UNIVERSITI PUTRA MALAYSIA

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ABSTRACT

Wound Healing Property of Gallic Acid-Loaded Graphene Oxide (GAGO) Nano-Formulation on Diabetic Murine Fibroblast

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Introduction: Diabetes mellitus (DM) is a chronic disease caused by insufficient insulin produced by the pancreas or ineffective use of insulin in body, which could cause delayed in wound healing. Wound happens when the integrity of the body tissues such as skin is damaged. Fibroblasts in skin dermal layer is vital to produce the connective tissue needed for wound healing. Gallic acid (GA) is a phenolic acid, which exists in most plants and it has been shown to have antioxidant features which enhance wound healing. However, when administered *in vivo*, GA has a short half-life. Nanomaterials are small in size, which is helpful to study the drug mechanisms and improve therapeutic effect. Graphene oxide (GO) is made up of a single-atom-thick layer of graphene sheets. GO properties including its physical (light), strength, thermal, and electrical conductivity make them ideal for active drug delivery. **Objective:** This study aims to assess the wound healing property of newly formulated Gallic Acid-loaded Graphene Oxide (GAGO) on diabetic murine fibroblast, with comparison to its native compounds, pure GA and pure GO. **Methodology:** Scratch assay was performed to assess the progress of wound closure on 3T3-L1 diabetic murine fibroblast-treated with GAGO nano-formulation at eight different concentrations (0μM-250μM). Photos were taken daily up to 72hr from each group and analysed using ImageJ software. **Results:** All treatment groups demonstrate concentration-dependent effects. Complete wound closure was observed in all treatment groups when treated with concentration <100μM. Although pure GA shows better wound closure at concentrations 100-200μM, it is toxic at 250μM to the cells. Interestingly, unlike pure GA and pure GO, GAGO significantly maintained its wound healing property up to 250μM. **Discussion:** Detachment of 3T3-L1 cells-treated with pure GA and pure GO, but not GAGO, observed at higher concentrations might be due to the toxicity effect of the pure compounds. **Conclusion:** These findings suggest that compared with pure GA and pure GO, encapsulation of GA on GO nano-carrier reduces the toxicity of this new formulation in diabetic fibroblast-treated cells. GAGO nano-formulation is comparable to pure GA while it improves wound healing performance when compared to pure GO.

Keywords: Diabetes mellitus, wound healing, gallic acid, graphene oxide, nano-formulation

ABSTRAK

Sifat Penyembuhan Luka Formulasi Nano Grafena Oksida yang Dimuatkan Asid Gallic (GAGO) pada Fibroblas Diabetik Mencit

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Pengenalan: Diabetes mellitus (DM) adalah penyakit kronik yang disebabkan oleh insulin yang tidak mencukupi yang dihasilkan oleh pankreas atau penggunaan insulin yang tidak berkesan dalam badan, yang boleh menyebabkan kelewatan dalam penyembuhan luka. Luka berlaku apabila integriti tisu badan seperti kulit rosak. Fibroblas dalam lapisan dermis kulit adalah penting untuk menghasilkan tisu penghubung yang diperlukan untuk penyembuhan luka. Asid Gallic (GA) ialah asid fenolik, yang wujud dalam kebanyakan tumbuhan dan ia telah terbukti mempunyai ciri antioksidan yang meningkatkan penyembuhan luka. Walau bagaimanapun, apabila ditadbir secara *in vivo*, GA mempunyai separuh hayat yang pendek. Bahan nano yang bersaiz kecil dapat membantu dalam pengkajian mekanisme ubat dan peningkatkan kesan terapeutik. Grafena Oksida (GO) terdiri daripada lapisan grafena setebal atom tunggal. Sifat GO termasuk fizikal (cahaya), kekuatan, terma dan kekonduksian elektrik menjadikannya sesuai untuk penghantaran ubat aktif. **Objektif:** Kajian ini bertujuan untuk menilai sifat penyembuhan luka Grafena Oksida (GAGO) yang dimuatkan Asid Gallic yang baru dirumus pada fibroblas murine diabetes, dengan perbandingan dengan sebatian aslinya, GA tulen dan GO tulen. **Metodologi:** Ujian calar dilakukan untuk menilai kemajuan penutupan luka pada fibroblas diabetik mencit 3T3-L1 yang dirawat dengan formula nano GAGO pada lapan kepekatan berbeza (0μM-250μM). Foto diambil setiap hari sehingga 72 jam dari setiap kumpulan dan dianalisis dengan menggunakan perisian ImageJ. **Keputusan:** Semua kumpulan rawatan menunjukkan kesan yang bergantung kepada kepekatan. Penutupan luka lengkap diperhatikan dalam semua kumpulan rawatan apabila dirawat dengan kepekatan <100μM. Walaupun GA tulen menunjukkan penutupan luka yang lebih baik pada kepekatan 100-200μM, ia adalah toksik pada 250μM kepada sel. Menariknya, tidak seperti GA tulen dan GO tulen, GAGO mengekalkan sifat penyembuhan lukanya dengan ketara sehingga 250μM. **Perbincangan:** Detasmen sel 3T3-L1 yang dirawat dengan GA tulen dan GO tulen, tetapi bukan GAGO, diperhatikan pada kepekatan yang lebih tinggi mungkin disebabkan oleh kesan ketoksikan sebatian tulen. **Kesimpulan:** Penemuan ini mencadangkan bahawa berbanding dengan GA tulen dan GO tulen, enkapsulasi GA pada pembawa nano GO mengurangkan ketoksikan formulasi baru ini dalam sel yang dirawat fibroblas diabetes. Formulasi nano GAGO adalah setanding dengan GA tulen sementara ia meningkatkan prestasi penyembuhan luka jika dibandingkan dengan GO tulen.

Kata kunci: Diabetes mellitus, penyembuhan luka, asid gallic, grafena oksida, formulasi nano

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
Anti-GAD	Antibodies to glutamic acid decarboxylase
ATCC	American Type Culture Collection
CDC	Centers for Disease Control and Prevention
COX-2	Cyclooxygen-ase-2
DKA	Diabetic ketoacidosis
DG	Dodecyl gallate
DM	Diabetes mellitus
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DFU	Diabetic foot ulcer
EC	Epicatechin
ECG	Epicatechin gallate
ECM	Extracellular matrix
EGC	Epigallocatechin
EGCG	Epigallocatechin gallate
EGF	Epidermal growth factor
FFA	Free fatty acids
FAK	Focal adhesion kinases
FCS	Fetal calf serum
FGF2	Fibroblast growth factor 2
GA	Gallic acid
GAGO	Gallic Acid-Loaded Graphene Oxide
GCG	Gallocatechin gallate
GDM	Gestational diabetes mellitus
GGA	Gelatin-gallic acid
GO	Graphene oxide
HG	Hexadecyl gallate
IA-2	Islet Antigen 2

IFN-γ	Interferon-γ
IL-1	Interleukin-1
IL-1β	Interleukin 1β
IL-6	Interleukin-6
IL-10	Interleukin-10
IDDM	Insulin-dependent diabetes mellitus
IGF-1	Insulin-like growth factor-1
MG	Methyl gallate
MMPs	Metalloproteinases
MRI	Magnetic resonance imaging
NF-κB	Nuclear factor κB
OG	Octyl gallate
PBS	Phosphate Buffer Solution
pDNA	Plasmid DNA
PET	Positron emission tomography
PG	Propyl gallate
rGO	Reduce graphene oxide
ROS	Reactive oxygen species
rpm	Resolutions per minute
SEM	Standard error of mean
SSD	Silver sulfadiazine
TCC	Total contact cast
TG	Tetradecyl gallate
TGFβ	Transforming growth factor-β
TNF- α	Tumor necrosis factor
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
VEGF	Vascular endothelial growth factor

CHAPTER 1

INTRODUCTION

1.1 Background

Diabetes mellitus (DM) is a chronic disorder due to the pancreas producing inadequate insulin or the body's ineffective use of insulin (World Health Organization, 2021). According to statistics of International Diabetes Federation (2021), Malaysia is one of the five countries for the largest number of diabetes patients in the Western Pacific Region. In 2021, 4.4 million Malaysian aged between 20 to 79 years are living with diabetes which caused USD 1090.7 dollars in health expenditure per person. It is expected that the number of diabetic patients and health expenses will further increase in the future. According to World Health Organization (2021), the risk of foot ulcers and infection will rise as wound healing is impaired, eventually leads to amputation. Hence, DM become a major public health concern in Malaysia.

Several studies have been done on understanding the roles of fibroblasts in various tissues during wound healing. Fibroblasts in the skin dermal layer play an important role in the production of the connective tissue needed for wound healing. For example, fibroblast secrete growth factors, collagens, cytokines and other components of extracellular matrix (ECM) (Addis et al., 2020). Meanwhile, migration and proliferation of fibroblasts will start the proliferative phase in the healing process.

Wound occurs when the integrity of the body tissues such as skin, mucous membranes, and organ tissues is damaged. There are four phases involve in normal wound healing; (1)

hemostasis, (2) inflammatory, (3) proliferation and (4) remodeling. In diabetic patients, wound healing still undergoes hemostasis (Shamiya et al., 2021). However, during inflammatory phase macrophages stay for a long time the site of the injury, which lead to increase amount of pro-inflammatory cytokines and high levels of reactive oxygen species (ROS), leading to persistent inflammation. Consequently, wound healing proses is interrupted and proliferation and remodeling phases could not take place, which results in impaired wound healing.

Various drugs and compounds have been used to test wound healing ability. One of these drugs is gallic acid (GA). GA is a phenolic acid and exists in most of the plants. It is a well-known natural antioxidant and has great wound healing properties (Yang et al., 2016). However, GA has a short half-life when administered *in vivo* (Khan et al., 2019). As a result of this limitation, an individual may need to spend more money to use this drug as a treatment.

In order to increase the therapeutic effect of GA and contribute to the study of drug mechanisms, nanotechnology have been used (Ghafor et al., 2020). GA compound was loaded to a nanocarrier, Graphene Oxide (GO) to form a nano-formulation called Gallic Acid-loaded Graphene Oxide (GAGO). These nanomaterials are small in size and can improve the drug delivery (Ghafor et al., 2020). GO is made up of a single-atom-thick layer of graphene sheets and has a large surface area to load the other drug. Its properties such as its physical (light), strength, thermal, and electrical conductivity make them ideal for active drug delivery.

1.2 Problem Statement

The problem statement of this study is that many Diabetes Mellitus (DM) patients have stalled chronic wounds due to hyperglycemia. There is a limit of available therapies for impaired wound healing in DM patients due to the side effect and cost effectiveness.

1.3 Justification

The justifications of this study are:

- GAGO is a potential therapeutic agent to improve wound healing.
- GAGO improve therapeutic effect and decrease drug toxicity (Cheng, Z. et al, 2012).

1.4 Objective

1.4.1 General Objective

The general objective of this study is to assess the wound healing property of GAGO nano-formulation on diabetic murine fibroblast, with comparison to its native compounds, pure GA and pure GO.

1.4.2 Specific Objective

a) To evaluate the wound closure of diabetic murine fibroblast cell line (3T3-L1) with the treatment of pure GA, pure GO and GAGO.
b) To compare the wound closure of diabetic murine fibroblast cell line (3T3-L1) using GAGO treatment with the wound closure of diabetic murine fibroblast cell line (3T3-L1) using pure GA and pure GO treatment.

1.5 Hypothesis

It is hypothesized that the GAGO nano-formulation will accelerate wound closure in diabetic fibroblast-treated cells.

CHAPTER 2

LITERATURE REVIEW

2.1 Diabetes Mellitus (DM)

2.1.1 Type 1 Diabetes Mellitus (T1DM)

Type 1 Diabetes Mellitus (T1DM) is known as insulin-dependent diabetes or juvenile diabetes. In Malaysia, there are 10,184 diabetic patients enrolled in the National Diabetes Registry suffering from T1DM in 2020 (Chandran & Zakariah, 2021). According to the Centers for Disease Control and Prevention (CDC) (2022), approximately 1 in every 10 diabetic patients suffered T1DM. It is commonly presenting in children or adolescents (Sapra & Bhandari, 2021) and starts at the earlier age of life. In some cases, T1DM can begin at birth and gradually increasing with age, before reaching its peaks at the ages of 4 to 6 and 10 to 14. The incidence of T1DM in children of both sexes is equal.

T1DM is caused by the autoimmune destruction of pancreatic β cells which lead to the insufficient or absent of the insulin in the body (Paschou et al., 2018). Insulin is required in converting glucose to glycogen, which is stored in fat, liver and muscle cells to prevent the blood sugar level from being too high. Lack of insulin will lead to an increase in blood glucose level. Over time, this causes several complications such as Diabetic ketoacidosis (DKA), amputations, retinopathy, neuropathy, nephropathy, strokes, heart disease, and blindness. T1DM patient are prone to develop other autoimmune diseases such as celiac disease and thyroid disorders. The destruction of pancreatic β cells can be caused by genetic, environment and immunologic factors. For non-immune type 1 diabetes, this condition is very uncommon (Moini, 2019). T1DM also known as idiopathic (type 1B)

diabetes, which is more fulminant disease. It is also secondary to pancreatitis diseases. This is a common insulin deficiency that occurs in Asian and African descendants in different degrees.

2.1.2 Type 2 Diabetes Mellitus (T2DM)

Type 2 Diabetes Mellitus (T2DM) is another subtypes of DM, which is also called non-insulin-dependent DM or adult-onset diabetes. It is caused by insulin resistance and relative insulin deficiency (Ginter & Simko, 2013). According to National Diabetes Registry Report 2020, 1,687,384 of Malaysian were suffered T2DM (Chandran & Zakariah, 2021) with ratio of 9 in every 10 diabetic patients (Moini, 2019). T2DM commonly affecting middle-aged and elderly people with long-term hyperglycemia, and generally presents in individuals over 45 years of age (Sapra & Bhandari, 2021; Goyal & Jialal, 2018). However, there are increasing pattern in T2DM cases in children, adolescents, and younger adults due to obesity, physical inactivity, and energy-dense diets. (Sapra & Bhandari, 2021). Males are more likely to have T2DM than females (Moini, 2019).

Sapra & Bhandari (2021) stated that the useful early identification of T2DM is fasting glucose levels and HbA1c testing. Most T2DM patients have a certain tissue sensitivity level to insulin (Moini, 2019). Many organs of the body can influence insulin resistance and chronic hyperglycemia associated with T2DM. Most of T2DM patients have family history of diabetes, with at least one parent with the disease. In the case if one of the twins is affected, there is 90% chance of the other twins to have T2DM as well (Moini, 2019).

GDM patient are at higher risk of developing T2DM. The interaction effect between genetics and lifestyle are complicated in T2DM (Sapra & Bhandari, 2021). T2DM can lead to health complications such as retinopathy, cataracts, neuropathy, hypertension, heart disease, gastroparesis, chronic kidney disease, and steatohepatitis biliary disease (Moini J, 2019).

2.1.3 Other Types of DM

Besides T1DM and T2DM, there are also other types of DM including gestational diabetes mellitus (GDM), neonatal diabetes, and steroid-induced diabetes. Based on National Diabetes Registry Report 2020, there are 0.06% of diabetic patients have other diabetes besides T1DM and T2DM in Malaysia (Chandran & Zakariah, 2021). GDM is a condition that leads to hyperglycemia in pregnancy (Goyal & Jialal, 2018) due to inability to metabolize carbohydrate because of insulin deficiency or insulin resistance (Moini, 2019). GDM disappeared after the birth of the child. The risk factors of GDM include obesity, older age, excessive gestational weight gain, stillbirth, a family history of diabetes, or history of congenital anomalies in previous children (Goyal & Jialal, 2018).

Neonatal diabetes is a monogenic mutation, which develops in the first 6 months after birth. This disease is rare and only happens in one of every 100,000 to 500,000 live births (Moini, 2019). It can be divided into two forms which are permanent and transient. The permanent form does not disappear but the transient form dissipates during infancy, and may reoccurring in later life. Both forms of neonatal diabetes are genetically inherited from either parent of the infant. According to Hwang & Weiss (2014), steroid-induced

diabetes refers to disease with abnormal elevated in blood glucose which is related to the use of glucocorticoids in a patient with or without a prior DM history. Glucocorticoids causes β cell dysfunction (glucose sensitivity and insulin secretion) and insulin resistance in other tissue on glucose metabolism.

2.1.4 Pathophysiology of DM

Environmental-genetic factors are considered as triggering factors for cell-mediated destruction of pancreatic β cells in patients with T1DM (Moini, 2019). Paschou et al. (2018) stated that T1DM is typically found in individuals with no family history and the coincidence rate between twins is 50% (Moini, 2019). Human Leucocyte Antigen (HLA) within the major histocompatibility complex (MHC) region are identified as the main genes predisposing in T1DM (Paschou et al., 2018). It is located on chromosome 6 and contributes about 40–50% of the genetic risk of T1DM development. Other genes that are also contribute to the genetic risk of T1DM development including insulin gene (Ins-VNTR, IDDM 2) polymorphisms on chromosome 1, and the cytotoxic T lymphocyte-associated antigen-4 gene (CTLA-4) on chromosome 2. Genetic associations such as AIRE, TPN22, STAT3, FoxP3, IFIH1, ERBB3 and HIP14 are also considered as genetic factors that lead to T1DM. At present, the reason for the existence of genetic predisposition is still not fully understood (Moini, 2019).

Viruses such as enterovirus, rubella or coxsackievirus B and nutrients such as cow's milk are the environmental factor that causes T1DM (Paschou et al., 2018). These viruses can cause cross-reactivity against pancreatic islet proteins and activate the immune response

(Moini, 2019). As a result, the production of proinflammatory cytokines is activated and may contribute to indirect effects. In addition, pancreatic β cells infected with enterovirus could cause persistent infections, thus triggering innate and adaptive immune responses which lead to functional damage and cell death. Enteroviruses can stimulate cytotoxic T-cell activation and increase autoreactive thymocytes' number by infecting thymic epithelial cells. Beta lactoglobulin in milk may trigger antibodies that interfere with the regulation of T-cell, causing immune reactions to pancreatic β cells.

There are three immunologic factors that cause T1DM, including immune tolerance (central, peripheral, Tregs), cellular immunity and humoral immunity (Paschou et al., 2018). In T1DM, the pancreatic β cells destruction happens through apoptosis. Interferon that is secreted by helper T lymphocytes will activate macrophages and stimulates the inflammatory cytokines secretion such as interleukin-1 (IL-1) and tumor necrosis factor (TNF-α) (Moini, 2019). This leads to more apoptosis and destruction of β cell. For humoral immunity, the autoantibodies produced by the disordered immune response may precede the T1DM clinical manifestations for several years (Paschou et al., 2018). Ellis et al. (1998, as cited in Pachou et al., 2018) stated that about 70% and 60% of T1DM patients had anti-GAD and IA-2 autoantibodies during diagnosis. Insulin autoantibodies may be produced during the destruction of active islet cell and β cells (Moini, 2019).

On the other hand, in the earlier stage, the insulin secretion in T2DM patients is increased to maintain normal glucose levels (Goyal & Jialal, 2018). Over time, the change of β cells cause the insulin secretion failing to maintain glucose homeostasis, thus leading to

hyperglycemia. Most of the T2DM patients are obese or have a high percentage of body fat in the abdominal area. The insulin resistance of T2DM is closely related to the dysfunction of β cells (Goyal & Jialal, 2018). An imbalance between available insulin and the required amount is caused by insulin resistance in the target tissue, relative insulin deficiency or both, which leads to the increase of blood sugar (Moini, 2019). Insulin resistance leads to the inability to inhibit the production of endogenous glucose in the liver, a failure of glucose uptake and glycogen synthesis in skeletal muscles after meal and the inability for the inhibition of hormone-sensitive lipase activation in adipose tissues. This causes excess breakdown of triglyceride in adipocytes as well as excess circulating FFAs.

There are a few epidemiological determinants for T2DM including genetic factors, demographics, lifestyle, and metabolism and intermediate-risk categories. Comparing with T1DM, T2DM has stronger genetic characteristics. Genes are interrelated, such as those encoding β cell mass or function, proinsulin and insulin molecular structure, insulin receptors, hepatic glucose synthesis, glucagon synthesis, and how the cells respond to insulin stimulation. Genetic abnormalities are caused by epigenetic changes that have taken place in several generations under the influence of the environment.

Aging is one of the T2DM risk factors that related to demographics (Moini, 2019). Other risk factors of T2DM that associated with lifestyle are obesity, diet, physical activity and stress. Obesity and aging usually cause insulin resistance which is multifactorial (Sapra & Bhandari, 2021). Patients with T2DM are generally overweight and show the signs of

insulin resistance, including acanthosis nigricans. Acanthosis nigricans are hyperpigmented, velvety patches on the skin of the neck, armpits or groin. A diet high in carbohydrates, sugar and cholesterol can lead to some conditions such as hypertension, high cholesterol and high triglyceride level increase the risk of getting T2DM (Moini, 2019).

2.2 Wound Healing

2.2.1 Introduction to Wound Healing

Wound happens when the integrity of the body tissues such as skin, mucous membranes, and organ tissues is damaged (Herman & Bordoni, 2022). Wound can be classified through several ways, which are the injury mode, exposure to external environment, wound depth, healing time, and potential infection risk (Chhabra et al., 2017). Wound can be formed through mechanical force, thermal, chemical and others. Mechanical force injuries can be further classified into blunt force injuries, sharp force injuries and firearms injuries.

Wounds in which the skin layer is injured by exposing the underlying tissues are known as open wounds, while the wounds that are not severe and the underlying tissues is injured are called closed wound (Chhabra et al., 2017). Besides, wound depth can be divided into partial thickness and full thickness. Partial-thickness wounds are the wound that are injured only at the skin epidermal layer or from epidermis to dermis and the dermis at least partially intact to form new epidermis for wound closure. For full-thickness wounds,

the wounds will fully damage until the underlying layer of the skin. Muscle, adipose tissue, bone and tendon may be exposed.

Wound healing can be classified into primary, secondary and tertiary wound healing (Chhabra et al., 2017). In primary wound healing, glue, sutures, Steri strips or staples are used for wound approximation (Percival, 2002). Blood coagulate and then, inflammation takes place. Granulocytes such as neutrophils are activated in one day to remove bacteria and debris from the wound. Re-epithelization happens within one to two days (Chhabra et al., 2017). After 48 hours, macrophages will replace neutrophils, which will disappear. Maximal neovascularization occurs in day 5. Fibroblast continued to accumulate and proliferate after 14 days. Scar were formed after 30 days.

Chhabra (2017) stated that the repair process in secondary healing is more complex due to a large number of cell losses. Compared with the primary wound healing, the inflammation in secondary wound healing is more intense. More granulation tissues will be produced. The wound has also contracted more. The delayed primary wound healing after four to six days due to the intentional interruption of secondary wound healing and mechanically closing the wound is known as tertiary wound healing. Granulation tissue is prior to this process.

2.2.2 Phases of wound healing

Wound healing is a complicated process, involving the interaction of varies cell types, growth factors, cytokines, and extracellular matrix (ECM). There are four phases of

wound healing including hemostasis, inflammation, proliferation and remodeling (Bai et al., 2020).

During hemostasis phase, the body starts its emergency repair system to stop the wound from bleeding. Platelets aggregation occurs. Thrombin causes the fibrin plug formation, which enhances the platelet clots to form a stable clot (Al-Amer, 2022). The growth factors are released in this phase (Gomes et al., 2017). It can last for two to three hours (Bai et al., 2020). In the inflammation phase, wound debridement happens (Gomes et al., 2017). Cells, such as macrophages, neutrophils, monocytes, migrate from the bloodstream, passing through the tissues and reaching the wound site to destroy bacteria and remove debris (Ezhilarasu et al., 2020). Typically, this phase lasts for hours to days (Bai et al., 2020).

Once the wound is cleaned up, the wound will enter a proliferation phase. The migration and proliferation of endothelial cells and fibroblasts facilitate angiogenesis and the synthesis of ECM (Gurtner et al, 2008, as cited in Bai et al., 2020). Keratinocytes will differentiate to restore the epidermal layer's function (O'toole, 2008, as cited in Bai et al., 2020). Migration of epithelial cells lead to reepithelization to take place (Guo & Dipietro, 2010, as cited in Bai et al., 2020). Proliferative phase usually lasts days to weeks (Gomes et al., 2017). During the remodeling phase, fibroblast convert into myofibroblast (Gomes et al., 2017). The wound is closed. Scar is formed and revised. The tensile strength has increased in general (Patel et al., 2019). This phase usually lasts weeks to several months (Gomes et al., 2017).

2.2.3 Wound Healing in Diabetes

Unlike normal wound healing, the wound healing of diabetic patients is delayed and impaired. This is because it can interrupt all four phases of wound healing, including haemostasis, inflammation, proliferation and remodeling, thus having a long-term negative effect on life's quality, morbidity and mortality (Patel et al., 2019). There are a massive number of macrophages accumulating in the diabetic wounds for a long time (Ezhilarasu et al., 2020). In diabetes wounds, the excessive macrophages secrete higher number of pro-inflammatory cytokines such as tumor necrosis factor α (TNFα), interleukin 6 (IL-6) and interleukin 1β (IL-1β), which leads to a persistent inflammatory response (Ezhilarasu et al., 2020; Spampinato et al., 2020). The release of pro-inflammatory cytokines including IL-1β and TNF-α as well as matrix metalloproteinase-9 (MMP-9) elevated while the release of anti-inflammatory signals such as CD206, growth factor-1 (IGF-1), transforming growth factor-β (TGFβ) and interleukin-10 (IL-10) decreased, resulting in the abnormal fibroblasts and keratinocytes apoptosis (Ezhilarasu et al., 2020). Meanwhile, these causes reduced angiogenesis.

Hyperglycemia causes the epithelial cells to dysfunction of new vessels formation (Spampinato et al., 2020). Hyperglycemia is related to the blood vessels that are stiffer, which will lead to slow circulation and dysfunction of microvascular, resulting in decreased oxygenation of tissues (Dinh et al., 2011, as cited in Spampinato et al., 2020). The changes of blood vessel observed in diabetic patients also explain the decrease of white blood cell migration into wounds, which becomes more susceptible to infections

(Greenhalgh, 2003, as cited in Spampinato et al., 2020). The hyperglycemia itself can damage the function of white blood cells. The density of capillaries in diabetic wound is not enough. Besides, the wound hypoxia aggravation also can be caused by the imbalance of the ratio of angiogenic factors such as TGF-β, fibroblast growth factor 2 (FGF2), vascular endothelial growth factor (VEGF), angiopoietins and angiostatic factors including thrombospondins, angiostatin and endostatin, resulting in the amplification of inflammation (Bai et al., 2020). The decrease of fibroblast differentiation into myofibroblast causes the decrease of ECM mechanical tension (Ezhilarasu et al., 2020).

Impaired diabetic wound healing leads to a complicated pathophysiology including immune, vascular, neuropathic, and biochemical components (Greenhalgh, 2003, as cited in Spampinato et al., 2020). Peripheral neuropathy causes numbness at the area of wound and decrease the feeling of pain. This results in the chronicization of wounds, which will not be observed immediately and treated properly. The features described are especially related to lower limbs, especially feet, which are more susceptible to even small wounds, so they are more likely to be chronic. Furthermore, motor and sympathetic functions alterations result in foot deformation, raised plantar pressure and excessive skin dryness that can be further beneficial to unobserved small wounds and cracks. In addition, impaired diabetic wound healing also leads to non-healing wound involving variety of complications that related to psychiatric stress and depression. For example, these complications include walking difficulty, functional limitations and infection such as septicemia, cellulitis, gangrene, osteomyelitis and abscess.

2.2.4 Current Treatment for Wound Healing in Diabetes

In 10 patients with limb amputations, 5 to 7 are caused by diabetic foot ulcer (DFU) (Kasiya et al., 2017). A large DFU cases need hospitalization and surgical intervention with amputation of the body part of interested (Spampinato et al., 2020). In worldwide, the report showed that one leg is amputated in every half a minute due to DFU (Kasiya et al., 2017). Three years after the first episode, the recurrence rate of DFU was more than 50% (Boulton et al., 2005). Therefore, the healing of damaged diabetic wounds become a major healthcare problem (Spampinato et al., 2020). In addition, impaired wound healing in diabetes also an economic burden to patients and their family due to its costly medical cost for the treatment and management of diabetic foot disease (Raghav et al., 2018).

Currently, there are generally available treatments for impaired diabetic wound healing including dressings, debridement, off-loading pressure, and infection treatment (Rosyid, 2017). Dressings is a traditional treatment method commonly used in the diabetic wound treatment to protect and promote wound healing (Bai et al., 2020; Rosyid, 2017). They have some advantageous features such as moisture, antimicrobial properties, promoting re-epithelization, further preventing wounds and exudate wicking (Gianino et al., 2018). Due to these features, they can maintain a moist environment, contribute to granulation tissue production and epithelialization, reduce the infection risk, accelerate wound healing, deliver growth factors, decrease scar formation, as well as mediate substances that prevent wound healing (Dinh et al., 2011; Spampinato et al., 2020). Dressings can be classified into various types including hydrogel, film, hydrocolloid, composite, foam, alginate and other absorptive dressings, namely negative pressure wound therapy (NPWT) (Galiano &

Mustoe, 2007). However, dressings also have some disadvantages, such as secondary injury, the long treatment process, physiological and psychological adverse effect on patient with diabetes (Bai et al., 2020).

Debridement refers to the action of removing foreign bodies, non-living substances, and unhealthy tissues that are difficult to recover from injury (Rosyid, 2017). It aims to turn the wound healing environment from chronic into acute. Wound debridement combines the wound bed preparation concept by decreasing bacterial load, enhancing healthy granulation tissue, controlling exudate and edema, and disposing necrotic tissue (Dinh et al., 2011). Debridement is performed by removing abnormally damaged base and wound edge tissue, including necrotic dermal tissue, hyperkeratosis of epidermis (callus), fragments and bacterial components that stop wound healing (Rosyid, 2017). It can be divided into five types, namely surgery, mechanics, enzymatic, biologics, and auto lithics. However, some clinical trials showed that only surgical debridement is effective. Surgical debridement is a kind of sharp debridement, which removes all necrotic tissue and bone. Special proteolytic enzymes including collagenase, papain or urea from papaya, fibrinolysis or DNAse, trypsin, streptokinase-streptodornase combination are used in enzymatic debridement while the proteolytic enzyme that secreted by sterile Lucilia sericata fly larvae is used in biologic debridement to dilute the dead tissue. Mechanical debridement carried out in physical by pressure irrigation, hydrotherapy, dry-wet dressing and lavage whereas autolytic debridement performed in nature which is healthy, moist and perfused ulcers.

A reduction of pressure on the ulcer is known as off-loading (Dinh et al., 2011). Generally, foot is the area where ulcers occur due to high pressure of the foot. Off-loading aims to avoid tissue trauma and to help the wound heal. Dinh et al. (2011) listed that there are a variety of available modalities to help in pressure reduction such as contact casts (TCCs), off-loading shoes, felted foam dressings, complete non-weight bearing, orthotics and short-leg walkers. When bacteria infect diabetic wounds, the treatment of infection is important. Primarily, infection is diagnosed according to the clinical conditions such as edema, erythema, discharge from pus, pain, warmth and softness (Webster et al., 2012, as cited in Rosyid, 2017). The society of America has classified the infection into 3 categories based on infectious diseases, namely mild infections, moderate infection and severe infection.

There are also other treatments such as antidiabetic drugs, growth factors, stem cells, preventive surgery, skin perfusion restoration, local wound care treatment, metabolic control, co-morbidity, biomaterial-based treatment (Bai et al., 2020). Some standard treatments only able to control the symptoms of impaired wound healing and their effective treatment of diabetic wound healing is still limited.

2.3 Gallic Acid (GA)

2.3.1 Introduction of GA

In the plant kingdom, the most plentiful phenolic acid is a 3,4,5-trihydroxybenzoic acid, also known as gallic acid (GA) (Kahkeshani et al., 2019). Its chemical formula is $C_6H_2(OH)_3COOH$. The structure of GA is composed of three phenolic hydroxyl groups,

an aromatic ring and a carboxylic acid group, forming a planar molecule (Badhani et al., 2015). It is a colorless or yellowish crystalline compound (Kahkeshani et al., 2019). There are two types of GA derivatives namely ester and catechin derivatives (K. Wang et al., 2020). Alkyl esters are the most ordinary ester derivatives of GA. It consists of dodecyl gallate (DG), hexadecyl gallate (HG), methyl gallate (MG), octyl gallate (OG), propyl gallate (PG), and tetradecyl gallate (TG). The main catechin derivatives includes epicatechin (EC), epigallocatechin (EGC), epigallocatechin gallate (EGCG), epicatechin gallate (ECG), and gallocatechin gallate (GCG). They consist of carbochains of different lengths attached to the carboxyl group. (J. Gao et al., 2019).

GA can be obtained from plant sources such as oak bark, gallnuts, sumac, and others (Ghafor et al., 2020). Besides, GA is also found in drinks including wine and tea (Badhani et al., 2015). A few external stimuli, including chemical stressors, UV radiation and microbial infections, can affect the amount of GA and the composition of phenolic compounds in plant tissues. Grape variety, processing method, and storage are the factors that affect the composition of phenolic compounds in wines and grape juices. In addition, the isolation of GA from various plant species including Punica spp. and Quercus spp. can be done through a variety of chromatographical methods (Kahkeshani et al., 2019). Tannase, a type of glycoprotein esterase is used for the hydrolytic breakdown of tannic acid to form GA (Fernandes & Salgado, 2016, as cited in Kahkeshani et al., 2019).

According to Gao et al., 2019), GA and its derivatives are widely used in food, cosmetic, pharmaceutical industries, dyeing, printing industries and medical uses. They are in

treating many diseases, such as cancer, inflammation, liver diseases, cardiovascular diseases, and neurodegenerative diseases (Shabani et al., 2020). However, GA has its limitation. Its half-life is short when it is administered *in vivo* (Ghafor et al., 2020). In order to improve its application, GA with unique features can be modified and loaded to a drug carrier like graphene oxide (GO). GA has a special conformation, forming intramolecular and intermolecular hydrogen bonds with three adjacent aromatic phenyl groups. It has strong chelating abilities with many inorganic ligands and proteins.

2.3.2 Therapeutic effect of GA

Wang et al. (2020) stated that GA, a polyphenol compound, which is a good source of natural health care products beneficial to human health. GA and its derivatives are widely used in various industries such as food, pharmaceutical, cosmetic, dyeing, and printing industries, as well as medical uses (Gao et al., 2019). In the food industry, they can be used as additives in many foods, including beverages, baked and fried food, condiments and candies, because their free radical scavenging and antioxidant properties can inhibit the oxidation and rancidity of oils and fats (Kahkeshani et al., 2019). They also use as flavoring agents and preservatives in the food industry.

GA is considered to have many therapeutic activities. Various studies have reported that GA has anti-melanogenic properties, anti-inflammatory properties, anti-allergy properties, antimicrobial properties, antiviral properties, anti-carcinogenic properties, antioxidant properties, anti-diabetic properties, and anti-aging properties (Badhani et al., 2015; Gao et al., 2019; Kahkeshani et al., 2019; Yang et al., 2016). Anti-melanogenic and anti-

tyrosinase activities of GA make it a potential skin protecting agent (Badhani et al., 2015). It inhibits the activity of tyrosinase, preventing in the activity of hyperpigmentation. Kim (2006) studied the anti-melanogenesis and anti-oxidation characteristics of GA, which showed that GA is a tyrosinase inhibitor and its effect was stronger than that of the standard anti-tyrosinase agent kojic acid. Besides, a study done by Panich et al. (2012) reported that GA had the protective effect on UVA-mediated melanogenesis.

The anti-inflammatory mechanisms of phenolic compounds are usually considered to be due to their ability to restore antioxidant enzyme activities, regulate inflammation induced by cytokines and kill free radicals (Samad & Javed, 2018). The natural polyphenol GA plays a potential anti-inflammatory role in medicine by inhibiting the activation of p65-NF-κB and IL-6/p-STAT3Y705. Fu et al. (2015) study showed that GA is an effective ingredient in treating allergic contact dermatitis. It can downregulate the release of the pro-inflammatory and inflammatory mediators, including tumor necrosis factor-α (TNF-α), cyclooxygen-ase-2 (COX-2), interleukins, nuclear factor κB (NF-κB) and interferon-γ (IFN-γ), preventing excessive responses of inflammation (Gao et al., 2019). A study done by Kim et al. (2006) showed that GA can be used as anti-allergic agent to block the release of histamine, preventing hypersensitivity.

Furthermore, GA has antibacterial effects on many pathogens such as *Staphylococcus aureus, Streptococcus mutans, Escherichia coli,* etc. and can inhibit the adherence, motility and biofilm formation of pathogen (Gao et al., 2019). GA also showed antifungal activity against *Fusarium semitectum, Alternaria altternata* and *F. fusiformis* (Badhani et

al., 2015). Through the antioxidant/pro-oxidant balance modulation, GA can exert its cytotoxicity and anti-tumor effects (Kahkeshani et al., 2019). Its anti-carcinogenic properties can suppress tumour angiogenesis (Lu et al., 2010). It can also decrease the expression and activity of matrix metalloproteinases, thus inhibiting invasion and metastasis (Kahkeshani et al., 2019).

A study done by Rahimifard et al. (2020) reported that GA can improve the level of mitochondrial complexes I, II, and IV, making it suitable to be the antioxidant agent. Due to its a low molecular weight, GA has a strong antioxidant activity (Shabani et al., 2020). The ability of GA to increase the secretion of insulin in pancreatic islet cells makes it an antidiabetic agent (Rahimifard et al., 2020). GA is a potential anti-aging agent as it can reduce the activity of β-galactosidase and cell cycle arrest in G2/M phase (Rahimifard et al., 2020).

In addition, GA also has the potential of neuroprotective, cardioprotective, hepatoprotective and nephroprotective (Badhani et al., 2015). Due to its antioxidant properties, GA is used to develop treatments for neurodegenerative diseases, namely Alzheimer's and Parkinson's diseases treatment (Gao et al., 2019). The neuroprotective effects of GA reduce β -amyloid peptide accumulation and density to treat Alzheimer's diseases (Shabani et al., 2020). As for cardioprotective properties of GA, harmful oxidative consequences of myocardial infarction are reduced because of its antioxidant capacity (Kahkeshani et al., 2019). GA can enhance the antioxidant enzymes activity and increase non-enzymatic antioxidant agents' level.

2.3.3 GA in Wound Healing

Skin is the outermost human body layer, which is vulnerable to mechanical injury and damage. There are three main layers of skin namely the epidermis, dermis and hypodermis. They are composed of various cells, tissues and vascular system in the skin. Wound is an injury that leads to the breakage of body tissues. The wound can be formed by a cut, a fall, or a severe impact. Skin have the ability to repair the wound. Wound healing process happen immediately after the wound is produced. It may be affected by many factors, and if it fails to heal in normal period, it will develop into a chronic wound.

A phenolic acid, GA exists in the fruits, wild flowers and leaves of many plants (Yang et al., 2016). It has anti-diabetic, anti-inflammatory, anti-oxidant, anticancer, and anti-aging properties. Various *in vitro* and *in vivo* study had been carried out to prove that GA can aid in wound healing. A study done by Thi et al. (2020) investigated the effect of gelatin-gallic acid (GGA) hydrogels in wound healing efficacy. Cells can be protected by hydrogels from oxidative damage due to their ROS-scavenging properties. It also promotes the process of wound healing by healing skin with high quality. The study proved that GA improved re-epithelialization and wound remodeling of the treated group compared to the other groups. Besides, Singh et al. (2020; as cited in Comino-Sanz et al., 2021) conducted a comparative study on the wound healing activity of the ethanol extract from Terminalia fruit and its active ingredient, GA in experimentally induced diabetic animals. The result showed that the wound healing activity in both healthy and diabetic

rats are enhanced by the fruit extract of Terminalia bellerica and its dynamic chemical component, GA. However, GA was reported to have higher wound healing effect.

Another *in vitro* study done by Yang et al. (2016) to study the GA effect on wound healing in normal and hyperglucidic human keratinocytes and fibroblasts. They found that GA can upregulate the expression of antioxidant genes, which showed its antioxidant properties. It can protect human keratinocytes from oxidative stress. It triggers the migration of cell in fibroblasts to promote wound healing in both normal and hyperglucidic condition. It activates healing factors including focal adhesion kinase (FAK) or c-Jun N-terminal kinase (JNK) by inducing the phosphorylation of healing factors. Elk-1, a transcription factor, can be activated by extracellularsignal-regulated kinases (Erk) phosphorylation, which is related to the regulation of matrix metalloproteases (MMP)-2 and -9 and lead to the induction of cell migration. Based on the results, GA is a potential wound healing agent to treat wounds, which result from metabolic complications.

2.4 Graphene Oxide (GO)

2.4.1 Introduction of Graphene

Graphene is a kind of carbon-based compound which is composed of single or multi-layer graphite films (Sheshmani & Fashapoyeh, 2013, as cited in Shamsi et al., 2020). The graphite films are composed of honeycomb lattice structure. Its thickness is about 1 nm. Graphene are able to wrap up into 0D fullerenes, roll into 1D nanotubes as well as stack into 3D graphite (Geim & Novoselov, 2007). Graphite is used for the production of graphene via chemical vapor deposition (CVD), mechanical or chemical methods (Choi

et al., 2010; Whitener & Sheehan, 2014, as cited in Zaaba et al., 2017). It is composed of a few graphite layer (Priyadarsini et al., 2018). Many studies on graphene have been carried out for 60 years (McClure, 1956; Slonczewski & Weiss, 1958; Wallace, 1947, as cited in Geim & Novoselov, 2007). It is generally used to describe varieties of carbon-based materials properties (Geim & Novoselov, 2007). There are some members of the graphene family. They are graphene oxide (GO), reduced graphene oxide (rGO), graphene sheets and layered graphenes such as few layered graphenes and multilayered graphene (Acik et al., 2010; Sanchez et al., 2012, as cited in Priyadarsini et al., 2018).

Nowadays, graphene has become a hot topic, and it is a kind of material with great application potential (Zaaba et al., 2017). Its structure makes it larger in diameter, surface area, thickness, conductivity and stiffness (Priyadarsini et al., 2018). It also has many advantageous properties, such as high mechanical strength, molecular barrier abilities, electrical conductivity, high thermal conductivity, high current, density, chemical inertness, optical transmittance and very high hydrophobicity (Chen et al., 2008; Choi et al., 2010; Cui et al., 2016; Kuilla et al., 2010; Lee et al., 2008, as cited in Priyadarsini et al., 2018; Smith et al., 2019). In addition, many studies have investigated that due to its high elasticity, high carrier mobility, electromechanical modulation, ferromagnetism, and spin transport, graphene has become a very promising material, which can be used as a robust atomic-level scaffold in the new nanomaterials design (Rana et al., 2011). Graphene is applied in molecular drug delivery, biosensing, cancer treatment, bioimaging and an assuring platform in tissue engineering because of its biocompatibility and quick functionalization Shen et al., 2012; Wang et al., 2011 (Goenka et al., 2014; Kuila et al.,

2011; Kulshrestha et al., 2014, as cited in Priyadarsini et al., 2018). In the bone repair or organ regeneration field, graphene-based materials are utilized (Priyadarsini et al., 2018).

Varies studies have shown that a promising alternative drug delivery nanocarrier is graphene (Maity et al., 2014). Its large hydrophobic surface makes it suitable for drug adsorption. Moreover, it is usually inert towards biological systems. Nevertheless, Dreyer et al. (2010) (as cited in Maity et al., 2014) found that strong graphene–graphene interactions of graphene resulted in poor solubility of water. When using original graphene, several challenges need to be solved, because van der Waals interaction leads to the difficulty of bottom-up synthesis and agglomeration in solution (Kuilla et al., 2010; Zhu et al., 2010, as cited in Smith et al., 2019). Graphite or other carbon sources can synthesize the compounds that are structurally similar to graphene via a top-down method as an alternative method to perform numerous original graphene advantages. Functionalized oxygen groups also being imbued to the surface.

2.4.2 Graphene Oxide (GO)

Graphene oxide (GO) is a kind of the graphene derivative. It is the oxidized form of graphene (Hummers & Offeman, 1958; Dreyer et al., 2009; R. K. Singh et al., 2016, as cited in Singh et al., 2018). It is synthesized by oxidation of graphite powder through an improved Hummer method. (Marcano et al., 2010, as cited in Abdul Ghafor et al., 2020). It has a mixed structure bearing a variety of oxygen-containing various functional groups like epoxy (>O), hydroxyl (-OH), carbonyl (C=O) and carboxylic (-COOH) groups (Compton & Nguyen, 2010; Gao, 2015, as cited in Singh et al., 2018). GO is one of the

most popular graphene-based products due to its various favorable characteristics (Shamsi et al., 2018).

Due to the strength, physical (optical), electrical conductivity and good thermal of GO, the properties and application characteristics of its nanoparticles such as polymer composites, sensors, "paper" materials, field effect transistors, energy-related materials and biomedical applications are tremendous (Geim & Novoselov, 2007; K. Yang et al., 2013, as cited in Abdul Ghafor et al., 2020). The structure of GO structure allows it to be more hydrophilic on its basal planes and edges (Sheshmani & Fashapoyeh, 2013, as cited in Abdul Ghafor et al., 2020). This helps to form a water-stable suspension and easy to exfoliate into single-layer sheets. The hydrophobic free surface π electrons can form π-π interactions on the basal plane of GO to be used for the loading of drug (Guo et al., 2011, as cited in Shamsi et al., 2018). The hydrophobic molecules can be stabilized by amphiphilic sheet-like molecule in the solution.

GO is suitable for medical applications as it is fluorescent (Abdul Ghafor et al., 2020). Some studies have been conducted previously to prove the antimicrobial activity of GO against gram-positive bacteria and gram-negative bacteria (Hu et al., 2010; Santos et al., 2012; Shamsi et al., 2018, as cited in Abdul Ghafor et al., 2020). Moreover, GO is also being studied for targeted drug delivery in cancer therapy due to its low toxicity (Chang et al., 2011, as cited in Abdul Ghafor et al., 2020).

In a medium, the features of GO such as amphiphilic and planar structure allow it to bind hydrophobic and/or hydrophilic biomolecules to avoid it from being unstable (Hu et al., 2011; Rana et al., 2011; Maity et al., 2014, as cited in Dorniani et al., 2016).The surface area of GO is large, which allows more drugs to be loaded on its surface. Furthermore, research done by Naxin et al. (2015) and Shi & Ye (2015) (as cited in Dorniani et al., 2016) investigated that GO are able to provide good dispersion, easy functionalization and high drug loading efficiency due to its large surface area and oxygen-containing functional groups including carboxyl group, epoxy group and phenolic hydroxyl group.

2.4.3 GO in Wound Healing

Wound healing is the naturally occurring physiological reaction in response to tissue damage (Wallace et al., 2021). Wound healing is a complex process, which involves the interaction among many cell types, cytokines, mediators and the vascular system. Vascular contraction and platelet aggregation play an important role in preventing wound bleeding, and then the influx of various inflammatory cells will release the mediators and cytokines to promote angiogenesis, thrombosis and re-epithelialization. The process of forming new blood vessels from pre-existing vasculature is known as angiogenesis (Mukherjee et al., 2015, as cited in Thangavel et al., 2018). This is a main focus of explaining the role of GO in wound healing.

The potential of GO in promoting angiogenesis in wound healing has aroused great interest in biomedical applications (Rehman et al., 2019). Various in-vivo and in-vitro angiogenesis assays study done by Mukherjee et al. (2015) (as cited in Rehman et al.,

2019) to observe the antigenic property of GO and reduced graphene oxide (rGO) showed that GO and rGO exhibit the property of pre-antigenic based on their concentrations. They have proved that the intercellular reactive oxygen species concentration is increased by GO and rGO. This will trigger the biomechanical machinery responsible for angiogenesis. Other studies found that low GO and rGO concentrations can stimulate intracellular reactive oxygen species and reactive nitrogen species synthesis to induce angiogenesis (Mukherjee et al., 2015; Waiwijit et al., 2014, as cited in Rehman et al., 2019). Further research on this mechanism is needed in order to clarify the unknown behind the previous studies.

2.4.4 Biomedical Applications of Graphene and GO

Graphene is thought to be the finest and most durable monolayer capable of independent existence (Goenka et al., 2014). The advantages properties of graphene and GO such as 2D structure, low toxicity and large surface area make them ideal for various biomedical applications. With its potential applications in biomedical fields such as drug delivery, cancer treatment, tissue engineering, antibacterial, gene therapy, biomedical imaging and biosensor, astounding GO has been continuously attracting attention and used in many research (Chung et al., 2013; Singh et al., 2018).

The 2D structure of graphene, as well as the presence of delocalized surface electrons, can be used for effective drug loading via hydrophobic interactions and π–π stacking. GO with low toxicity is excel than various types of anticancer drugs as it only targets tumors instead of normal healthy cells (Yang et al., 2011). Dorniani et al. (2016) studied on the

anticancer nanodelivery system using graphene oxide (GO) as nanocarrier for an active anticancer agent gallic acid (GA). y. In this study, GA was released from the designed anticancer nanocomposite (GOGA) in phosphate buffered saline (PBS) solution at pH7.4 in a sustained manner. The result showed that GOGA nanocomposite could inhibit the growth of cancer cell without affecting the growth of normal healthy cell.

Furthermore, graphene's large surface area enables high density bio-functionalization through the modification of both covalent and non-covalent surface (Goenka et al., 2014). It has excellent mechanical properties such as high elasticity, flexibility, strength as well as the ability to tailor a variety of functionalities on flat surfaces, which makes it a potential reinforcement material in hydrogels, biodegradable films, electrospun fibers and other scaffolds for tissue engineering. In addition, graphene has been proved to be effective in binding single-stranded DNA rather than double-stranded DNA. It is also able to protect oligonucleotides from being cut by enzymes (Zhang et al., 2012). These favorable characteristics of graphene were studied for the applications of gene delivery. For example, it commonly used PEI-functionalized GO for the delivery of plasmid DNA (pDNA).

Graphene and GO have been studied using a variety of molecular imaging techniques, including magnetic resonance imaging (MRI), optical, photoacoustic, and radionuclide-based such as positron emission tomography (PET) imaging (Zhang et al., 2012). Graphene-based nanomaterials with large surface area and versatile chemistry can be used for multimodality imaging, where the same agent can be detected at the same time.

Moreover, good water dispersibility, biocompatibility, high affinity for specific biomolecules and the coexistence of both hydrophobic and hydrophilic nature of GO are the advantages of GO in developing an advanced level of biosensors.

CHAPTER 3

MATERIALS AND METHODS

All apparatus and equipment used in these studies were obtained from the Cell Signaling, Biochemistry and Physiology laboratories in Faculty of Medicine and Health Sciences, Universiti Putra Malaysia (UPM).

3.1 Materials and Chemical Reagents

3.1.1 Equipment

The laboratory equipment that used in this study including carbon dioxide (CO_2) incubator (RS Biotech, UK) , water bath (Memmert, Germany), benchtop centrifuge (Hettich, Germany) , Class II Biological Safety Cabinet (Esco, Singapore), inverted microscope (Leica Microsystems, Germany), chiller (Thermo Fisher Scientific, United State), -80°C freezer (RS Biotech, UK), sonicator, weighing balance, vortex, autoclave sterilizer (Tomy, Japan) and oven (Lab Companion, Korea) were provided by

3.1.2 Chemicals

Dulbecco's Modified Eagle Medium (DMEM) media (with L-Glutamine and sodium pyruvate, Trypsin-EDTA (ethylenediaminetetraacetic acid), penicillin and streptomycin, were purchased from Nacalai Tesque (Kyoto, Japan). Fetal calf serum (FCS) was obtained from Tico Europe (Netherland), trypsin (GeneDireX, Inc., United States), phosphate buffered saline (PBS) tablets (Oxoid) was acquired from ThermoFisher Scientific (UK).

Dimethyl sulfoxide (DMSO) (Pierce), trypan blue solution, silver sulfadiazine (SSD) was obtained from Sigma-Aldrich (USA).

3.1.3 Compounds

Gallic acid (GA) was purchased from Sigma-Aldrich (USA).. Graphene oxide (GO) and gallic acid-loaded graphene oxide (GAGO) were supplied by Ashraful Hadi Abdul Ghafor from Universiti Putra Malaysia (UPM).

3.1.4 GO Synthesis

Graphite powder is used to synthesize GO through an improved Hummer's method. 360mL H_2SO_4 and 40mL H_3PO_4 were mixed to produce a 9:1 ratio mixture. The mixture is added to a 100mL beaker containing 3g of graphite power and stir at 300rpm. The pH of the mixture was measured and adjusted with NaOH and HCl to 4.71. Then, 18 g of $KMnO_4$ was added gradually and slowly to the mixture while stirring at temperature of 40°C. After the mixture was fully dissolved, the mixture was heated to 50°C and the stirring was continued for 12 hours. After stirred for 12 hours, the mixture was cooled down at room temperature for 2 hours. Then, the mixture was poured onto 400mL ice bath. After the ice melt, 3mL of H_2O_2 was slowly added into the mixture. The mixture was centrifuged at room temperature, 4000rpm for 15 minutes. Then, the supernatant was removed. The suspension was washed with 200mL dH_2O, 200mL HCL and 200mL ethanol successively to remove impurities by centrifugation method (4000rpm, 15min, room temperature). 200mL diethyl ether was added into the suspension. PTFE membrane (50mm diameter and 0.45µM pore size) and Buchner flask with vacuum pump were used

to filter the suspension obtained. The GO obtained left to dry overnight at room temperature (Ghafor et al, 2020).

3.1.5 Preparation of GAGO Nano-Formulation

0.25g of pure GA and 0.05g of pure GO were dissolved in 50mL of distilled water. The mixture was measured and adjusted to pH 4.71, before continuedly stirred for 16 hours. Any unreacted GA was then removed from the mixture through centrifugation (1073xg for 15 min) and washed thoroughly in distilled water. The suspension was stored in a 40℃ oven overnight. After the suspension dried, it was stored in a glass tube (Ghafor et al, 2020).

3.2 Cell Culture

3.2.1 Cell lines

Diabetic murine fibroblast cell line (3T3-L1) used in this study was purchased from American Type Culture Collection (ATCC) (Rockville, USA) and grown in DMEM medium supplemented with 10% of FBS and 100µg/mL penicillin-streptomycin at 37°C in a humidified atmosphere of 5% CO_2 incubator.

3.2.2 Thawing Cells

Complete media DMEM was pre-warmed before using it. The cells in vials were thawed in a water bath for 2 minutes. After thawing the cells, the cell suspension from the vials was quickly diluted into 4mL DMEM complete media in a 15mL centrifuge tube and the cell suspension was resuspended. The cell suspension in the 15mL centrifuge tube was

centrifuged at 130xg, 25°C for 8 minutes. The supernatant was removed and discarded. 1mL new DMEM complete media was added to the pallet of the cells and resuspend. 10µL of trypan blue solution was dropped on a parafilm. 10µL of cell suspension was mixed with 10µL of trypan blue solution on the parafilm. Then, 10µL of the mixture was put on the hemocytometer to check cell viability. After checking cell viability, 1mL of the cell suspension was transferred into a T25 flask containing 5mL of DMEM complete media.

3.2.3 Cell Counting

Cells counting was performed by using a bright-line haemocytometer (Hausser Scientific, Horsham, PA, USA). 10µL of trypan blue solution was dropped on a parafilm. 10µL of cell suspension was mixed with the trypan blue solution on the parafilm. Then, 10µL of the mixture was put on the hemocytometer chamber to check cell viability. Live and dead cells were counted. Cell counting was calculated using the formula below:

$$\text{Cells counting} = \frac{(\text{Total in 4 quadrants}/4) \text{ x } 10^4 \text{ x } 2^{\text{x}}}{1 \text{ mL}}$$

x = dilution factor

Cell viability was calculated by using the following formula:

$$\text{Cell viability (\%)} = \frac{\text{Live}}{\text{Live } + \text{ Dead cells}} \times 100\%$$

3.2.4 Cell Subculture

When the cells reached 80-90% confluence, subculture was carried out to allow the cells for further growing. First, the culture media was removed and discarded. The cells were washed with PBS and the PBS was discarded. 3mL of 1X Trypsin solution was added into a T75 flask and the flask was incubated in a 37°C CO_2 incubator for 5-10 minutes to facilitate cell detachment and dispersal. The cells were observed under an inverted microscope until cell layer was dispersed. 9mL of DMEM complete media was added to the flask to stop trypsin reaction and the cells were aspirated by gently pipetting. Then, cell suspension was transferred into centrifuge tube and the tube was centrifuge at 130xg, 25°C for 8 minutes. After centrifugation, the supernatant was discarded and the cells was resuspended in 1mL DMEM complete media. The new culture flask was placed in a 37°C CO_2 incubator.

3.2.5 Cryopreservation of Cells

Cryopreservation was carried out to store cells at an extremely low temperature (-80°C) to prevent cell death and damage. First, the culture media was removed and discarded. The cells were washed with PBS and the PBS was discarded. 3mL of 1X Trypsin solution was added into a T75 flask and the flask was incubated in a 37°C CO2 incubator for 5-10 minutes to facilitate cell detachment and dispersal. The cells were observed under an inverted microscope until cell layer was dispersed. 9mL of DMEM complete media was added to the flask and the cells were aspirated by gently pipetting. Then, cell suspension was transferred to centrifuge tube and the tube was centrifuge at 130xg, 25°C for 8 minutes. After centrifugation, the supernatant was discarded and the cells was

resuspended in 900µL DMEM complete media. The cell suspension was transferred into the storage vial. 100µL DMSO was slowly added drop by drop to the vial and mixed well. Then, the vial was stored in Mr Frosty in -80°C freezer.

3.3 Preparation of Treatment Solution for *In Vitro* Assays

To prepare the stock solutions of GAGO and pure GO, GAGO film or GO powder was added with of sterile ultrapure water to make up concentration of 1000µM. GAGO and GO solutions were then sonicated for 1hr to break the intermolecular interactions and speed up the dissolution. Meanwhile, stock solution of GA was prepared by mixing GA powder with of sterile ultrapure water Next, GA solution was vigorously vortexed for at least 20 minutes to ensure complete dissolution of all GA powder. The final working concentrations for GAGO, pure GA and pure GO were prepared at 5, 10, 20, 50, 100, 200, 250 and 500µM. Prior to each experiment, working concentrations for GAGO, GA and GO were freshly prepared from stock solution and vortexed or sonicated for at least 15 minutes before they were being diluted in complete media.

3.4 Scratch Assay

Diabetic murine fibroblast (3T3-L1) cells were seeded into 6-well plate. The cells were incubated in CO_2 incubator at 37°C overnight to allow cells grow and attach on the surface of the plate. After the cells reached 80-90% confluence, sterile 200µL tip was used to create a scratch on a confluent cell monolayer. The media containing detached cells were removed at the area of scratch using pipette. 1mL of phosphate buffered saline (PBS) was used to wash the cells in each well of the 6-well plate. Then, each well was treated with

eight different concentrations (0µM, 5µM, 10µM, 20µM, 50µM, 100µM, 200µM, 250µM) of either pure GA, pure GO, or GAGO. Treatment with silver sulfadiazine (SSD) was used as a positive control and untreated cells (media only) was considered as negative control. Inverted microscope (Leica DMi1, Germany) with digital camera was used to observe and take picture for the wound closure. The wound closure was monitored and photographed at 0 hour, 24 hours, 48 hours, and 72 hours. This experiment was carried out in triplicates (n=3). The results were analyzed using ImageJ software. The percentage of the wound closed area was calculated using the following formula:

$$\text{Wound Closure (\%)} = \frac{\text{Measurement at 0h} - \text{measurement at 24h/48h/72h}}{\text{Measurement at 0h}} \times 100\%$$

An increase in the percentage of the closed area indicated the migration of cells.

3.5 Statistical Analysis

GraphPad Prism version 8.0.1 software (GraphPad Software, La Jolla California USA) was used to analyze the differences between experimental groups using two-way analysis of variance (ANOVA) and followed by Tukey's test for multiple comparison. Data was presented as mean ± error of the mean (SEM). The p-value less than 0.05 (<0.05) was considered to be statistically significant. All experiments were repeated three times independently ($n \geq 6$).

CHAPTER 4

RESULTS AND DISCUSSION

4.1 The Effect of GAGO, GA and GO Treatment on Wound Closure in 3T3-L1 Fibroblast Cells

The purpose of this study is to assess the wound healing property of GAGO nano-formulation on diabetic murine fibroblast cell line (3T3-L1), and compare it with its native compounds, pure GA and pure GO. The 3T3-L1 cell line was used in this study as it is the fibroblast, which is the cell type that is mostly found in connective tissues (Li & Wang, 2011). Its functions, such as secretion of cytokines, growth factors, collagen and other extracellular matrix (ECM) components, play an essential part in tissue repair from the late inflammatory phase to the completely final epithelization of the wounded tissue (Addis et al., 2020). It is also in charge of organizing the ECM's constituents and producing the majority of the collagen and elastin. In addition, 3T3-L1 cell line is widely used as a cell culture model of insulin resistance in adipocytes (Rossi et al., 2020). These cells offer a cell culture system, which provides the foundation for an insulin-resistant state's molecular description (Knutson & Balba, 1997).

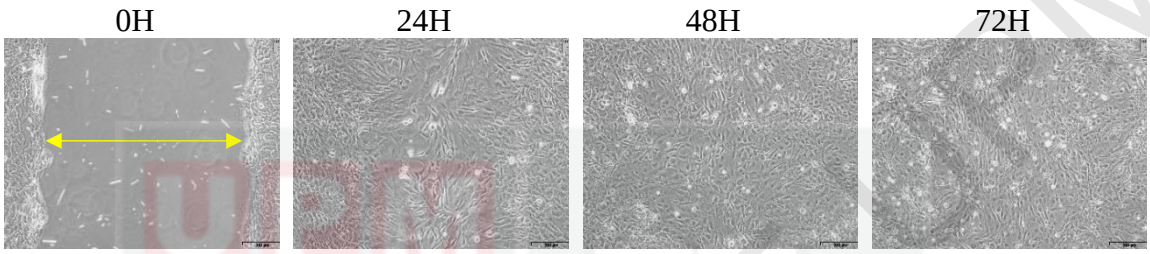
In this study, scratch assay was carried out to evaluate and compare the wound closure of 3T3-L1 cells with the treatment of pure GA, pure GO and GAGO nano-formulation at eight different concentrations ranged between 0μM and 250μM. Scratch assay is a simple, cheap and fast method used to study cell migration. This assay can observe the morphology and movement of cell throughout the study and adjust the test conditions according to the study purposes (Grada et al., 2017). It is crucial to scratch the wound

gently to prevent the detachment of cell, avoid scratching too slowly to prevent crooked line and scratch the wound in a consistent way, where the tip angle is 90° to the well plate surface to form a wound of similar size in each well. The range of treatment concentration was selected based on the safe concentration of GAGO nano-formulation stated in Ghafor et al. (2020) study, which is between the ranges of 0-150 µg/mL. Preliminary study had done showed the 3T3-L1 cells unable to perform scratch assay at the concentration of 500µM treatment due to the detachment of the cells. Hence, the range of the treatment concentration was fixed at the ranges of 0µM to 250µM.

The wound closure was assessed and evaluated daily up to 72 hours at three different time points (24, 48 and 72 hours). Untreated group (0µM) was considered as a negative control, while cells treated with 1% of silver sulfadiazine (SSD) was used as a positive control in this study. Figure 4.1A showed the scratch assay images of 3T3-L1 cells treated with 1% SSD for 0, 24, 48 and 72 hours. The yellow arrow shows the size of wounded area. Majority of the scratched wound of 3T3-L1 cells treated with 1% SSD closed after 24 hours. The width of the wound gap was measured using ImageJ software. Each photo was measured at 3 locations (top, middle and bottom) to obtain the average measured value for data analysis. Two-way ANOVA was used to compare between two independent variables, followed by Tukey's test for the comparison between control group (untreated group) and other treatment concentration and/or between two treatment groups. The graphs were plotted according to the percentage of wound closure with the various ranges of treatment concentration used. Figure 4.1B showed the wound closure of the 3T3-L1

cells treated with positive control (1% of Silver sulfadiazine, SSD) and negative control (0µM). Both controls are comparable.

(A)



(B)

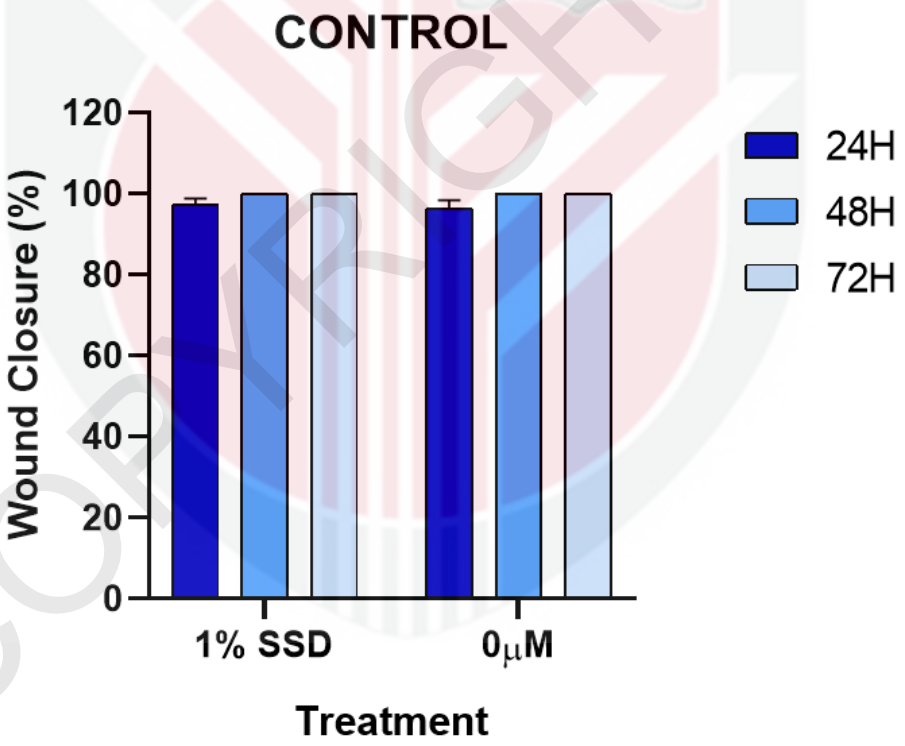


Figure 4.1: (A) Scratch assay images of 3T3-L1 cells treated with 1% of silver sulfadiazine (SSD) at 0, 24, 48 and 72 hours. The yellow double-headed arrows show the distance of wounded area (cell-free area) overtime (0, 24hr, 48hr and 72hr). Scale bar, 200µm (100x magnification). **(B)** Percentages of the wound closure of 3T3-L1 cells treated with positive control (1% of Silver sulfadiazine) and negative control (0µM) at 24, 48 and 72 hours. Data were expressed as mean ± standard deviation of mean (SEM).

Figure 4.2 showed the scratch assay images of 3T3-L1 cells treated with GAGO nano-formulation. From the observation, small GAGO fragments were attached on treated cells. The number of GAGO fragments attached to the treated cells was increased with the concentrations. None of the concentration was able to close the wound gap after 24 hours. The lower concentrations of GAGO treatment including 0μM, 5μM, 10μM and 20μM had a fully wound closure after 48 hours. During 72 hours post treatment, 50μM and 100μM of GAGO treatment closed the scratched wound of 3T3-L1 treated cells. The scratched wound at the concentrations of 200μM and 250μM GAGO nano-formulation were migrated slowly and unable to fully close after 72 hours.

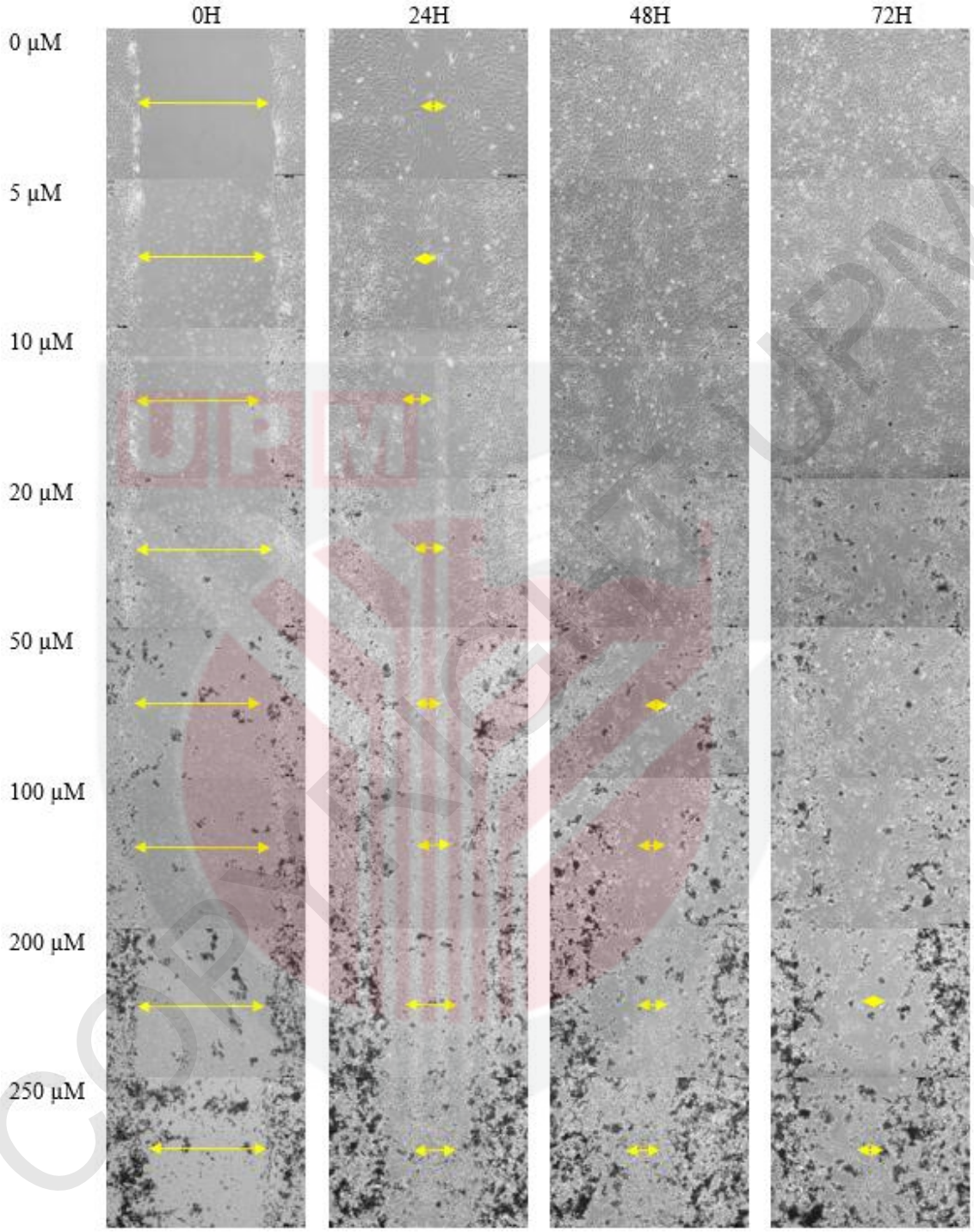


Figure 4.2: Scratch assay images of 3T3-L1 cells treated with Gallic Acid-Loaded Graphene Oxide (GAGO) nano-formulation at 0, 24, 48 and 72 hours. The yellow double-headed arrows show the distance of wounded area (cell-free area) overtime (0, 24hr, 48hr and 72hr). Scale bar, 200µm (100x magnification).

As shown in Figure 4.3A, the wound closure of 3T3-L1 cells treated with pure GA at various concentration such as 0µM, 5µM, 10µM, 20µM, 50µM, 100µM, 200µM and 250µM were observed. None of the concentration was able to close the wound gap after 24 hours. The scratched wound of 3T3-L1 cells treated with 0µM to 100µM pure GA was closed after 48 hours. The cells treated with 200µM of pure GA was migrated and closed the wound gap after 72 hours. At a high concentration of 250µM pure GA, the cells shrunk and detached from the well plate after 72 hours, resulting in the wider wound. Thus, there was no wound closure for 250µM of pure GA treatment. With the increase of time and concentration, more floating cells can be observed in the well plate.

In Figure 4.3B, three graphs showed the comparison between the wound closure percentages of 3T3-L1 cells treated with GAGO nano-formulation and pure GA after 24, 48 and 72 hours. From Figure 4.3B 24h graph, when the concentration of 3T3-L1 cells treated with pure GA was less than or equal to 50µM, it was comparable to that of the untreated group. The pure GA concentration more than or equal to 100µM was significant to the untreated group. There had no wound closure in a high concentration of 250µM pure GA. The concentration of 5µM to 50µM pure GA has almost similar wound closure at 24-hour timepoint while the percentage of wound closure decreased in the higher concentrations range of 100µM to 250µM. This is because the higher concentration of pure GA increases the toxicity to the 3T3-L1 cells. 250µM of pure GA is toxic to the 3T3-L1 cells because the cells cannot migrate and demonstrated the sign of cell death which is the detachment and floating of the cell in the culture medium (ATCC, n.d.). The wound

gap became wider, resulting in 0% wound closure. The induction of toxicity based on concentration-dependent manner was consistent with previous study.

In addition, according to Yang et al. (2016) study, the results indicated that pure GA can improve the wound closure rate significantly and promote the wound healing of fibroblast in hyperglucidic condition through cell migration. This is in contradiction with previous study as the findings of this study showed that after 24 hours, the untreated groups migrated faster and wound closure was better than other GA treatment groups. Moreover, during 48-hour post treatment (Figure 4.3B 48h), the concentrations of 100µM and 200µM of pure GA were comparable to control group while the wound closure of 250µM pure GA was remained at 0%. For 72-hour post treatment (Figure 4.3B 72h), similar observation as 48-hour post treatment can be observed in all the concentrations except 200µM, which can close the gap, as compared with 48-hour post-treatment.

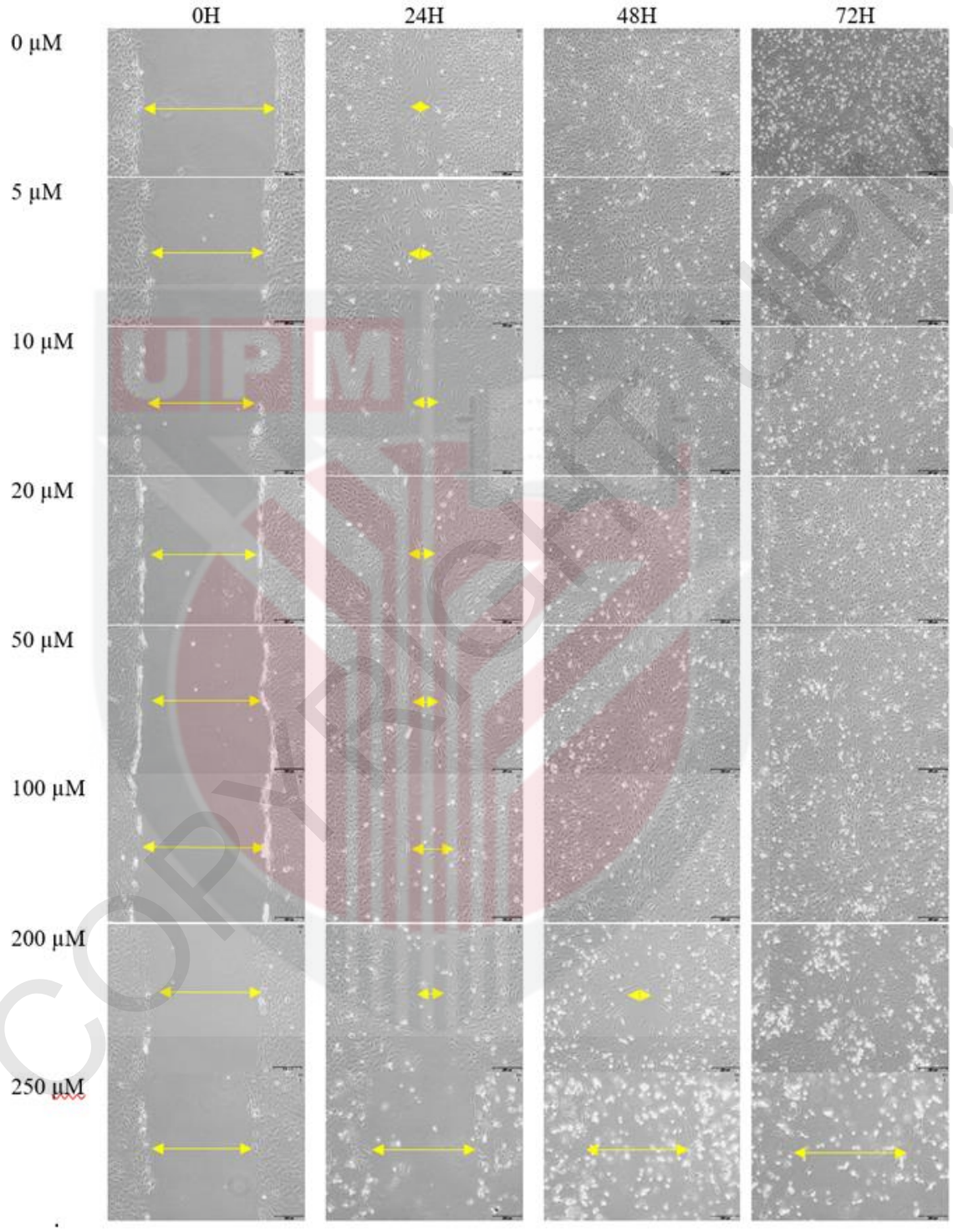
For the encapsulation of GA on GO nano-carier treatment (Figure 4.3B 24h), only GAGO treatment with lower concentration such as 5µM was comparable to the negative control group, and the GAGO treatment with other concentrations showed significant difference to the negative control group. Interestingly, 3T3-L1 cells treated with 250µM of the encapsulation of GA on nano-formulation were able to migrate and close approximately 50% of wounds after 24 hours. The increased treatment concentration causes the decreased of wound closure percentage until 20µM and then GAGO nano-formulation concentration range of 20µM to 200µM have almost similar wound closure percentages after 24 hours. Although the wound closure of 100µM seems higher than the wound

closure of other concentration, it was statistically shown that the GAGO nano-formulation concentration range of 20µM to 200µM were comparable to each other. For 48-hour and 72-hour post treatment, when the concentration increased, the percentage of wound closure decreased. This is because GAGO treatment induces the toxicity to the cells according to time and concentration-dependent manner (Ghafor et al., 2020). From the 48-hour graph (Figure 4.3B 48h), when the concentration was below or equal to 50µM, GAGO nano-formulation managed to completely close the wound gap and were comparable to control group. The encapsulation of GA on nano-formulation at concentration more than or equal to 100µM was significant to control group. During 72-hour post treatment, 100µM of GAGO nano-formulation able to close the wound gap while similar observation as 48-hour post treatment can be observed in GAGO treatment with higher concentrations including 200µM and 250µM.

In the comparison with its native compound, pure GA, GAGO was statistically comparable to pure GA in most of the concentration except 50µM and 200µM pure GA treatment has better wound closure. Interestingly, it seems to have better wound closure than pure GA at 250µM as GAGO treatment showed more than 60% of wounds were closed, which indicated that GAGO nano-formulation reduced the toxicity of pure GA, resulting in the migration of cells and the reduction of cell-free area. GA is able to alter the surface morphology of GO by masking the sharp edge on the GO structure, thus reducing the toxicity effect of GAGO nano-formulation (Ghafor et al., 2020). Besides, the encapsulation of GA on GO nano-carrier contributes to slow release of the GA. Therefore, the cells will not be exposed to the tested concentration at one time, thus reducing the

toxicity of GA. None of the treatment groups managed to close the scratched gap after 24 hours. For the 48-hour graph (Figure 4.3B 48hr), when the concentration was below or equal to 50µM, both treatment groups managed to completely close the wound gap. Both treatments have almost similar observation as 48-hour post treatment after 72 hours. The study showed that GA were released from the delivery system after 48 hours (Mahboob et al., 2020). Therefore, the scratched wound of 3T3-L1 cells treated with GAGO treatments started to close after 48 hours. Based on these 3 graphs, all treatment groups demonstrated concentration-dependent effects.

(A)



(B)

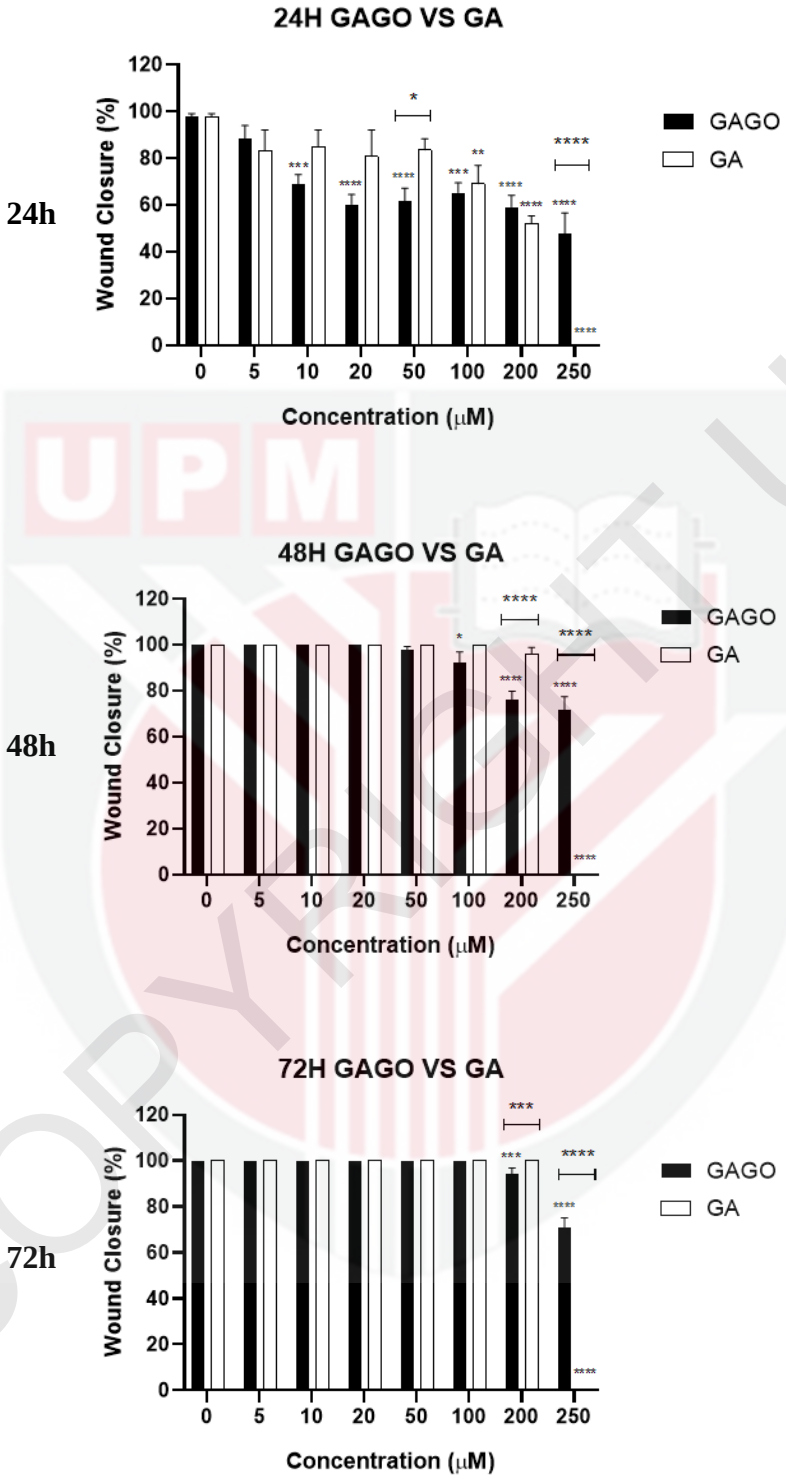


Figure 4.3: (A) Scratch assay images of 3T3-L1 cells treated with pure gallic acid (GA) at 0, 24, 48 and 72 hours. The yellow double-headed arrows show the distance of wounded area (cell-free area) overtime (0, 24hr, 48hr and 72hr). Scale bar, 200μm (100x magnification). **(B)** Percentages of the wound closure of 3T3-L1 cells treated with different concentrations of Gallic Acid-Loaded Graphene Oxide (GAGO) and pure gallic

acid (GA) ranged between 0µM – 250µM at 24, 48 and 72 hours. Data were expressed as mean ± standard deviation of mean (SEM). The results were analyzed using two-way ANOVA and Tukey's multiple comparison test. The significant difference of p-value equal to 0.0332 represents *, equal to 0.0021 represents **, equals to 0.0002 represents ***, and less than 0.0001 represents ****, when compared with the control group (untreated cells) and/or between two treatment groups.

On the other hand, the scratch assay images of 3T3-L1 cells treated with pure GO were observed in Figure 4.4A. It can be observed that small GO fragments were attached to treated cells. The lower the concentration of pure GO used, the less the small GO fragments attached to the treated cells. None of the concentration was able to close the wound gap after 24 hours. The scratched wound of 3T3-L1 cells treated with 0µM, 5µM, 10µM and 20µM of pure GO were closed at 48 hours post treatment. The cells treated with 50µM of pure GO migrated and closed the wound gap after 72 hours. The scratched wound at the concentrations of 100µM and 200µM pure GO were migrated slowly. However, the wound unable to close after 72 hours. The shrunk and detached cells treated with 250µM pure GO treatment was not migrated. The size of wound gap for 250µM pure GO treatment increases after 72hours due to the detachment of the cells. As with pure GA treatment, it can be observed that the number of floating cells increases with time and concentration.

Figure 4.4B showed the comparison between the wound closure percentages of 3T3-L1 cells treated with GAGO nano-formulation and pure GO ranged between 0µM to 250µM at 24, 48 and 72 hours. It can be seen from Figure 4.4B 24hr that pure GO treatment with concentration lower than or equal to 20µM was comparable to the negative control group. The pure GO treatment concentration above 50µM was significant to the negative control

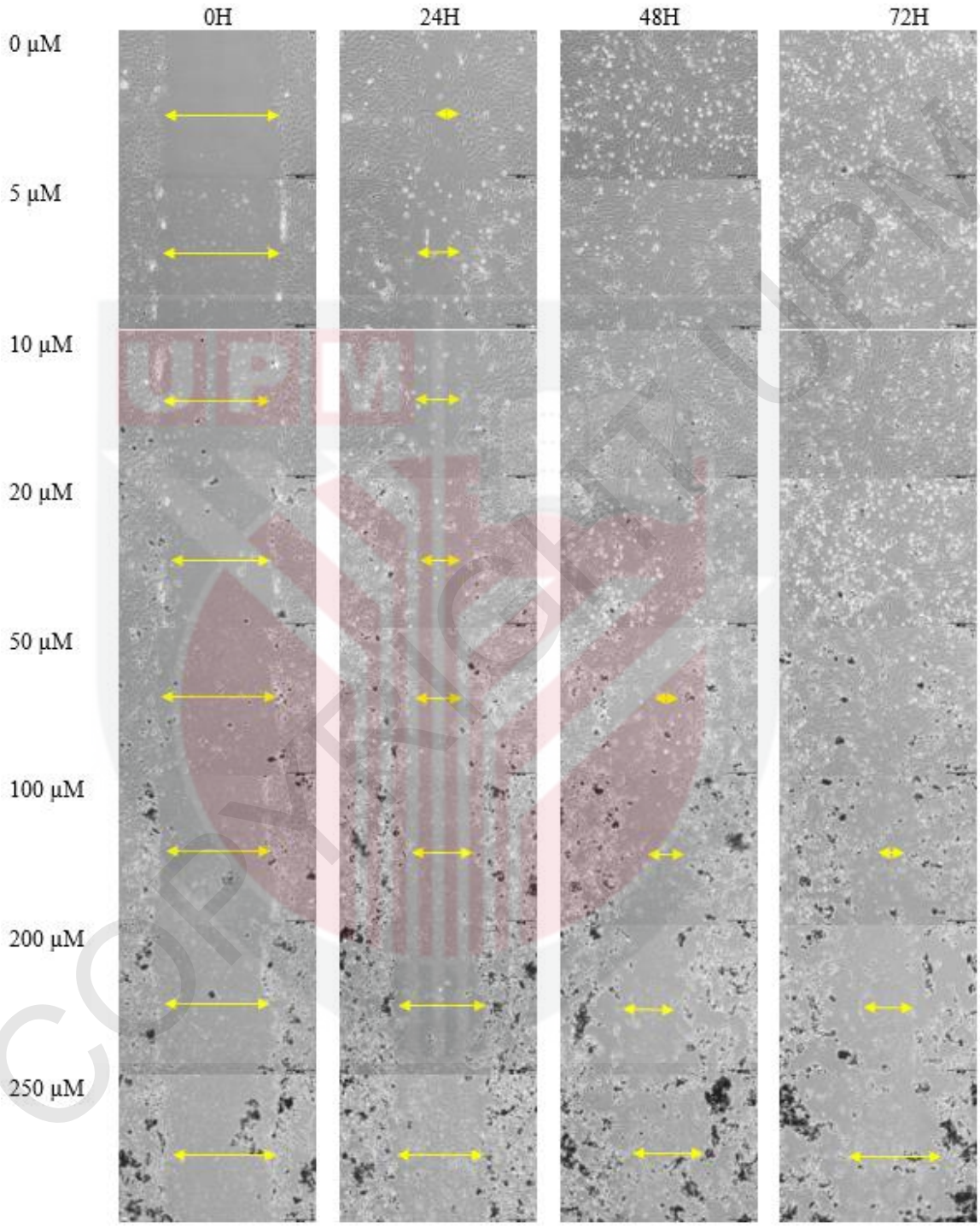
group. GO treatment groups were unable to close the gap at 24 hours post treatment. Furthermore, pure GO with the concentration below or equal to 20µM managed to fully close the gap and were comparable to untreated group after 48 hours. Pure GO treatment with 50µM unable to fully closed the wound gap and was comparable to the negative control. Pure GO treatment with concentration higher than or equal to 100µM showed significant to the untreated group. The wound gaps of the GO treatment groups with the concentrations less than 100µM were completely closed while pure GO treatment with 200µM closed approximately 50% of the wound gap after 72 hours. The wound closure percentage for 250µM pure GO treatment was reduced to zero after 72 hours.

Some studies have proved that pure GO has the properties of wound healing. Solimanal. (2021) study showed that GO-cellulose nanocomposite can improve endothelial cell migration both *in vitro* and *in vivo* study in rats. In Figure 4.4B 24hr, 5µM of pure GO has slightly lower wound closure percentage than 10µM of pure GO during 24-hour post treatment, but the two are statistically comparable. However, from 10µM to 250µM of pure GO, the increase of pure GO concentration causes the decrease of wound closure percentage. This is due to the GO toxicity increase in higher concentration, causing the detachment of the cells and lead to cell death after 72 hours. This finding was consistent with previous research, which pure GO demonstrated concentration-dependent toxicity. Comparing with other concentrations, the high pure GO concentration of 200µM migrate slowly after 72 hours. Besides, Deepachitra et al. (2014) had done a study about using GO incorporated collagen–fibrin biofilm (CFGO) as wound dressing material in both *in vitro* and *in vivo* studies. This study has shown that the CFGO treated wounds healed faster

than those treated with control and CF. However, in this study, the negative control group has higher wound closure than pure GO treated wound.

When comparing GAGO nano-formulation and pure GO, both treatment groups were comparable except the concentration of 200µM, which showed a significance between pure GO and GAGO treatment groups after 24 hours. Most of the GAGO nano-formulation concentrations were comparable to pure GO except the high concentrations such as 100µM, 200µM and 250µM after 48 hours. At the concentration of 50µM, there is no significance difference between pure GO and GAGO treatment groups and the wound treated with pure GO was nearly 100% closed. Intriguingly, the newly-formulated GAGO nanoparticle has better wound closure than pure GO at concentration 100µM, 200µM and 250µM as the nano-formulation of GAGO reduced the toxicity of pure GO, resulting in the migration of the cells to increase the wound closure percentage of the cells. However, both treatment groups with the concentration higher than 100µM were unable to close the wound after 72 hours. There were significant between pure GO and GAGO treatment groups with the concentration of 200µM and 250µM.

(A)



(B)

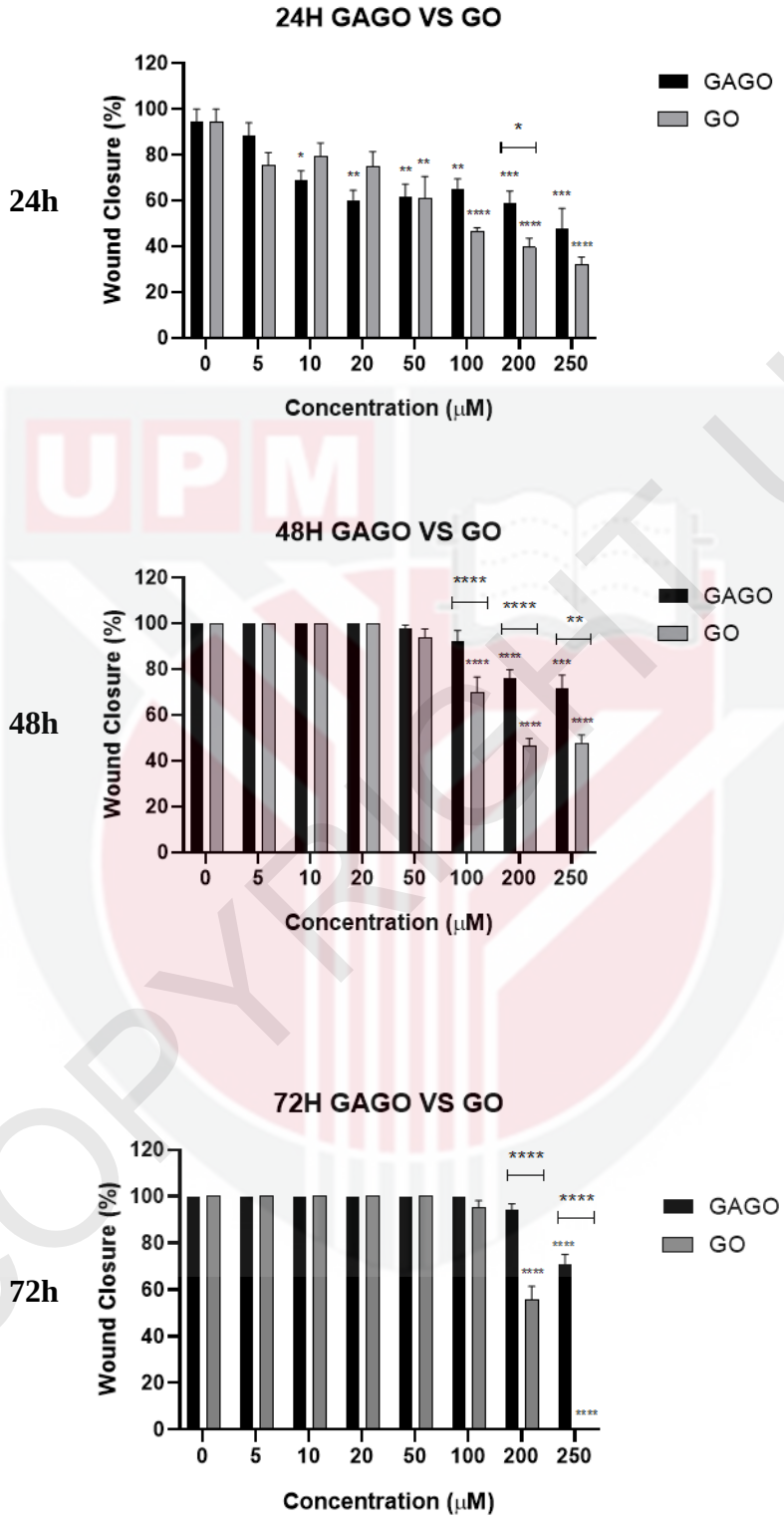


Figure 4.4: **(A)** Scratch assay images of 3T3-L1 cells treated with pure graphene oxide (GO) at 0, 24, 48 and 72 hours. The yellow double-headed arrows show the distance of

wounded area (cell-free area) overtime (0, 24hr, 48hr and 72hr). Scale bar, 200μm (100x magnification). **(B)** Percentages of the wound closure of 3T3-L1 cells treated with different concentrations of Gallic Acid-Loaded Graphene Oxide (GAGO) and pure graphene oxide (GO) ranged between 0μM – 250μM at 24, 48 and 72 hours. Data were expressed as mean ± standard deviation of mean (SEM). The results were analyzed using two-way ANOVA and Tukey's multiple comparison test. The significant difference of p-value equal to 0.0332 represents *, equal to 0.0021 represents **, equals to 0.0002 represents ***, and less than 0.0001 represents ****, when compared with the control group (untreated cells) and/or between two treatment groups.

There are also some problems faced during the study. The scratch assay images of 3T3-L1 cells treated with pure GO and newly-formulated GAGO nanoparticles were hard to observe especially in high concentration due to the black compound fragment attached on the cells. The pure GO and GAGO nano-formulation were freshly prepared and sonicated before using them. In order to solve the difficulty in observing the cells, it is suggested to wash the cells with PBS solution before viewing them. Moreover, although the study done by Yang et al. (2016) showed that the improvement of wound healing treated with GA in hyperglucidic condition might not be due to cell proliferation but due to accelerated cell migration, there was also the possibility of cell proliferation in this study as we did not starve the cell or add cell proliferation inhibitors. To solve this problem, it is suggested to use the proliferation inhibitor such as mitomycin c in the study to further proved that the wound closure of cells is caused by cell migration.

CHAPTER 5

CONCLUSION AND FUTURE RECOMMENDATIONS

5.1 Conclusion

The present study indicates that pure GA, pure GO and GAGO nano-formulation demonstrated concentration dependent manner on 3T3-L1 treated cells. The study finding indicated that the wound healing property of GAGO nano-formulation was comparable to its native compound, pure GA in diabetic fibroblast-treated cells. However, at high concentration of 250µM, the encapsulation of GA on GO nano-carrier reduces the toxicity as compared with pure GA in diabetic fibroblast-treated cells. Besides, when compared with pure GO, the encapsulation of GA on GO nano-carriers showed higher percentage of wound closure than pure GO. Hence, it has the potential to improve wound healing performance and reduces the toxicity of this new formulation in diabetic fibroblast-treated cells.

5.2 Future Recommendations

Although *in-vitro* study has advantages in term of the ethical consideration and able to evaluate the newly-formulated GAGO nanoparticles directly in an adjustable condition, there are also some limitations, which make it difficult to study the complex cell line's functions *in vivo* systems involving various cells and tissues. Hence, further study is recommended to perform *in vivo* study for the evaluation of the wound healing effects of GAGO nano-formulation to further understand the wound healing effect and the effectiveness of GAGO nano-formulation in diabetic model. Besides, second

recommendation is to correlate the GAGO nano-formulation with its effective concentration through antioxidant assay.

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