structural proteins entail collagen, elastin, and laminin, while proteoglycans such as dermatan sulfate and hyaluronan, normally consist of multiple

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glycosaminoglycan chains that branch off a linear protein core (Schultz, Ladwig, & Wysocki, 2005) (**Figure 2.1**).

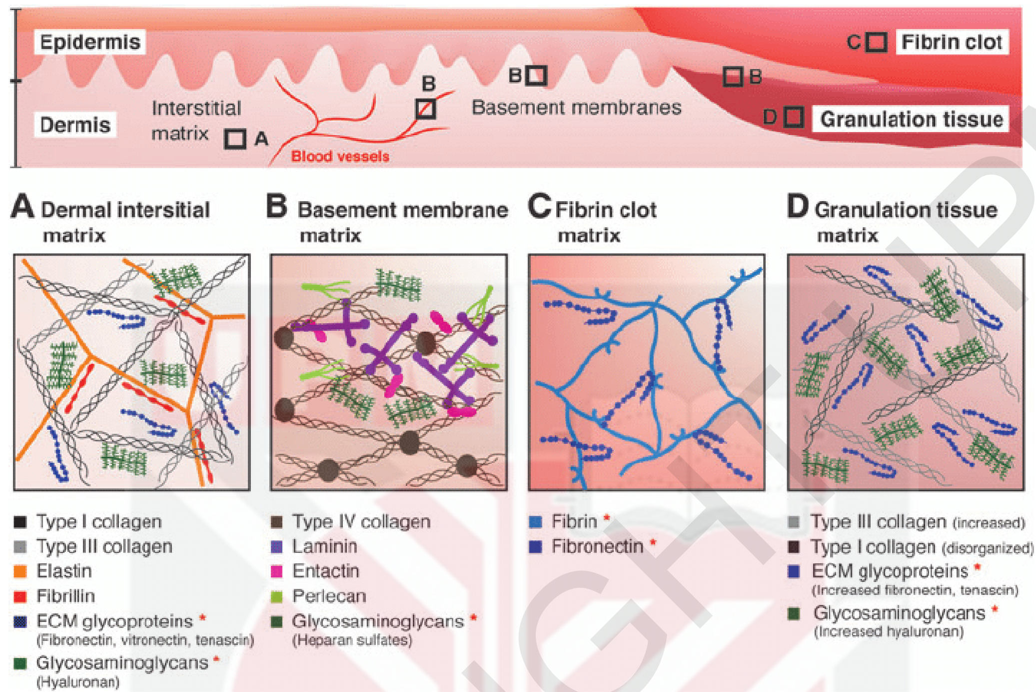

Figure 2.1: ECM compositions in healthy skin during wound healing.

(Briquez, Hubbell, & Martino, 2015)

2.1.2 Mechanism of Inflammation

According to Schultz et al. (2005), chronic wounds contain significant levels of inflammatory cells, resulting in elevated protease levels and tend to degrade the ECM components, growth factors and receptors that are necessary for healing. This statement can be supported by a study that addresses the pathophysiology of any injury or inflammation and potential strategies to regulate the immune system as well as facilitate acute wound skin regeneration and improve the closure of chronic wounds (Larouche, Sheoran, Maruyama, & Martino, 2018). An inflammatory response after injury is both normal and essential in

restoring homeostasis to the tissue. The phases of skin wound healing which include hemostasis, inflammation , proliferation and maturation, result from a cascade of events, largely mediated by immune cells and signaling molecules (Larouche et al., 2018). These phases will be discussed further.

2.2 Wound Healing

2.2.1 Phases of Wound Healing

Wound healing is meant by an automatic biological series of events that the body sets when skin is disfigured. It consists of complex and dynamic stages of regenerating missing cellular structure of layers of tissues. Because the wound healing stages are linear, progress of wound is depending on the patient's internal and external conditions, whether severe or mild. This cascade of healing can be grouped into four distinct yet overlapping phases: hemostasis, inflammation, proliferation and maturation phases (Guo & DiPietro, 2010). Some authors varied the name of stages, so differences in the phase descriptors are used. For instant, proliferation is mentioned as granulation and maturation phase also called as remodelling phase.

The first phase of haemostasis starts immediately after injury, with the wound being closed by clotting process. When the blood flow is restricted by the constriction of blood vessel, the platelets hold together to close the gap in the blood vessel wall. A study stated that inflammatory cytokines and growth factors such as transforming growth factor (TGF)-B, platelet derived ground factor (PDGF), fibroblast growth factor (FGF), and epidermal growth factor (EGF) will be released in the clot and surrounding wound tissue during this phase (Guo & DiPietro, 2010). Homeostasis stage of wound healing occurs very quickly. The platelets

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bind to the surface of a sub-endothelium within seconds of the breach of the epithelial layer of a blood vessel.

Once the injured blood vessels leak causing localized swelling, that's when the promotion of inflammatory phase begins. T lymphocytes migrate into the wound site following sequential infiltration of inflammatory cells such as neutrophils and macrophages. Some reports suggest that delayed T-cell penetration and reduced T-cell concentration in the wound site are correlated with impaired wound healing while others have indicated that CD4+ cells (T-helper cells) play a positive role in wound healing and that CD8 + cells (cytotoxic T cells) play an inhibitory role in wound healing (Park & Barbul, 2004). Inflammation is a common part of the cycle of wound healing and is only troublesome when prolonged or severe.

The focus of the third phase, the proliferative phase is to fill and seal the wound once the wound is cleaned out. During this phase, epithelial cells start to migrate across the wound bed until the wound is fully covered with epithelium. Moreover, new network of blood vessels must be developed so that the granulation tissue can remain healthy and get adequate oxygen and nutrients. Myofibroblasts cause the wound to contract by grasping the edges of the wound and drawing them together using a similar mechanism to that of smooth muscle cells. Epithelisation happens more easily when the wounds are kept moist and hydrated. Correct occlusive or semi-occlusive dressing is therefore important to maintain proper humidity in the tissue to maximize epithelialisation.

Maturation phase or remodelling phase occurs when the new tissue slowly gains strength and resilience. This stage is when the collagen remodels and matures. There is an increase of tensile strength of tissue layers as well. During previous phase, collagen becomes disorganized and the wound becomes thick. However, collagen is aligned along tension lines

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during the maturation process, and water is reabsorbed to allow the collagen fibres to lie closer together and to cross-link. Cross-linking collagen decreases scar thickness and improves the wound's surface region too.

2.2.2 Factors Affecting Wound Healing

All four stages must occur in proper sequence and time frame for a wound to heal successfully. Many factors may lead to one or more stages of intervention, resulting in impaired and graceless wound healing. It is stated in the previous study that impairment of wound healing could occur due to local or systemic factors. The local factors discussed include supply of oxygen, infection and present of foreign bodies. Meanwhile, aging, sex, stress, failure of circulation, obesity, diabetes, medication such as steroid or anticancer drugs, alcohol intake, smoking habit, immunosuppressed state, nutrition and other type of systemic diseases become the systemic causes that impair wound recovering (Abiko & Selimovic, 2010).

Local factors are the factors that directly affect wound characteristics themselves (Guo & DiPietro, 2010). Among the factors mentioned above, this study most probably would like to focus on oxygen factor since we are focusing on the antioxidant status of potential phytoconstituents towards ability of wound healing on normal and diabetic cells. Certain factors such as aging and diabetes can lead to impairment of vascular flow, hence responsible for the stage of poor oxygenation of the tissue. This is because a sufficient amount of oxygen is essential for efficient wound healing (Guo & DiPietro, 2010). In normal condition, reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) and superoxide (O_2) are needed to conduct essential processes involved in the treatment of injury. However, this claim was contended where ROS can increase during hypoxia and hyperoxia, thus an

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elevated level of these ROS overcome the good effect and cause more tissue damage (Rodriguez et al., 2008).

People who suffer diabetes, one of the systemic conditions, vulnerable to developing chronic non-healing diabetic foot ulcers (DFUs), estimated to occur in 15% of all diabetes patients (Guo & DiPietro, 2010). Acute and chronic wound will be discussed onwards and displayed in **Figure 2.2**. Diabetic wounds include many dysregulated cellular functions, such as defective T-cell immunity, leukocyte chemotaxis defects, phagocytosis, and bactericidal ability, and fibroblast and epidermal cell dysfunctions. Those defects are responsible for insufficient bacterial clearance and repair delayed or damaged in people with diabetes (Loots et al., 1998; Sibbald & Woo, 2008).

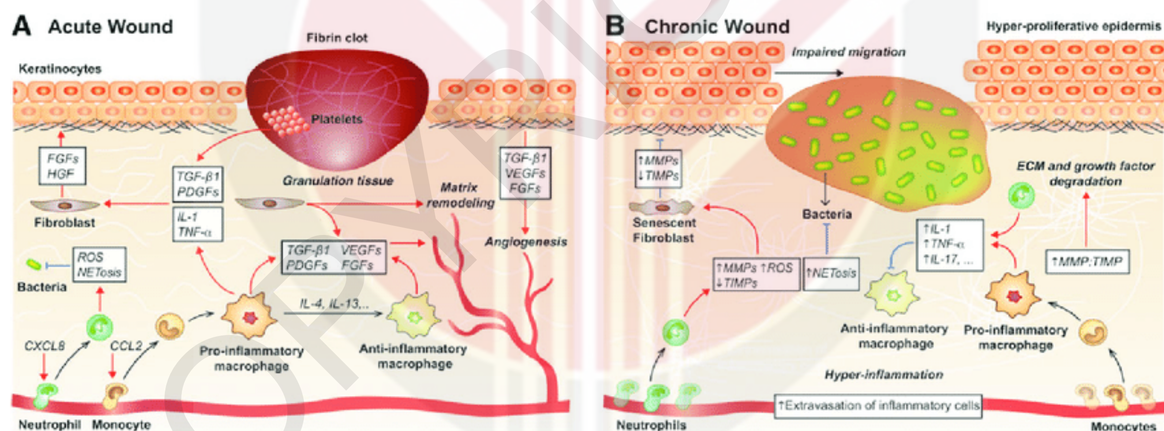

Figure 2.2: Diagram of the immune mechanisms in acute and chronic wound healing.

(Larouche et al., 2018)

2.2.3 Acute Wound

Wound with compromised treatment, includes prolonged acute wounds and chronic wounds. These two forms of wounds have typically failed to progress through normal healing

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stages. However, there is no generally accepted or known definition of what constitutes an acute or chronic wound (Fletcher, 2008). The first type of wound is acute wound. Acute wound is wound which was there for a shorter period of time. Acute wound progresses through typical wound healing stages; hemostasis, inflammation, proliferation and remodelling and shows strong signs of healing within four weeks (Alberts et al., 2019). Major categories of acute wound are abrasions, lacerations, incisions, burns and puncture wounds (Tejiram, Kavalukas, Shupp, & Barbul, 2016).

2.2.4 Chronic Wound

Chronic wound is associated with nearly severe disorders that lead to an impaired recovery. The prolonged inflammatory stage has been suggested to cause increased levels of proteases such as MMPs, elastase, plasmin and thrombin, which kill components of the ECM and damage growth factors and their regeneration. A deficiency or imbalance in growth factors, cytokines and/or MMPs inside wounds has been hypothesized to facilitate the establishment of chronic injuries (Bennett & Schultz, 1993). Moreover, chronic wound disorders such as diabetes, peripheral vascular insufficiency, thrombocytopenia, venous insufficiency, or previous radiation are causes of insufficient development of clots which delay the normal healing processes (Spear, 2013). When it comes to this type of wound, it is clear that the attempt to transform a chronic wound into a healing wound is to try to identify the root cause of failure to progress in a timely manner through the stages of healing.

2.3 Diabetes Mellitus

2.3.1 Introduction to Diabetes Mellitus

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More than 100 recognized physiological factors lead to healing deficiencies in diabetes sufferers (Brem & Tomic-Canic, 2007). Those factors include reduced or impaired growth factor development (Falanga, 2005; Galkowska, Wojewodzka, & Olszewski, 2006), angiogenic response (Falanga, 2005; Galiano et al., 2004), macrophage function (Maruyama et al., 2007), collagen accumulation, epidermal barrier function, granulation tissue quantity (Falanga, 2005), keratinocyte and fibroblast migration and proliferation, amount of epidermal nerves (Gibran et al., 2002), bone cure, and balance between accumulation of extracellular matrix components and their remodelling by matrix metalloproteinases (Lobmann et al., 2002).

2.3.2 Type-1 Diabetes Mellitus (DM)

There are 4 categories of diabetes which include Type-1 DM, Type-2 DM, gestational DM, and also other specific types (Gardner, 2011), but only 2 types that are frequently discussed. Looking at Type-1 DM, it is characterized by loss of insulin-producing pancreatic islet beta cells, leading to insulin deficiency (Facts & Diabetes, 2011). It is called juvenile-onset diabetes, because it mostly begins in infancy. In other word, it occurs when the body assaults antibodies on pancreas. The liver is impaired and is not producing insulin. This is because the bulk of Type-1 diabetes is immune-mediated in that a T-cell-mediated autoimmune attack results in the loss of beta cells and thus insulin (Rother, 2007).

2.3.3 Type-2 Diabetes Mellitus (DM)

Type-2 diabetes is defined by insulin resistance, which can be associated with a relatively poor insulin secretion (Gardner, 2011). Insulin receptor is thought to be involved in the deficient sensitivity of body tissues to insulin. The exact faults, however, are not understood. Cases of diabetes mellitus resulting from a known defect are individually classified. Type-2 diabetes is the most common form of mellitus diabetes (Facts & Diabetes,

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2011). In the past 20 years, it has become more prevalent in children and teenagers, mainly because more young people are overweight or obese. Around 90% of diabetes patients have Type-2. One of the literature indicates that healing is delayed for many reasons in some conditions, such as diabetes, starvation, ageing and immunocompromised situations (Rasik & Shukla, 2000).

2.4 Wound Healing in Fibroblast Cell

2.4.1 Introduction to Fibroblast Cell

In cell culture, fibroblasts are cells that are commonly used, either as temporary primary cell culture or as transformed cell lines permanently. These cells are the most familiar connective tissue in animals, having an elongated morphology with a fusiform shape (Duval, 2011; Virchow, 1858). Fibroblasts have recently become cell sources for use in disease modelling following cell reprogramming because it is easily accessible in the body (Fernandes et al., 2016). Apart from that, study of wound healing in keratinocytes and fibroblasts was done in normal and hyperglucidic condition previously (D. J. Yang et al., 2016). Due to that, diabetic fibroblasts were used to mimic diabetic mechanism of wound closure. These studies have found the similar results where fibroblast lines obtained from patient biopsies were used to explain the pathogenesis of several diseases (Villegas & McPhaul, 2005).

2.4.2 Normal fibroblast (3T3 Cell lines)

The designation '3T3' refers to the abbreviation '3-day transfer, inoculum 3×10^5 cells'. Initially, this cell line was developed from the primary mouse embryonic fibroblast cells cultivated by the specified protocol, the '3T3 protocol'. 3T3 cells developed by George

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Todaro and Howard Green in 1962, where these two scientists obtained their 3T3 cells from Swiss albino mouse embryo tissue (Todaro & Green, 1963). Previously, this normal mouse fibroblast cell lines 3T3, treated with different concentrations gallic acid, graphene oxide and gallic acid-loaded grapheme oxide nanocomposite, have been used as an successful drug delivery targeting device for cancer therapy (Dorniani et al., 2016). Besides, they were also used in keratinocyte production, with 3T3 cells secreting favourable growth factors for these types of cells.

2.4.3 Diabetic fibroblast (3T3-L1 Cell lines)

3T3-L1 is a line of cells derived from (mouse), 3T3-L1 cells used in biological adipose tissue study. 3T3-L1 cells have a fibroblast-like morphology but the cells transform into an adipocyte-like phenotype under suitable conditions. In any cultivation study, by day 4, the majority of 3T3-derived cells had resulted in cell death. By comparison, on day 8, 3T3-L1 derived cells displayed typical characteristics of megakaryocyte lineages in different marker analyses (Matsubara, Suzuki, Ikeda, & Murata, 2010). As diabetic fibroblast mimics the hyperglucidic condition in wound of human, complication in wound healing can be said to be due to inflammation-related cellular damage at the healing site. Since diabetic wounds have significantly lower rates of many growth factors, the focus of these factors was on improving wound repair (Matsuda et al., 1998).

2.5 Antioxidant Properties

2.5.1 Oxidative Stress

The pathogenesis of lifestyle-related diseases like atherosclerosis, hypertension, diabetes mellitus, ischemic diseases and malignancies is well known to include oxidative

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stress (Bhattacharyya, Chattopadhyay, Mitra, & Crowe, 2014). Oxidative stress is identified as harmful since radicals free of oxygen attack biological molecules such as lipids, proteins and DNA (Sharma, Jha, Dubey, & Pessarakli, 2012). Nevertheless, oxidative stress also plays a useful role in the physiological adaptation and in intracellular signal transduction regulation. Oxidative stress is characterized as a condition in which oxidation exceeds the antioxidant systems in the secondary body, resulting in a loss of balance (Betteridge, 2000).

2.5.2 Antioxidant Activity in Relation to Oxidative Stress & Diabetes

Previous study about antioxidant status in relation to oxidative stress in Type-2 diabetes mellitus stated that, diabetes mellitus is a disease in which oxidative stress is increased. The study found a significant decrease in total antioxidant status among patients with diabetes and a significant increase in malondialdehyde (MDA) levels compared to controls (Jamuna Rani & Mythili, 2014). The same authors also claimed that oxidative metabolism activates reactive oxygen species (ROS). Apart from that, oxidative stress is known as the amount charged for oxygen used. Over-generation of ROS in this oxidative stress has pathological consequences toward protein, lipid and DNA damage (D'Autréaux & Toledano, 2007). Therefore, hyperglycemia condition induces a high number of free radicals in diabetes diseases (Johansen, Harris, Rychly, & Ergul, 2005; Saxena, Srivastava, Kale, & Baquer, 1993).

2.5.3 Antioxidant Therapies for Wound Healing

Normal wound healing physiology relies on the low levels of ROS and oxidative stress. Over-exposure to oxidative stress can lead to impaired wound healing. Antioxidants are therefore meant to help reduce wound oxidative stress and thus speed up wound healing.

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It is said in a research that an abnormal low levels of antioxidants were related to impaired wound healing (Schäfer & Werner, 2008). Furthermore, antioxidants are effective mediators in controlling the damage that biological molecules such as DNA, protein, lipids and body tissue can potentially suffer in the presence of reactive species (Fitzmaurice, Sivamani, & Isseroff, 2011).

Concerning the association of the antioxidant status with the state of oxidative stress in clinical samples, the measurement of individual levels of antioxidants may yield conflicting results. For example, some papers report low antioxidant status in cancer samples, interpreting this as a loss of their protective capacity due to high oxidative stress, whereas others interpret the results of high antioxidant rates as an adaptive response mechanism for detoxifying harmful metabolites associated with oxidative stress. In order to resolve these prejudices, it is advisable to assess the total antioxidant status by simultaneously assessing all antioxidants, without excluding their interactions. In conclusion, the use of antioxidants for wound healing is still in its earlier phase and this study will make the role of antioxidants in wound healing better established.

2.6 Antioxidant Assays

2.6.1 Reactive Oxygen Species (ROS)

Reactive oxygen species or ROS known as signalling molecules, such as superoxide anion, hydroxyl radical and singlet oxygen, are free radicals that require oxygen and are mostly formed by the mitochondria. These molecules have been shown to be involved in signal transmission during oxidative stress that can damage muscle tissue, inhibit output and induce apoptosis (Hekimi, Wang, & Noë, 2016; Moungjaroen et al., 2006; Musumeci, Maria

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Trovato, Imbesi, & Castrogiovanni, 2014). Some studies have documented that accumulation of ROS can activate the pathway to apoptosis by altering the potential of the mitochondrial membrane. This transition affects the mitochondrial oxidation channel and can contribute to the release of cytochrome c from the mitochondria to the cytoplasm (Gao et al., 2015; Xiong et al., 2015).

ROS biologically have a 'positive' and 'evil' side (Patel, Rinker, Peng, & Chilian, 2018). Firstly, optimum number of ROS is required for redox homeostasis to work as well as being useful in the cardiovascular and immune system. In addition, ROS modulates cell proliferation and apoptotic pathways to ensure proper cell cycle regulation and programmed cell death. Other than that, ROS can inhibit protein phosphatases by cysteine oxidation, and maintain adequate growth factor signal levels. These molecules are essential in activating antioxidant genes through mechanisms such as the response element PI3K-NFE2-like2 (Nrf2)-antioxidant (ARE). Partly, they also play roles in the expression of transcription nuclear factor-kB and in immune system generally (Kohchi, Inagawa, Nishizawa, & Soma, 2009).

Excessive amounts of ROS may, however, results in pathologies ranging from autoimmune diseases, cardiomyopathies and other inflammatory diseases (Li et al., 2013). The most emphasized goal of this analysis is to pay attention to the effects of ROS that cause oxidative damage. In addition, the existence of nitrotyrosines can change protein confirmation and function such as neurofilaments and actin, leading to pathologies such as atherosclerosis, myocardial ischemia and irritable bowel syndrome (Beckman & Koppenol, 1996). These free radicals cause several complications inside a cell, resulting in oxidative mitochondrial damage or cell apoptosis as mentioned in the previous paragraph.

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Additionally, high-concentration ROS can be particularly harmful to humans. When the ROS amount is higher than the defence mechanisms, many cellular functions can be affected by damaging nucleic acids, oxidizing proteins and causing lipid peroxidation (Sánchez, 2017) (**Figure 2.3**).

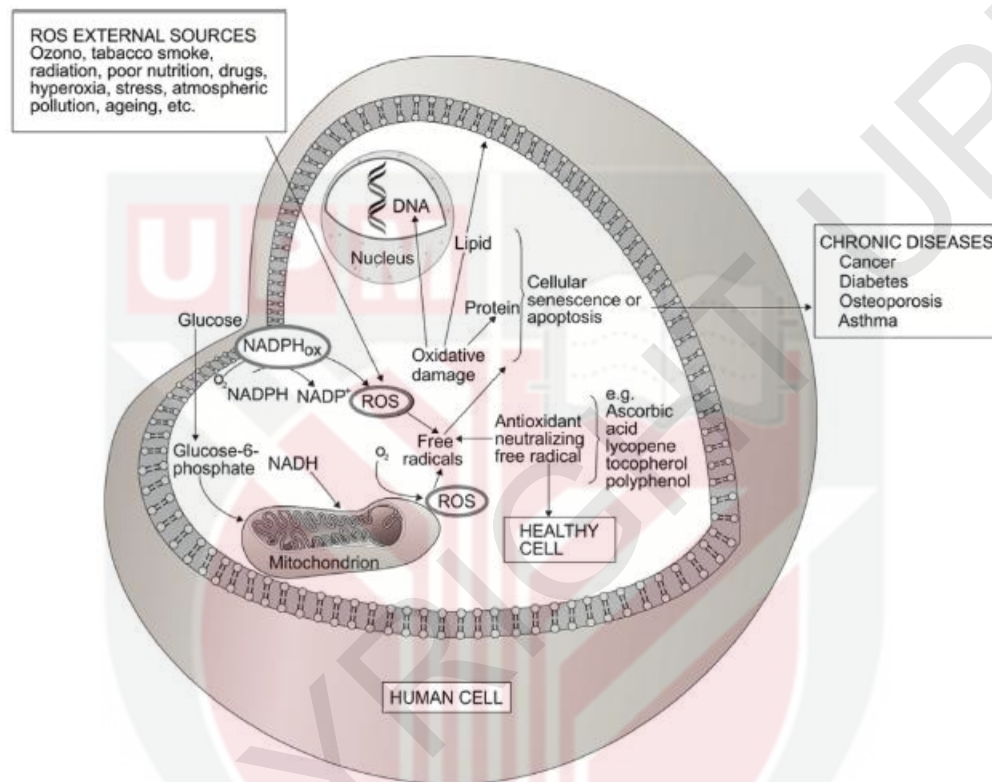

Figure 2.3: Mechanism of free radical generated from internal and external sources in human cell.

(Sánchez, 2017)

According to Hui Xu and friends (2018), ROS, the most common free oxygen radicals, is taking part in a series of physiological and pathological skin processes. In placing more emphasis, D'Autréaux & Toledano (2007) claimed that low ROS levels are required during the wound healing process for cell signalling, primarily for angiogenesis maintenance, which indicates that these physiological low levels of ROS are necessary for maintenance of

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skin functions and metabolism. Meanwhile, excessive development of ROS or impaired detoxification of aggressive molecules may cause oxidative stress, which has been described as an important feature in chronic, non-healing wounds pathogenesis (Sun et al., 2018).

Previous research conducted a report on the effect of species-mediated oxidative stress on mitochondrial apoptosis activation by reactive oxygen. The study results showed that H_2O_2-generated ROS significantly increased mitochondrial oxidative stress by decreasing superoxide dismutase, catalase and glutathione peroxidase activities and the lipid peroxidation (Li et al., 2018). To sum up, there are many other studies that suggest crucial role antioxidants play in improving pathophysiological conditions. That is why compounds with strong antioxidant properties are added to reduce this excessive output of ROS, which could be harmful to *in vitro* cells.

2.6.2 Lipid Peroxidation

The mutagenic effects of ROS are not limited to DNA damage but may also involve an attack by ROS molecules on proteins and lipids. Oxidative damage to lipids in cells , for example, initiates lipid peroxidation, leading to the production of a variety of mutagenic products that could alter cellular functions and increase cancer initiation or progression (Barrera, 2012; Cejas et al., 2004). As a result, lipid peroxidation assay is also important as measurement of oxidative stress level. Lipid peroxidation was commonly used as an indicator of the damage caused by ROS to cell membranes.

Malondialdehyde (MDA) is among the best studied end-products of polyunsaturated fatty acid peroxidation in clinical samples and is often used to estimate oxidative stress conditions (Cheeseman & Esterbauer, 1990). Other than that, previous study explained that 4-hydroxy-2-nonenal (4-HNE) is the major product of lipid peroxidation which can be detected. And it has been shown that its expression is correlated with the wound healing cycle using

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mouse models (Gupta, Singh, & Raghubir, 2002). This can be supported by Gasparovic and colleagues, claiming that LPO's final products are reactive aldehydes such as 4-hydroxyalkenals, and other related α , β-unsaturated aldehydes. 4-HNE, MDA, and acrolein are the most inestigated aldehydes that result in increased membrane fluidity, cytosol efflux and protein loss. (Gasparovic, Jaganjac, Mihaljevic, Sunjic, & Zarkovic, 2013).

Lipid-derived aldehydes are more stable than ROS, so they can diffuse across membranes and reach targets far from the initial oxidative injury site. Many of them are also known as second messengers of free radicals, due to their stability and biological activity (Guéraud et al., 2010; Zarkovic, 2003). Skin is very susceptible to a number of redox reactions as it is the heaviest, largest organ with the most complex functions. Therefore, it is important to maintain equilibrium between oxidants and antioxidants.

2.7 Potential Phytoconstituent

2.7.1 Compounds of High Antioxidant Property

The oxidants have been known to cause signals for reactions that are essential to wound healing. However, millimolar oxidant concentrations are able to deplete the source of antioxidants in the wound and cause more unintended harm to the tissue (J. Shi, Yu, Pohorly, & Kakuda, 2003). Several animal and human studies have already shown the efficacy of these compounds in promoting healing, as interest in the use of antioxidants continues to grow. Hence, few papers had suggested that, based on several scientific studies, certain compounds are best listed as having strong antioxidant properties as well as reducing the concentration of free radicals. The compounds include superoxide dismutase, vitamin E, α-

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lipoic acid, metronidazole, elemental antioxidants, β-glucan, curcumin, , epigallocatechin gallate (EGCG), proanthocyanidins, propolis, and honey (Fitzmaurice et al., 2011).

2.7.2 Introduction to Gallic Acid

Gallic acid (GA) is a 3, 4, 5-trihydroxybenzoic acid (Ng et al . , 2004), another phenolic compound found mainly in gallnuts, sumac, witch hazel, apples, bark of oak and green tea. It has antioxidants, anti-tyrosinases, antimicrobials, anti-inflammatory, anticancer and neuroprotective properties (Bastianetto, Krantic, Chabot, & Quirion, 2011; Krogh, Yunes, & Andricopulo, 2000; Phiriyawirut & Phaechamud, 2012; Shahrzad, Aoyagi, Winter, Koyama, & Bitsch, 2001). This claim can also be supported by similar research done by Mansouri et al. (2014). Furthermore, GA is also used as a baseline in antioxidant research due to its positive antioxidant behaviour, and has free radicals that scavenge activities (Phiriyawirut & Phaechamud, 2012).

2.7.3 Gallic Acid, Diabetes Mellitus and Wound Healing

By traditional medicines, GA-containing polyphenols have been widely used to treat many chronic skin disorders such as vitiligo and psoriasis, and are also considered to be of medicinal benefit by wound healing and to have anti-inflammatory effects when used topically (Korkina, Mikhal'chik, Suprun, Pastore, & Dal Toso, 2007; Tse, Cheng, Che, Zhao, & Lin, 2007). Given that these plant-extracted treatments are readily accessible and relatively safe, there is growing interest in the use of natural compounds to prevent and counter skin diseases (L. Korkina, De Luca, & Pastore, 2012). Therefore GA has been introduced in skin

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care products due to the frequent use of GA as a standard antioxidant assay (Hansen & Just, 2001).

GA is also known in relation to this analysis to have important protective effects on the progression of the disease in animal models consistent with Type-1- and Type-2-diabetes. Yazdanpanah and his research colleagues said chronic ulcers, a common complication of diabetes, can lead to delayed and impaired healing of skin wounds (Yazdanpanah, Nasiri, & Adarvishi, 2015). Previous study had investigated the effect of GA on DM-induced delayed wound healing in both keratinocytes and fibroblasts with high glucose-containing medium to mimic diabetes (D. J. Yang et al., 2016). According to the report, they found that in mild and high glucose environments, GA has antioxidant properties and effects on cell migration, suggesting that GA has curative potential for chronic DM wound.

Moreover, the findings showed in the same observation-based study that GA accelerates wound closure in both normal and diabetic environments. Jointly, the beneficial effects of GA on wound repair in hyperglucidic conditions strongly indicate that GA may be a potential therapeutic agent for wound repair (D. J. Yang et al., 2016). In addition, this hallmark of diabetes contributes to increased oxidative stress and cell damage, and inhibits wound healing (Marrotte, Chen, Hakim, & Chen, 2010; Tamura et al., 2003). Yang et al. (2016) demonstrated that GA shows increased radical scavenging behaviour in skin cells to combat oxidative stress. Such findings strongly suggest GA exercises its antioxidant role through direct upregulation of antioxidant gene expression.

2.7.4 Graphene Oxide as Nanocarrier

It is proved that graphene oxide (GO) has been used as an active anti-cancer nanocarrier and antioxidant agent to GA in anticancer nanodelivery technology (Dorniani et al., 2016). Another study also reported that selective drug delivery for cancer therapy

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(Akhavan, Ghaderi, Aghayee, Fereydooni, & Talebi, 2012), biosensing, and photothermal cancer therapy was investigated for GO. It is because it has a relatively large surface area relative to many other materials, large functional surface groups, good photothermal properties and low cytotoxicity (Goenka, Sant, & Sant, 2014; Zhang, Nayak, Hong, & Cai, 2012). Better efficiency was also seen over graphene oxide alone (λ=1064 nm), resulting in impressive infected wound healing. (Bernal-Chávez, Nava-Arzaluz, Quiroz-Segoviano, & Ganem-Rondero, 2019).

Apart from that , the wide surface area and the functional GO groups containing oxygen such as phenol hydroxyl, epoxy and carboxylic groups make it ideal for high efficiency and good dispersion of drugs (Ma et al., 2015; P. Shi & Ye, 2015). The claim can be supported by Sahne, Mohammadi, & Najafpour (2019) in their paper. Graphene's key advantage over other nanomaterials is its ultra-high surface area and hybridized carbon sp^2 region, which makes it an effective drug carrier to load large quantities of drug molecules on both sides of the single atom layer sheet (Y. Yang, Asiri, Tang, Du, & Lin, 2013). This research has found similar results where theoretically, the specific surface area of graphene is about 2630 m^2g^{-1}, which is very large (Zhu et al., 2010).

Other than that, a research showed that an electrochemical immunosensor used functional GO as a carrier for ultrasensitive detection of phosphorylated protein in the multienzyme labelling amplification strategy (X. Yang et al., 2011). There are several other studies that highlighted GO's features as a flexible nanostructure to be implemented in this growing field of nanobiotechnology. This use of nanocarriers is a technique to increase the bioavailability and water-solubility of active compounds. Hence, it can be inferred that this successful application of GO has laid the ground for the construction of a promising nanoplatform for biomedical studies, in particular in cell growth regulation, stem cell

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differentiation, FET/FRET sensors, cancer treatment, mass spectrometry analysis (Chung et al., 2013) and of course in the drug or gene delivery systems.

2.7.5 Nanoparticle-based Approach for Wound Healing

The range of nanosystems proposed is very broad, including the use of very different materials, natural or synthetic, ranging from polymers or lipids to microorganism-derived systems. With the aim of enhancing systems performance, seeking to combat several of the problems that arise in a wound, especially when it is chronic, these materials have been combined to create nanocomposites or scaffolds. This field was positively influenced by nanotechnology, leading to tissue regeneration, encouraging wound closure and preventing potential infections (Bernal-Chávez et al., 2019). Looking at an example, some authors found that nanoparticles loaded with curcumin that were prepared using a sol-gel-based polymerization method would eventually reduce inflammation, promote reepithelization and collagen deposition (Bernal-Chávez et al., 2019).

There was another research that has the same function as silver nanoparticles to heal the wound that can control biofilms. This has important implications particularly in chronic wounds, where biofilms can compromise wound healing due to their antimicrobial tolerance (Barbosa et al., 2016). Topical application of this nanoproduct speeds up the normal and diabetic wound healing due to the activating properties of certain antioxidants, namely EGCG and α-lipoic acid (Bernal-Chávez et al., 2019). A literature found that silver nanoparticles decorated with phytochemicals may be potential candidates for skin regeneration and wound healing (Dubey et al., 2015). Therefore, all the nano-transporters are a reasonable choice for achieving those objectives.

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This *in vitro* study focuses on GA's ability to see the possible underlying mechanism combined with GO, as Nagpal et al. (2012) had demonstrated that GA's nanoparticle drug delivery system could enhance antidepressant-like activity in Swiss male albino mice. The justification for designing drug delivery systems is to enhance the drug's therapeutic efficacy and reduce its harmful side effects by maximizing the volume and length of the medication near the target cells and the drug exposure to non-target cells (Nagpal, Singh, & Mishra, 2012). In conclusion, developments in nanotechnology may lead to the production of several components, such as nanomaterials, nanoengineering processes, growth factor and wound healing target stem cell therapy (Hamdan et al., 2017).

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CHAPTER 3

METHODOLOGY

3.1 Cell lines

3T3 and 3T3-L1, normal and diabetic murine fibroblast cells, respectively, were obtained from American Type Culture Collection (ATCC), Manassas, United States of America.

3.2 3T3 & 3T3-L1 Murine Fibroblast Cell Lines

3.2.1 Preparation of Fresh DMEM Medium

500ml of incomplete high glucose Dulbecco's Modified Eagle's Medium (DMEM) purchased from Nacalai Tesque (Kyoto, Japan) media was aseptically prepared in the biosafety cabinet. Subsequently, 50µl of 10% of Fetal Bovine Serum (FBS) and 5µl of 1% of penicillin-streptomycin antibiotics were added to have a complete growth medium and stored at 4°C. A quality control of the complete growth medium was then conducted to check for contamination. Briefly, 3ml of the complete growth medium was transferred into a T25 cell culture flask and incubated at 37°C in a humidified atmosphere with a 5% CO_2 concentration for 24 hour, followed with observation under microscope. A free contamination full growth medium is defined with no observed unknown floating cell or microorganism growing in the medium, as well as no changes in colour.

3.2.2 Cell Culture

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All procedures were performed in a class II Biosafety Cabinet (BSC) to ensure the cells were grown in sterile environment. 3T3 and 3T3-L1 murine fibroblast cell lines were grown in complete growth medium DMEM (complemented by 10 % FBS and 1% penicillin-streptomycin antibiotics). Later, the cells were grown using T75 cell culture flask incubated at 37°C in humidified atmosphere with an incubator concentration of 5% CO_2. Using inverted microscope, the cells were monitored daily to check whether the cells are growing healthily and ensuring there is no contamination. When the cells reached a confluence of 75% to 85%, cell passage was performed.

3.2.3 Cell Passaging

Cell passage or subculture of cells is a process that keeps cells alive and growing by transferring and splitting the confluent cells from the current culture flask into a few new culture flasks added with a complete growth medium supplement. When the cultivated 3T3 and 3T3-L1 cell lines converged from 75% to 85%, the cells were subcultured into two new fresh T75 cell culture flasks. After discarding the old medium from the flask, the flask was washed twice, with 4ml of PBS. The remaining death cells that were attached in the flask were washed away and discarded with PBS. To remove the adherent cells from the surface of the flask, 3ml of 0.05% Trypsin solution was transferred to the cell culture flask. The trypsinized flask was then incubated for 5 minutes in a fully humidified 37°C incubator at a concentration of 5% CO_2. The flask must be placed flat in the incubator ensuring complete coverage of the monolayer. The flask was gently tapped after taking out the flask from the incubator to ensure complete detachment of cells from the flask surface. Afterwards, under the inverted microscope, the flask was examined to ensure that all cells were separated from the surface. To stop trypsin activity, 1 ml of complete media containing FBS was pipetted

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into the flask. To avoid the full trypsin operation as well as full cell detachment from the surface, the added medium resuspended in and out for three times to disperse around the flask. The entire content in the flask was then transferred to a 15ml falcon tube. The falcon tube was centrifuged at 4°C for 5 minutes, with a resolution of 1800 rpm. The solution's supernatant was discarded leaving the pellet in the tube of the falcon which is the cells. 1ml of fresh medium was added and resuspended within pellet cells. Upon completion of this process, cell counting was performed with a hemocytometer to ensure that $8x10^4$ cells /cm^2 of cells were grown into the new T75 cell culture flask and added up to 9ml of fresh complete medium. The culture flask was observed under the inverted microscope and incubated at a temperature of 37 ^{o} C in a fully humidified incubator with a concentration of 5% CO_2.

3.2.4 Cryopreservation of Cells

Firstly, select 75% - 85% of the confluence of monolayer culture with normal growth and contamination-free characteristics. The procedure was almost the similar as mentioned in **Subsection 3.2.3**. However, in this section, following collection of pellets after trypsinization, 100µl of cell pellet was resuspended with freezing medium which was diluted with the 5%-10% preservative dimethyl sulfoxide (DMSO) about 100µl in growth media supplemented with 1.8ml of 10% FBS and 1% penicillin-streptomycin antibiotics. This proportion is only for one cryovial. DMSO was suggested to put in last order to avoid cell shock or apoptosis as DMSO will injures the cells. Maximum of 2ml of the resuspended mixture was sucked and put in the vial before been wrapped with parafilm. The cryovial was then held under -30 ^{o} C for the first 24 hour, and then overnight or for long-term storage at -80°C of the nitrogen tank. Cryopreservation of cells is a method of freezing and storing cells

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at very low temperatures to limit the growth of cell lines and to reduce genetic changes in the continuous cell lines while preserving their viability for several months and years.

3.2.5 Stock Cell Thawing

Cell thawing is a method of defrosting and reviving cryopreserved cryovial cells that were deposited in the liquid nitrogen tank. The cell thawing principles are rapid which dilute the frozen cells in the vial and reseed them at high cell density to ensure a higher rate of success in re-growing cells. Due to the high toxicity of DMSO towards cell lines in vial at room temperature, the process has to be done quickly. Cryovials were easily thawed in a 37°C water bath for 1 minute after being removed from the nitrogen tank. Both warm hands should prewarm the cryovials to ensure the cryovials material is in liquid form. The cells were sucked and moved directly into a 15ml falcon tube containing 1ml of complete medium. For the collection of cell pellets, the falcon tube with cells was centrifuged at 1200rpm at 4°C for 5 minutes. The supernatant was discarded after centrifugation, and 1ml of fresh medium was added. Before being transferred into T75 culture flask containing 9ml of complete medium, the cells pellet was resuspended with media. The flask was then examined microscopically and incubated in a completely humidified incubator at 37ºC with 5% of CO_2.

3.2.6 Cell Counting

Cell counting is a way of quantifying the number of cells. Cell count was performed in culture media and expressed as a number of cells per volume unit using Tryphan blue dye exclusion test to ensure that the percentage of cell viability in culture is equal to or greater than 90%. 10µl of Tryphan blue dye was mounted on a paraffin film for serial dilution

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purpose. Serial dilution is done using cultured cell concentration. Serial dilution of 1:1 ratio meaning 10μl of suspended cells was combined with Tryphan blue dye 10μl. 10μl of the dye that containing cells was carefully loaded onto the hemocytometer chamber after completed dilution. The hemocytometer was then examined under the inverted microscope to count the viable cells that appeared as white colour, clear and looked like transparent. In contrast, non-viable cells appeared as dark blue due respectively to the loss of membrane integrity. Total number of viable cells can be expressed in percentages of cell viability which were calculated using the formula shown below:

Formula to calculate number of cells:

$$\text{Example} = \frac{n}{4}\ x\ 2^{df}\ x\ 10^4$$

$$= \frac{27}{4}\ x\ 2^2\ x\ 10^4$$

$$= 2.7\ x\ 10^5 \text{ cells}$$

From the counted cells, V_1 is calculated to know how much medium is needed to dilute the cells. Using formula:

$$M_1V_1 = M_2V_2$$

$$(2.7\ x\ 10^5)\ V_1 = (8\ x\ 10^4)\ (10\ 000\mu l)$$

$$V_1 = 2962.963\mu l \text{ cells}$$

Hence, 2962.963 μl cells needed for seeding. Subtracting 2962.963 μl cells from total volume of 10000 μl, given that, it needed 7037.04μl of medium to be added with.

3.3 Reactive Oxygen Species (ROS) Assay

3.3.1 Preparation of cell suspension for ROS assay

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The cell density used in this assay is about $2.5x10^4$ for normal and diabetic murine fibroblast cells, seeded in black 96-well plates. 3T3 and 3T3-L1 cells were grown in T25 cell culture flask, added with a full growth medium in fresh DMEM. Once the cells reached confluency of 70% to 80%, the cells were trypsinized for detachment of cells from the surface of the flask. The cells were then transferred into a 15ml falcon tube and centrifuged for 5 minutes at 1800rpm. The supernatant was discarded after centrifugation, and 1ml of complete medium was added to the cell pellet. Later, the pellet of cells was resuspended for several times by pipetting up and down gently. Cell counting was performed to assess the viability of the cells and their number. This is done by exclusion of the tryphan blue.

3.3.2 Seeding and incubation of 3T3 and 3T3-L1 cells

Prior to any treatment, 100 ml of $2.5x10^4$ cells of 3T3 and 3T3-L1 cell lines were seeded into each well of the black 96-well plate. Seeded cells were then incubated in the humidified incubator at a temperature of 37^oC, with a concentration of 5% CO_2 for 24 hours. The next day, those plates were ready for concentration treatment.

3.3.3 Treatment with GA, GO and GAGO

In this study, 3T3 and 3T3-L1 cell lines were treated with 3 different compounds; Gallic Acid (GA), Graphene Oxide (GO) and 9 different concentration of Gallic Acid-Loaded Graphene Oxide (GAGO). Following method mentioned in **Section 3.3.2**, the cells were treated with 200 µl of GA, GO and GAGO (0, 5, 10, 20, 50, 100, 200, 250 and 500µM) for 12 and 24 hours. Native GA and native GO were used as comparisons to GAGO nano

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formulation, and H_2O_2 was used as a positive control (100 µM) in this study. All treatments were conducted in triplicate, and in three independent experiments.

3.3.4 Loading of DCFHDA Reagent and Reading of ROS Results

To evaluate the oxidative status, all the content in each well were removed after 12 hours of post treatment by sucking them about 170µl before washing and rinsing with 100µl of PBS. Remaining cells were then incubated for 60 minutes with 100µl of 20µM DCFHDA diluted in DMSO. DCFHDA reagent is used to detect peroxyl radical and hydrogen peroxide. Before leaving in the 37 ^{o} C incubator, the plates were wrapped with aluminium foil as the DCFHDA reagent is sensitive to light. The nonpolar and non-ionic DCFHDA that crosses the cell membranes will be hydrolyzed to non-fluorescent 2′, 7′-dichlorofluorescein (DCFH) by intracellular esterases. After incubating them in humidified incubator with a concentration of 5% CO_2, the plates will be read by using fluorescent microplate reader at optical density of 485 excitation and 520 emission. The same procedures were repeated after 24 hours of post treatment.

3.4 Statistical Analysis

Mean ± standard mean error (SEM) was used to present data in triplicate from three independent experiments. A GraphPad Prism software version 8 was used for statistical analysis to assess the differences between groups by using Two-way Variance Analysis (ANOVA) followed by Tukey's post-hoc study for multiple comparisons. A p-value of less than 0.05 is considered significant.

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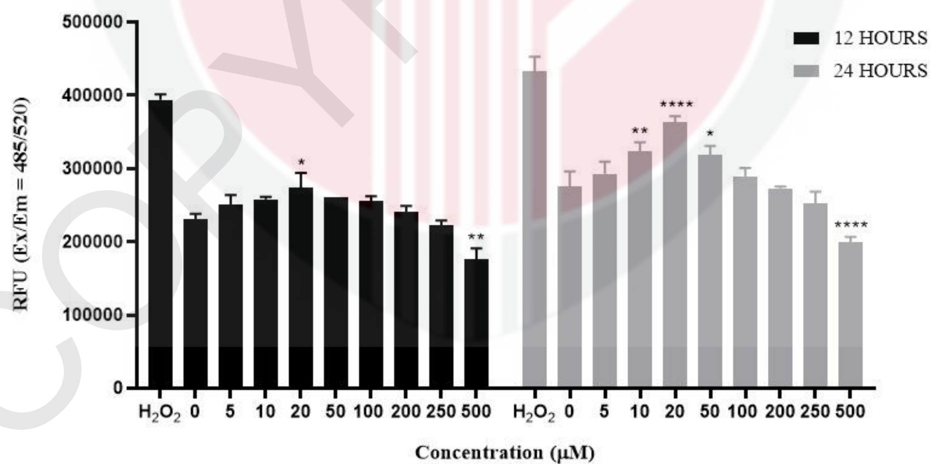
CHAPTER 4

RESULTS

4.1 Accumulation of ROS production on 3T3 and 3T3-L1 Cells upon post treatment

Previous investigation stated that the levels of intracellular ROS were taken in account by change in relative units of fluroscence (RFU) (Wang et al., 2018). The data were analyzed using multiple test comparisons. In all cell lines, positive controls were exposed to 100 µM H_2O_2 as stated in a study done by H. Alexander, S. Syed Alwi, L. Yazan et al. (2019). In general, combination of GA and GO nanoformulation enhanced antioxidant property on L1-treated cells better than 3T3-treated cells. From the overall graph, GAGO expressed better antioxidant level by observing low number of ROS.



(A)

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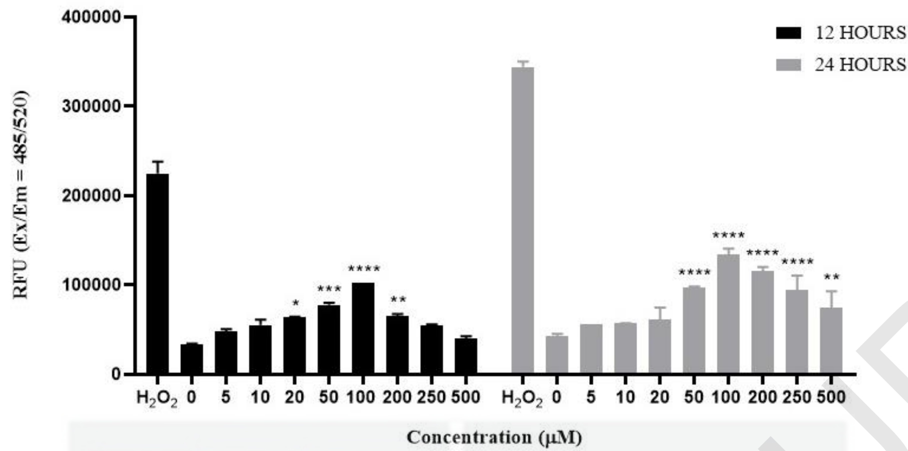
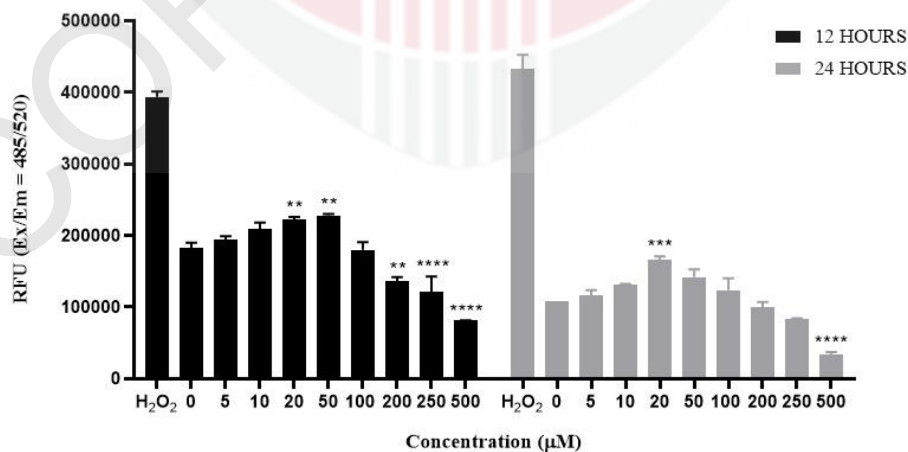


(B)

Figure 4.1.1: Gallic acid-loaded on 3T3 and 3T3-L1 cells effects on ROS production. Both cells types were incubated under normal condition for 12 and 24 hours. (**A**) 3T3, (**B**) 3T3-L1. All data are the mean ± S.E.M of three independent experiments. * $p < 0.05$, ** $p < 0.01$, ***p<0.001, ****p<0.0001 which is significantly different from the untreated group (0 µM concentration).



(A)

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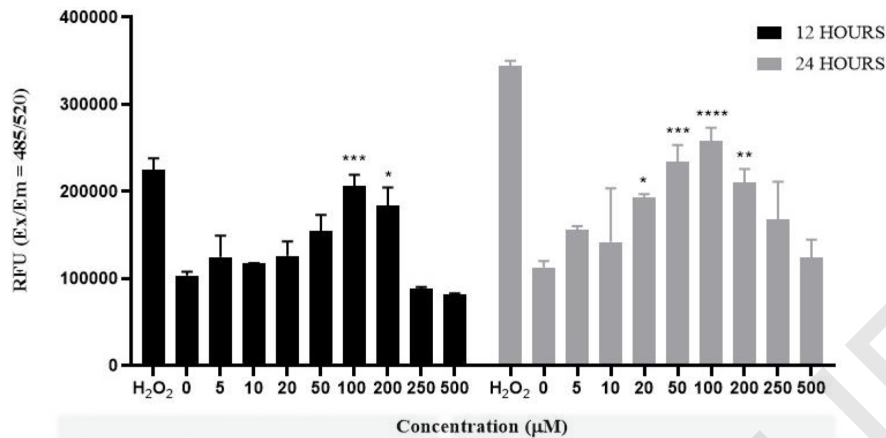
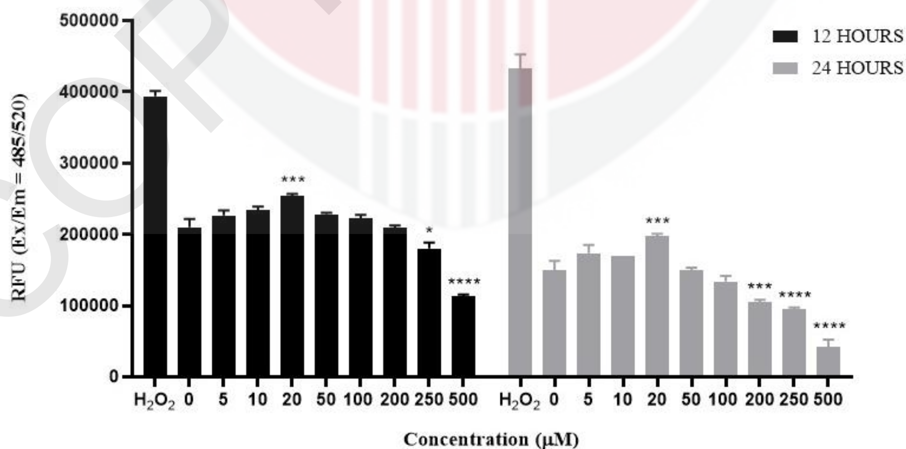


(B)

Figure 4.1.2: Graphene oxide-loaded on 3T3 and 3T3-L1 cells effects on ROS production. Both cells types were incubated under normal condition for 12 and 24 hours. (**A**) 3T3, (**B**) 3T3-L1. All data are the mean ± S.E.M of three independent experiments. * $p < 0.05$, ** $p < 0.01$, ***p<0.001, ****p<0.0001 which is significantly different from the untreated group (0 μM concentration).



(A)

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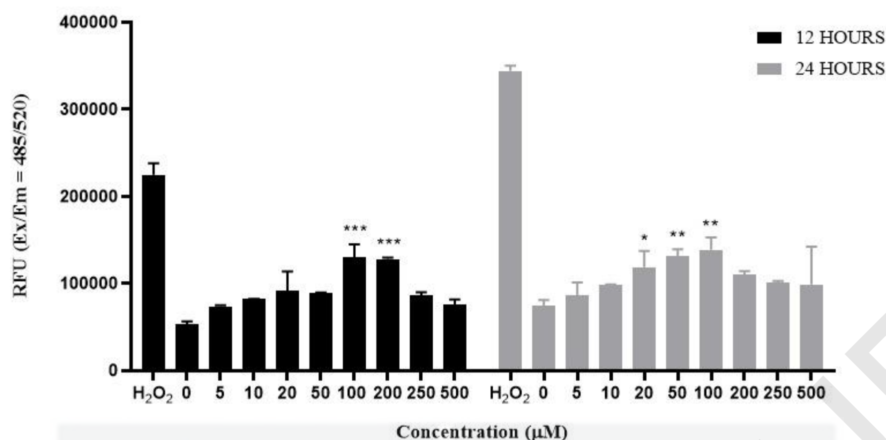

(B)

Figure 4.1.3: Gallic acid-loaded-graphene oxide treated on 3T3 and 3T3-L1 cells effects on ROS production. Both cells types were incubated under normal condition for 12 and 24 hours. (**A**) 3T3, (**B**) 3T3-L1. All data are the mean ± S.E.M of three independent experiments. * $p < 0.05$, ** $p < 0.01$, ***p<0.001, ****p<0.0001 which is significantly different from the untreated group (0 μM concentration).

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CHAPTER 5

DISCUSSION

5.1 ROS Assay

Normal skin layer consists of epidermis, dermis and subcutaneous layer. When there is deep cut or injury occur on skin that lead to bleeding and wound formation, it may reach until the dermis and subcutaneous layer (Guo, Xu, Ma, Huang, & Gao, 2010). So, platelet and fibrin play important role in clotting and healing the wounded area. Due to that, wound undergo through four stages of healing before reaching full recovery. The four stages are homeostasis, inflammation, proliferation and maturation (Alberts et al., 2019). The maturation or remodelling stage is the most important phase as it determines the strength and manifestation of the healed tissue.

However, the mechanisms are different in normal and diabetic wound healing. DM is a chronic hyperglycaemia condition affecting more than 124 million people worldwide. Impaired wound healing is one of the major complications in DM. Wound healing is a normal physiological process that occurs through a sequence of co-ordinated cellular and cytokine-mediated events that culminate in restoring tissue functional integrity. In normal wound healing, high number of fibroblast bring along VEGF (Vascular Endothelial Growth Factor) to initiate regrowth of epithelial cell.

While in diabetic wound, insufficient of angiogenesis which is the formation of new blood capillary, hypoxia condition, macrophage function as well as lower number of fibroblast delay the re-epithelization and wound closure. So, in this research, it focused on antioxidant property that relates to the rate of wound healing. When talking about antioxidant, somehow it has association with reactive oxygen species. In normal wound

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healing response, reactive oxygen species (ROS) plays a crucial role. They serve as secondary messengers to other immunocytes and non-lymphoid cells involved in the healing cycle and appear to be critical in organizing lymphoid cell recruitment to the wound site and successful tissue repair (Dunnill et al., 2017).

However, ROS also involves in signal transmission during oxidative stress that can damage muscle tissue, inhibit output and induce apoptosis. Some studies have documented that accumulation of ROS can activate the pathway to apoptosis by altering the potential of mitochondrial membrane (Hekimi, Wang, & Noë, 2016; Moungjaroen et al., 2006; Musumeci, Maria Trovato, Imbesi, & Castrogiovanni, 2014). That's why excessive amounts of ROS results in various diseases such as autoimmune diseases, cardiomyopathies and other inflammatory diseases (Li et al., 2013). It can be very harmful to humans. Therefore, the most top priority goal of this study was to pay attention to the effects of ROS that cause oxidative damage and delay wound healing.

Looking at ROS in diabetic patient, diabetic patient with vascular disorder, including obstructed vascular sclerosis, results in decreased circulation in the vascular circulation. Declining vascular circulation will cause hypoxia. Hypoxia is a condition that improves and enhances inflammatory reactions as well as increases the ROS level. These radical oxidant free or ROS will delays the healing of wounds. ROS also often caused by an increase in blood sugar level. This is how ROS works in diabetic patient's wound healing.

A paper written by Christopher Dunnill and his friends (2017) stated that low levels of ROS are essential in stimulating effective wound healing. Nevertheless excessive ROS release results in cellular damage and impaired wound repair. In conjunction with that, it is known that ROS are needed at low levels in cells to prevent oxidative damage. In order to ensure that these ROS signals do not reach damaging levels, it becomes a need to encounter it

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with antioxidant strategy. This is where GA was introduced in this study. GA, known as 3,4,5-trihydroxybenzoic acid is a type of phenolic acid, found in gallnuts, sumac, witch hazel, tea leaves, oak bark and other plants (EMBL-EBI, 2018).

GA is a well-known natural antioxidant that is basically a secondary polyphenolic metabolite. So this will eventually reduce number excessive of ROS (Yang et al., 2016). Previous study entitled Gallic Acid-loaded-Graphene Oxide Based Nanoformulation (GAGO) act as potential anti-bacterial agent against Staphylococcus *aureus* (Shamsi, Elias, Narti Edayu Sarchio, & Md Yasin, 2018). The results showed the effectiveness of GAGO as their treatment was likely attributed to the ability GO (the carrier), rather than GA itself. Regardless of those advantages of GA, it also has its own limitations. Therefore, the carrier was introduced to GA which is the graphene oxide (GO), and make it into nano formulation become GAGO.

Because of the limitations, this is the reason of why formulating new compound called GAGO become a must. By doing this, we can actually reduce the cytotoxicity that might give the adverse effect to the patient. The question rises in the present work whether the synthesized nano formulation, GAGO is toxic or not. This is because the aim is to load the antioxidant agent in the nano formulation as drug delivery system for faster wound healing therapy.

According to **Figure 4.1.1 (A)**, in normal cells treated with GA, it can be seen the pattern consistently increased from 0 µM of concentration until 20 µM and then ROS level drop as the dose become higher up until 500 µM indicating that this treatment works in dose-dependent manner. After 12 hours post treatment, there was significant different detected in comparing the concentration of 20 µM and 500 µM with untreated cells with $p = 0.0187$ and $p = 0.0016$ respectively. After 24 hours post treatment, GA showed significant different on 10

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μM (p = 0.0066), 20 μM (p < 0.0001), 50 μM (p = 0.0175) and 500 μM (p < 0.0001) concentration.

While for **Figure 4.1.1 (B)**, the induced cells showed only slight increase from 0 μM up until 100 uM and then declined on higher concentration suggesting that all of this compound GA, GO and GAGO started to release their therapeutic effect which is the antioxidant property consistently at 100 μM in diabetic cells. After 12 hours post treatment, there was significant different in 20 μM with $p = 0.0122$, 50 μM ($p = 0.0003$), 100 μM ($p < 0.0001$) and 200 μM ($p = 0.0082$). Later, GA expressed absolute significant different in most of the concentrations 12 hours later; 50 μM ($p < 0.000$), 100 μM ($p < 0.0001$), 200 μM ($p < 0.0001$), 250 μM ($p < 0.0001$) and in 500 μM ($p = 0.0077$).

It is said in a study that, elevated ROS, which are continually developed and maintained longer in cells, are specifically related to compromised persistent non-healing wound repair (Schäfer & Werner, 2008). That's why ROS level started to drop at 100 μM for 3T3-L1. And for 3T3, the compounds already show their effects as early as at 20 μM. Therefore, it was a bit late in 3T3-L1. And then, moving on to the carrier for this nano formulation, which is the GO. As compared to our GAGO, GO gives the best treatment for 3T3 as in **Figure 4.1.2 (A)** and posed significant different for the most of concentrations; 20 μM ($p = 0.0043$), 50 μM (p = 0.0011), 200 μM ($p = 0.0012$), 250 μM ($p < 0.0001$) and at 500 μM ($p < 0.0001$) during 12 hours post treatment and only showed significant different at 20 μM and 500 μM with $p = 0.0008$ and $p < 0.0001$, respectively.

While in 3T3-L1 or in **Figure 4.1.2 (B)**, ROS produced the highest in GO. After 12 hours of treatment, GO also expressed significant different at 100 μM ($p = 0.0009$) and 200 μM ($p = 0.0113$) concentrations. 12 hours later, GO showed significant different on concentration of 20 μM ($p = 0.0115$), 50 μM ($p = 0.0001$), 100 μM ($p < 0.0001$) and 200 μM

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(p = 0.0017) apparently. This is relatable with one of our team's paper (Ghafor, Elias, Shamsi, Yasin, & Sarchio, 2020), indicating that GO has the highest toxicity. This means GO is the least effective and GA is the best in treating diabetic wound. Previous study also had investigated the effects of GA on DM-induced delayed wound healing in fibroblast with high glucose-containing medium to mimic diabetic condition. And according to this study, it is reported that GA has antioxidant properties and effects on cell migration, indicating that this compound exhibits more healing potential for chronic DM wound (Yang et al., 2016).

Apart from that, when we look at the effects of our compound of interest, which is the GAGO nano formulation on normal cells as in **Figure 4.1.3 (A)**, when we combine GA and GO together, it improves the outcome with significant different at 20 µM (p = 0.0004), 250 µM (p = 0.0168) and 500 µM with $p < 0.0001$ when comparing with untreated cells after 12 hours post treatment. Meanwhile, after 24 hours, 3T3 cells showed significant different at 20 µM (p = 0.0001), 200 µM (p = 0.0002), 250 µM ($p < 0.0001$) and 500 µM ($p < 0.0001$). As for **Figure 4.1.2 (B)**, when GAGO treated on 3T3-L1, after 12 hours post treatment, there was significant different detected in the concentration of 100 µM and 200 µM with p = 0.0001 and p = 0.0002 respectively. After 24 hours post treatment, GAGO showed significant different on 20 µM (p = 0.0373), 50 µM ($p < 0.0042$), and 100 µM (p = 0.0013) concentration.

Zhang et al. (2016) stated in recent years, nanotechnology has been widely applied in drug delivery system due to the suitable method for site-specific and time-controlled delivery of bioactive agents. The nano size helps in improving solubility of hydrophobic drugs, increasing drug accumulation in tumors, enhancing the stability of therapeutic agents against chemical or enzymatic degradation, and decreasing cytotoxic side effects. Somehow GAGO lessen the toxicity of GO against 3T3-L1. At the same time, it maintains the effectiveness of

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anti-diabetic GA in this nano formulation in par with the normal fibroblast. It can be seen that all graph displayed bell-shaped graph in general.

Looking at 3T3 cell lines pattern, from 12 to 24hours, the ROS level decreased. This is because maybe the compounds give effects as early as 12 hours and then start killing the cells as in previous research, ROS started to increase for 3T3 cells after 24 hours post treatment eventhough the cells were treated with other compounds such as TQ and TQ-NLC (Alexander, Syed Alwi, Yazan, Zakarial Ansar, & Ong, 2019). While for L1, we can see ROS level inclined from 12 to 24 hours. It is proved in a study that, these nanoparticles started to show its effectiveness on drug delivery after 24 hours. Few sets of experiment were conducted up until 48 and 72 hours but it seemed the RFU is decreasing over time as the cells begin to undergo apoptosis.

And in my opinion, GA, GO and GAGO suggested to give minor therapeutic effect after 24 hours and onwards. In other word, the effects are significant over time. In conclusion, we can say that when we compared normal fibroblast, GA shows good effects and works better prominently in 3T3-L1. Meanwhile, GAGO exhibit better anti-oxidant level successfully by observing consistent low number of ROS production in both normal and diabetic fibroblast. Therefore, the reason for developing drug delivery systems is to increase the therapeutic effectiveness of the drug and its adverse side effects by increasing the volume and length of the medication between the target cells and the sensitivity to non-target cells (Nagpal, Singh, & Mishra, 2012).

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CHAPTER 6

CONCLUSION AND RECOMMENDATION

6.1 Conclusion

Graphene oxide (GO), which is highly toxic towards 3T3-L1 cells as low as 20 µM concentration, showed highly significant in RFU level as well as reduced percentage of cell viability. Hence, the non-toxic gallic acid (GA) showed significant antioxidant effects on 3T3-L1 induced cells. GO used in this study acts as drug carriers as they are expected to break the drug resistance from reaching the targeted region. Hence, the synthesized GAGO were expected to carry drug to the intended place and ensure the drug release in a controlled, at the intended place, improve therapeutic efficacy and minimize the side effects.

6.2 Future Recommendation

Although the objective of this study has been achieved, there are some recommendation which could be used to improve and further understand about the effects of GAGO on both normal (3T3) and diabetic (3T3-L1) fibroblast cells. These recommendations include:

- Determine the proper induction of oxidative stress on the 3T3 and 3T3-L1 cells. The different between normal cells and diabetic cells need to be at least two-fold different.
- Conduct *in vivo* study to relate the effectiveness of this nano formulation strategy with real wound healing properties on skin for obtaining new reliable data.

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