INVITRO EVALUATION OF GRAPHENE OXIDE ON CELL CULTURE GROWTH

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A RESEARCH PAPER SUBMITTED TO THE FACULTY OF VETERINARY MEDICINE,

UNIVERSITI MALAYSIA KELANTAN

IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE DEGREE OF DOCTOR OF VETERINARY MEDICINE

MAY 2022

UNIVERSITI MALAYSIA KELANTAN

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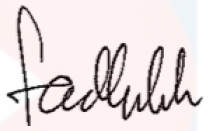
CERTIFICATION

It is now certified that we have read this study entitled "**Invitro evaluation of Graphene Oxide on Cell Culture Growth**" by **Zakiah Binti Marzuki (D17A0040).** It is satisfactory in terms of scope, quality, and presentation as partial fulfillment of the course DVT 5204 Final Year Project requirement.



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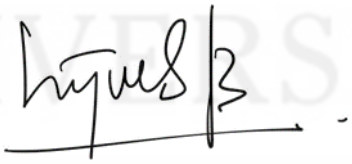

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ACKNOWLEDGEMENT

My most incredible gratitude goes to my supervisor, Dr. Nor Fadhilah Binti Kamaruzzaman, for her helpful guidance, willingness to support, and boundless knowledge and skills in developing this final year research period and thesis writing, especially during this final year of research period laboratory work. Special thanks are also given to Dr. Luqman Bin Abu Bakar as my co-supervisor for the project's assistance, expertise, support, and guidance during the research period, especially during the animal study. My highest thanks are also given to Associate Professor Dr. Rumaizi bin Shaari for sharing his unsurpassed knowledge, experience, and advice to associate with my projects.

I would also love to include my love and gratitude to Dr. Amalina, Dr. Brenda, Pn. Nani, Pn. Salma, En. Wan and the rest of the staff of FPV UMK for their unlimited support and guidance during laboratory and animal study throughout my research period.

Special thanks to Cik Elyn Amiela Bt Salleh acting as the research assistant for this project, known for his assistance and diligence during the research period.

Finally, I would love to thank Luqman Hadi, Shafiqah Syaza, Diyana, Aida, Faiz, Shafiq, alif, wan, paan, muqles, fakhrul, chan, izzaty, ashiqah, and the rest of the DVM class of 2017/2022 for their outstanding roles as my mental stabilizers during the final year project research period.

Thank You

DEDICATION

In the name of Allah, The Most Beneficent, The Most Merciful, and The Most Gracious

I humbly dedicate this thesis to

my all-time beloved family

supportive friends

&

members of Faculty of Veterinary Medicine, Universiti Malaysia Kelantan

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ABSTRACT

An abstract of the research paper was presented to the Faculty of Veterinary Medicine, Universiti Malaysia Kelantan, in partial requirement for the course DVT 5436 – Research Project.

The skin serves as a critical barrier, preventing hazardous foreign matter from entering the body. Due to this significance, graphene oxide has been used to regenerate skin via wound dressings or electronic skin monitoring. Wound dressings such as electrospun mats, hydrogels, and sponges, are the most effective way to accelerate wound healing, thus, graphene oxide has been incorporated into these types of dressing. Graphene Oxide has been studied to be used in hemorrhage control measures used in accident cases involving hemorrhage and uncontrolled bleeding and graphene oxide can create artificial extracellular matrices for tissue remodeling. It was mentioned that the integration of graphene-based flakes could support cellular proliferation leading to skin regeneration, thus providing wound healing. This research aimed to determine the concentration of graphene oxide that provides cell growth and the concentration of graphene oxide that causes toxicity cell growth in Vero cells. Based on the results from the determination of confluency cells on different types of concentrations of graphene oxide, the number of Vero cells increased parallel to the increase of the concentration of the graphene oxide.

Keywords: *Graphene Oxide, cell growth, tissue engineering, cytotoxicity, Vero cells*

ABSTRAK

Abstrak daripada kertas penyelidikan dikemukakan kepada Fakulti Perubatan Veterinar, Universiti Malaysia Kelantan untuk memenuhi sebahagian daripada keperluan kursus DVT 5436 – Projek Penyelidikan.

Kulit berfungsi sebagai penghalang bahan asing yang berbahaya daripada masuk ke dalam badan. Oleh sebab itu, kepentingan graphene oxide telah digunakan untuk menjana semula kulit melalui pembalut luka yang telah diolah. Pembalut luka seperti glectrospun tikar, hidrogel, dan span, adalah cara paling berkesan untuk mempercepatkan penyembuhan luka, oleh itu, graphene oxide telah dimasukkan ke dalam jenis pembalut ini. Graphene Oksida telah dikaji untuk digunakan dalam langkah kawalan hemorrhagel yang digunakan dalam kes kemalangan melibatkan pendarahan yang tidak terkawal dan graphene oxide boleh mencipta matriks ekstrasel tiruan untuk pembentukan semula tisu. Disebutkan bahawa penyepaduan kepingan berasaskan graphene boleh menyokong percambahan selular yang membawa kepada kulit penjanaan semula, dengan itu memberikan penyembuhan luka. Penyelidikan ini bertujuan untuk menentukan kepekatan graphene oksida yang menyediakan pertumbuhan sel dan kepekatan graphene oxide yang menyebabkan pertumbuhan sel ketoksikan dalam sel Vero. Berdasarkan keputusan daripada penentuan sel penumpuan pada pelbagai jenis kepekatan graphene oksida, bilangan sel Vero meningkat selari dengan peningkatan kepekatan daripada graphene oksida.

Kata kunci: *graphene oksida, pertumbuhan sel, kejuruteraan tisu, ketosikan, sel Vero*

1.0 INTRODUCTION

Skin injuries caused by surgery, trauma, diabetes, or persistent wounds are often encountered in clinics. (Li *et al.*, 2019). Wound healing treatments include using medications or the administration of bio activators, wound dressings, and autologous grafting of skin(Li *et al*., 2019). Applying a bio-activator does not provide adequate action time, while skin grafting may result in complications abrasions at the donor site. Additionally, conventional wounds dressings are ineffective at regulating the healing process.

This is a mechanism that inhibits the progression of skin regeneration. Additionally, continuous oxidative stress is detrimental to the skin. Regeneration emphasizes the importance of developing innovative wound dressings that efficiently regulate healing(Li *et al*., 2019).

A human investigation demonstrated that impregnated collagen dressings result in full wound closure in diabetic patients without causing hypersensitivity, are antimicrobial compatible, and are cost-effective (Amirrah *et al*., 2020). Despite over 60 years of use, there is no published investigation on the adverse consequences (Hornschuh *et al*., 2020).

On the other hand, when combined with chitosan to generate nanocomposite scaffolds, graphene has been used in hemostasis and wound healing applications (Choudhary *et al*., 2020). Graphene has developed into favorable because of its one-of-a-kind properties, such as a large surface area and superior mechanical tenacity (Novoselov *et al*., 2004). Additionally, graphene is known to exert antimicrobial qualities that become advantageous in assisting in the healing of wounds caused by, apart from its role in hemorrhage control, bacterial infection (Lu *et al.,* 2012). Apart from that, graphene can create synthetic extracellular matrices for the skin. Regeneration acts as a scaffold for cell adhesion and proliferation, promoting wound healing (Ding *et al*., 2015; Safina et al., 2020).

Gp has multiple functional groups, and GO is the highly oxidized version (e.g., hydroxyl, carboxyl, and epoxy groups). GO may easily be coupled with other biomolecules and biomaterials because of its functional groups. Furthermore, GO has an advantage over Gp in easy dissolution in water and other organic solvents because of the polar oxygen functional groups. Because of its surface functionalization, GO is less cytotoxic in several studies than graphene and its derivatives. The hydrophilic property of GO allows it to avoid aggregation in cell culture media due to its oxygen concentration. The aggregation phenomenon reduces nutrition delivery, causing oxidative stress and triggering the apoptotic pathway.

Furthermore, the oxygen functional groups of GO may be able to modulate protein adsorption in the extracellular matrix (ECM), resulting in improved cell adhesion and proliferation. Another Gp derivative, rGO, may be made by removing most of the oxygen-containing groups from GO and regaining its electrical conductivity capabilities, which have been demonstrated to boost neurogenesis. Furthermore, the sharp edges and oxygen-functional groups of GO may cause bacterial cell membrane rupture and oxidative stress, resulting in angiogenesis and osteogenesis increase. The antibacterial and antimicrobial characteristics of GO could make it a suitable material for tissue regeneration application.

2.0 RESEARCH PROBLEM

Surgery-related skin injuries, trauma, diabetes, and chronic wounds are often encountered in clinics. As we all know, conventional wounds Dressings are ineffective at regulating the healing process. Thus, it is important to repurpose the existing nanomaterials to promote healing. There is much research done on various substances with cell growth properties. It is noted that graphene oxide is a nanomaterial that can demonstrate antimicrobial activities but has not been effectively used in veterinary medicine. In this experiment, we investigated the impact of Graphene oxide on cell growth**.**

3.0 RESEARCH QUESTION

3.1 Does Graphene Oxide promote the following cell growth (Vero cells)?

3.2 What concentration can graphene oxide promote cell growth?

3.3 What concentration of graphene oxide can cause toxicity?

4.0 RESEARCH HYPOTHESIS

3.4 Graphene oxide can improve the growth of selected cell lining of the Vero cells.

3.5 Graphene oxide can be effective with a low toxicity effect.

5.0 OBJECTIVES

3.6 To determine the concentration of graphene oxide provides cell growth.

3.7 To determine the concentration of graphene oxide that causes toxicity cell growth.

6.0 LITERATURE REVIEW

6.1 Skin

6.1.1 Normal skin structure

The skin is mammals' biggest organ, acting as a physical barrier between them and their surroundings (König *et al*., 2007). Skin is composed of two separate layers, epidermis and dermis, at the microscopic level (Colville *et al*., 2015). The epidermis is the outermost layer of the skin, consisting of the stratum corneum, stratum granulosum, stratum spinosum, and stratum basale (Saxena *et al*., 2014). The epidermis comprises a stratified epithelium lined by a dense keratinocyte population that connects the dermal tissue via the basement membrane (Simpson *et al*., 2011). Apart from that, the dermis is a thick connective tissue densely packed with fibrillary proteins, collagen, and blood capillaries (Crichton *et al.,* 2017; Wei *et al*., 2017). Apart from providing circulatory support, the dermis layer comprises blood supply networks for nutrition delivery (Saxena *et al.,* 2014).

6.1.2 Skin injury and repair

Macrophages are involved in every stage of wound healing and orchestrate the process. Their functional phenotype is determined by the wound microenvironment, which changes during recovery, affecting the macrophage phenotype. During the early and brief inflammatory phases, macrophages perform pro-inflammatory functions such as antigen presentation, phagocytosis, and the production of pro-inflammatory cytokines and growth factors that aid in wound healing. In this phase, wound macrophages most likely exhibit the classically activated, or so-called M1 phenotype (Gurtner *et al*. 2008).

Following that, macrophages can alter the composition of the ECM via the release of degrading enzymes and the synthesis of ECM molecules during angiogenesis and remodeling (Mirza *et al*., 2009). This suggests that

alternatively activated macrophages play a critical role in this phase of wound healing. Macrophage dysfunction during the wound healing process can result in ulcers, chronic wounds, hypertrophic scars, and keloids. For the treatment of wound repair disorders, more information about macrophages' role in these conditions, particularly their functional phenotype, is needed to identify additional therapeutic opportunities (Mirza *et al*., 2009; Lucas et al., 2010).

6.2 Cell growth behavior on graphene oxide

6.2.1 Substrates for antibiotics

The GO and RGO articles demonstrated the ability of their surfaces to prevent bacterial growth. Grampositive and Gramnegative bacteria illustrate the inhibitory impact on GO and RGO nanowalls. RGO nanowalls had a more significant antibacterial effect than GO nanowalls due to the more efficient charge transfer of RGO with Bacterial cells. The antibacterial activity of graphene derivatives results from the oxidative stress caused by membrane rupture. On the other hand, one investigation revealed that the graphene surface encouraged rather than prevented bacterial growth. The contentious findings suggested that experimental settings could affect bacterial growth on GO.

6.2.2 Mammalian cell culture

Graphene derivatives were used as a culture medium for mammalian cells. For instance, the behavior of NIH-3T3 fibroblasts was examined on a variety of carbon nanomaterial-coated surfaces, including graphene oxide, rhodium guanidine oxide, and carbon

nanotubes. Carbon nanomaterial-coated substrates demonstrated increased biocompatibility and effectiveness of gene transfection. Graphene/chitosan hybrid films also showed potential for tissue engineering applications such as repair and enhancement of tissue functioning. Interestingly, the graphene surface increased neurite sprouting, and outgrowth compared to a regular polystyrene tissue culture plate.

6.2.3 Cell differentiation

The characteristic of differentiated mammary epithelial cells is an abundance of milk-specific components secreted in response to lactogenic hormones. It describes establishing a stable clonal cell line from primary bovine mammary alveolar cells (MAC-T) via stable transfection with the SV-40 large T-antigen. When grown on the plastic substratum, they exhibit epithelial cells' characteristic "cobblestone" morphology. Differentiation was induced by increasing cell-cell interaction in the presence of prolactin on a floating collagen gel. The differentiated phenotype was defined by an increase in the abundance of casein messenger RNA, an increase in the number and size of indirect immunofluorescent casein secretory vesicles in each cell, and an increase in the secretion of a.- and fl-casein proteins. This cell line is unique due to its clonal nature, immortality, and ability to differentiate and secrete casein proteins uniformly(Huynh *et al.*, 1991).

6.3 Soft tissue engineering

Tissue engineering, which first gained popularity in the 1990s to assist in regenerating injured tissues, necessitated the development of new biodegradable materials for scaffold creation. Unlike traditional techniques, which include tissue transplantation or permanent implant implantation, these biodegradable scaffolds serve as temporary constructs that promote cell growth and diversification while dissolving and being eventually replaced by new tissue.

6.3.1 Skin tissue engineering

The skin serves as a critical barrier, preventing hazardous foreign matter from entering the body(Zare *et al.*, 2021). Due to this significance, GOBMs have been used to regenerate skin via wound dressings (Zare *et al.*, 2021) or electronic skin monitoring(Zare *et al.*, 2021). Wound dressings, such as electrospun mats (Zare *et al*., 2021), hydrogels, and sponges, are the most effective way to accelerate wound healing; thus, GOBMs have been incorporated into these types of dressings, although other shapes (e.g., modified rGO nanosheets (Zare *et al*., 2021)) may also provide drastic novel treatment options. Tang and colleagues developed an inspired scaffold based on polydopamine-NGO (NGO) and chitosan (CS), and silk fibroin (SF) hydrogels (pGO-CS/SF). Electroactivity responded to electrical impulses and increased cytological behavior due to the page. Antioxidant activity inhibited cellular oxidation by eliminating excess reactive oxygen species (ROS) (Zare et al., 2021). The fact that rGO can significantly boost angiogenesis is a bonus, as this is highly advantageous for slow-healing/chronic wounds. Along with these uses, it is critical to understand GOBMs and their interactions with the skin, as this is a primary route of exposure for materials(Zare *et al*., 2021). The expertise

with skin regeneration stems from constructing a membrane from an FGO-based nanocomposite with a hydrophobic outer layer with low porosity to keep bacteria away from the skin and a hydrophilic inner layer with more excellent porosity seed stem cells and growth factors.

6.3.2 Stages in tissue recovering

Following the implantation of tissue-engineered constructions, the implant undergoes typically spontaneous vascularisation. This is partly due to the surgical technique inducing an inflammatory wound healing response. Furthermore, the seeded cells frequently induce hypoxia in the implant, which increases the release of angiogenic growth factors from the body*(Rouwkema et al.*, 2008). However, this induced vascular ingrowth is often insufficient to provide enough nutrition transport to the transplanted tissue's core cells. As a result, different vascularisation procedures are required to assure the life of extensive tissue-engineered grafts(Rouwkema *et al*., 2008).

Several methods for improving vascularisation are currently being researched. Scaffold design, angiogenic factor incorporation, in vivo revascularisation, and in vitro revascularisation are among them. Even though all of these tactics are theoretically possible. Scaffold design and angiogenic factor delivery, the first two techniques, rely on the ingrowth of host vasculature into the entire implanted construct. As a result, while these tactics can speed up the vascularisation process, it will still take many days to weeks for the implant's center to become perfused(Rouwkema *et al.*, 2008). Because the construct is microsurgically coupled to the host vasculature, in vivo revascularisation can theoretically result in immediate perfusion of a build following implantation at

the final site. However, a pre-implantation phase is required before implantation in the last place. The implant must rely on spontaneous angiogenesis from the surrounding vessels to enter the construct during this time. As a result, nutritional shortages are likely to arise at this point.

Because arteries must develop from the host into the construct until they reach the vascular network established in vitro, in vitro revascularisation does not result in instantaneous perfusion. The invading vessels can then anastomose with the existing vasculature, allowing blood to flow throughout the entire construct. Because host vessels do not have to grow into the whole build but only into its outer regions until the ingrowing plates meet the preformed vascular network, this method can dramatically reduce the time it takes to vascularise the implant compared to scaffold design angiogenic factor delivery (Rouwkema *et al.*, 2008).

6.4 The application of graphene Oxide nanoparticles in tissue engineering

Graphene oxide (GO) is a graphene derivative. The functionalization of graphene's sp2-bonded carbon to create 2D nanomaterials has demonstrated significant potential as nanocarriers for biological molecules and drug delivery(Li et al., 2019). The strong CC bonds confer on graphene superior mechanical properties, including Young's modulus of 1100 GPa and fracture strength of 130 GPa. Between two carbon atoms, the additional perpendicular p-orbital produces a -bond. The -bonds on both sides of the graphene planar structure form a massive delocalized conjugated system, which confers outstanding thermal and electrical conductivities on graphene.

Additionally, graphene's electrical structure enables further chemical manipulation. Through stacking, the highly concentrated electrons on the graphene

plane can interact with various proteins that contain aromatic structures. Additionally, they can be used in electrophilic reactions like click reactions, cycloadditions, and carbine insertion reactions. Hydrophobic graphene may absorb various organic compounds and polymers with high hydrophobicity through the van der Waals contact. Apart from the noncovalent stacking and van der Waals interactions mentioned previously, large oxygen functional groups can be used to functionalize GO with various molecules or biomolecules via noncovalent interactions such as hydrogen bonds and ionic interactions covalent bonds formed through chemical reactions. Thus, graphene and graphene oxide can be easily mixed with a range of bioactive materials to provide desired properties suitable for tissue engineering. This section describes and examines the mechanical, electrical, chemical, and other aspects of graphene and graphene oxide (GO) applications in tissue engineering.

6.5 Graphene oxide

Nano-sized carbon fibers, particularly graphene oxide, are the oxidized carbon layer that shows biocompatibility and has a reasonable solubility rate in water (Subbiah et al., 2014). Graphene has been studied to be used in hemorrhage control measures used in accident cases involving hemorrhage and uncontrolled bleeding (Choudhary et al., 2020). According to Safina et al. (2020), graphene oxide can create artificial extracellular matrices for tissue remodeling. It was mentioned that the integration of graphene-based flakes could support cellular proliferation leading to skin regeneration, thus providing wound healing. Furthermore, due to its structure, graphene, through the covalent linking of amino groups of chitosan with carboxyl groups of graphene oxide, can act as the cell scaffold for new tissue formation, as demonstrated in Figure 2.4 (Ding et al., 2015; Jasim et al., 2018).

The usage of graphene is similar to other nanomaterials, posing several toxicity effects. Potentially, graphene is toxic to normal cells by altering the normal physiological conditions of the cells (Makvandi et al., 2020). It is seen through studies that have been conducted in humans, as graphene produces an abundance of reactive oxygen species (ROS), it can pose risks in developing Alzheimer's disease, diabetes, atherosclerosis and is even able to promote carcinogenesis (Kirtonia et al., 2020; Leng et al., 2019; Shi et al., 2020; Zhang et al., 2019). Apart from cytotoxicity, graphene has been linked with genotoxicity, causing DNA cleavage (Xu et al., 2018). THE DNA REPAIR MECHANISM IS ACTIVATED by DNA cleavage leading to DNA damage. However, exposure to graphene has been observed to cause downregulation in the synthesis of DNA repair functions such as RAD51, ATM, and PARP1, thus affecting the DNA repair process. This failure to repair damaged DNA can lead to necrosis (Sasidharan et al., 2016).

7.0 MATERIALS AND METHODS

7.1 Graphene Oxide (GO)

Graphene oxide (GO) was obtained from Malaysia's GO Advanced Solutions Sdn. Bhd. GO was dried, weighed, and resuspended in water for stock preparation. GO was generated at various concentrations by dispersing GO nanoparticles in water, which functions as a dispersing medium, and then sonicating the suspension for one hour with probe sonication at a 50% strength to break down the GO to a monolayer sheet.

7.2 Vero Epithelial Cell Cultures

In 1962, a normal adult African green monkey's kidney tissue was used to start the Vero cell line. Verotoxins, virus detection in ground beef, effectiveness testing,

malaria research, media testing, vaccine development, protein expression, and mycoplasma testing are just a few of the uses for the cell line. The Vero cell line is also a good choice for transfection.

7.3 Complete Cell Culture Growth Medium

The complete cell culture medium was prepared by supplementing it with 10% fetal bovine serum (FBS) and 5% penicillin-streptomycin in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, United Kingdom). The material was sterilized and stored at 4 C for two weeks using a 0.22 m filter membrane.

7.4 Cultivation of mammalian cells

In this experiment, Vero cells were used. Before use, cells maintained at -80°C were gently thawed by gently stirring a vial of cells in a 37°C water bath. The vial contents were transferred to a centrifuge tube filled with 9 mL of pre-warmed complete medium and centrifuged for 5 minutes at 1000 pm. The supernatant was discarded to guarantee the complete elimination of the DMSO remnant. The cell pellet was resuspended in 5 mL of pre-warmed complete medium and added to a 10 mL pre-warmed full medium filled T75 culture flask. Cells were kept at 37°C for CO2 incubation.

When cells achieved 80–100% confluency, they were passaged and divided. The confluency of cells was determined using an inverted microscope (Olympus, Tokyo, Japan). The growth media in the flask was discarded before cell passage, and the cells were washed twice with sterile phosphate-buffered saline (PBS) to eliminate cell debris. Cells were then incubated for 5 minutes at 37°C in 4 ml of 0.5 mg/ml trypsin-EDTA solution (Sigma-Aldrich, Cambridge, United Kingdom). Before centrifugation at 1000 rpm for 5 minutes, trypsin was deactivated by adding

10 ml of growth medium. After removing the supernatant, the cells' pellet was resuspended in 10 ml of growth media.

7.5 Microscopic assessment of cell concentration

Cells were combined with trypan blue dye and placed on a hemocytometer for observation under a microscope to count them. Cells were measured using the formula C= Av 2 104 cells ml, where C equals cell concentration (cells/ml), Av equals an average number of cells counted in four corners, and two* equals dilution factor.

7.6 Preservation of the cell growth

They were grown to confluence and then detached from the flask using the previously described trypsinization procedure to preserve the cells. Centrifugation at 1000 rpm for 5 minutes was used to transfer the cell suspension into a tube. The pellet was resuspended in media (90% FBS, 10% DMSO) at a 5106 cells/ml concentration. DMSO was utilized as a cryoprotective drug in this study to protect cells from damage induced by extreme cold. The suspensions were stored overnight at -80°C freezers in cryovials (Fisher Scientific, Loughborough, United Kingdom) labeled with the date and passage number. The cryovials were transported to a cryogenic freezer holding liquid nitrogen at a temperature of -150°C for long-term storage.

7.7 Experimental Design

7.7.1 Determination confluency of Cell Culture Growth

To determine the confluency of the cell culture growth, post-treatment must be applied to the cell culture. Firstly, remove the old media. 1ml PBS was prepared to wash the cell culture. Then we add 0.5ml Trypsin

EDTA to detach the cell on the well. We then incubate the cells for 5 minutes. After incubation, all solutions in the well will be transferred into a 1.5ml microcentrifuge tube for each well. Then we spin down at 2000rpm for 2 minutes. After that, we remove the supernatant. Then add 100µL 2% DMEM or DMEM. Next, we pipette up and down a few times, and 100 µL trypan blue will be added and mixed well. Then we can count the cells.

7.7.2 Statistical analysis

Quantitative data were expressed as the mean of at least three replicated samples plus the standard deviation. ANOVA and the Student's t-test were used for statistical analysis (SPSS software 13.0, USA; GraphPad Prism 7.0, USA). Statistical significance was defined as a value of p 0.05.

8.0 RESULTS

Table 8.1: Microscopic evaluation of Vero cells treated with Graphene Oxide on Day 1.

The Volume of Graphene Oxide (µL)	Microscopic Evaluation
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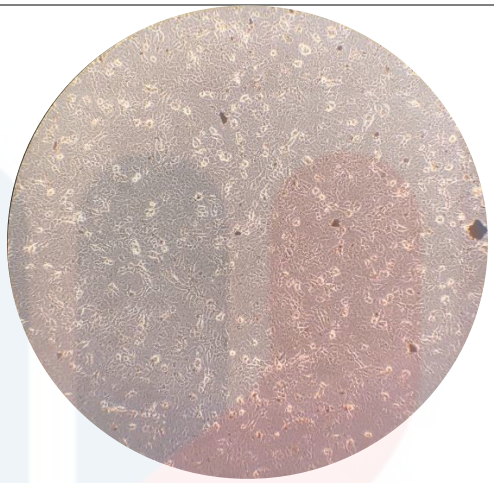
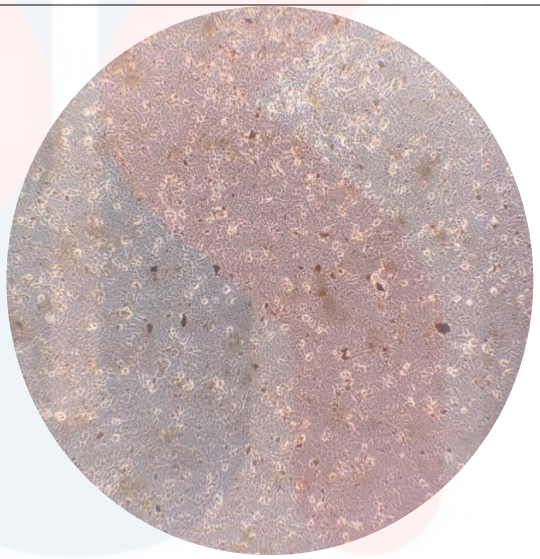
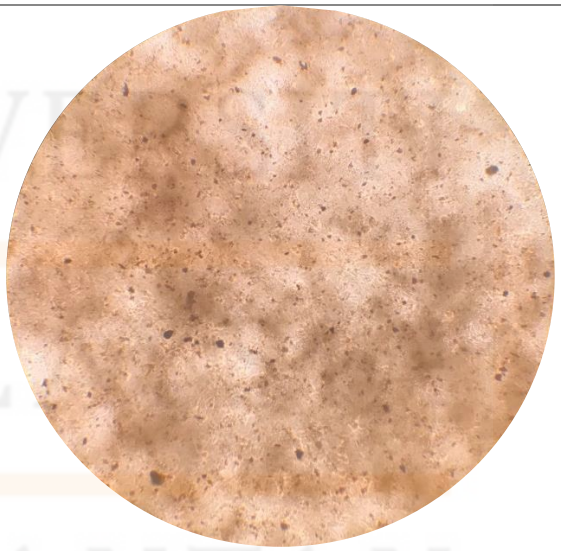
25	
50	
100	

Table 8.2: Microscopic evaluation of Vero cells treated with Graphene Oxide on Day 2

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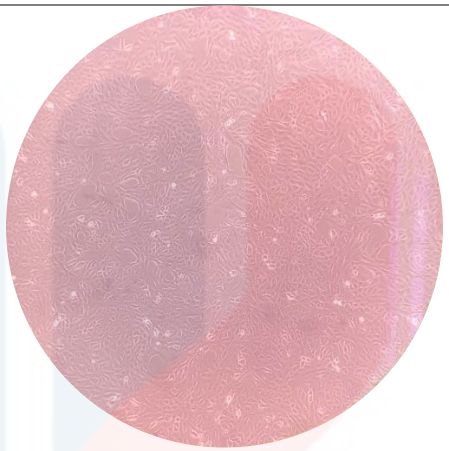
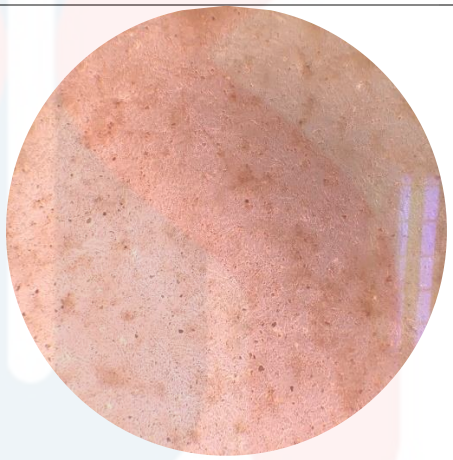
NT	
150	
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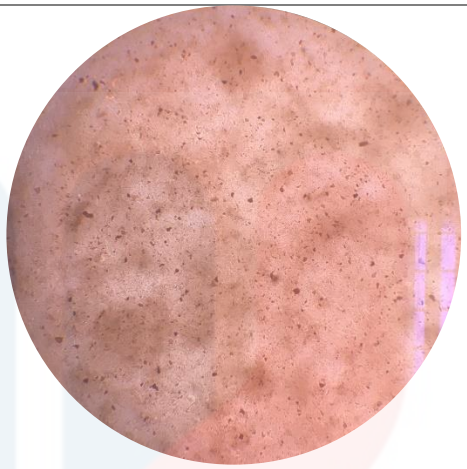
250	
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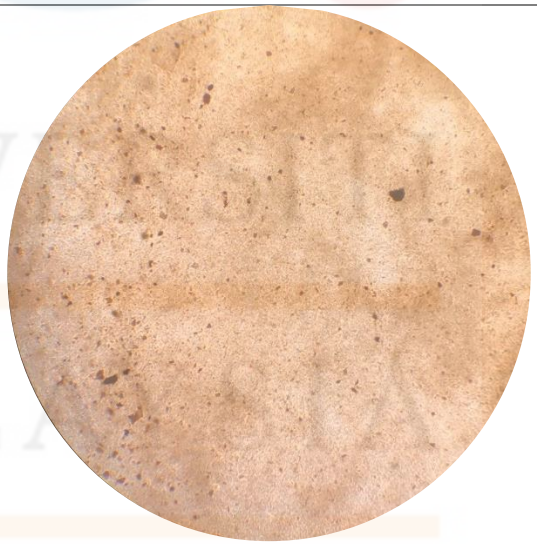
350	

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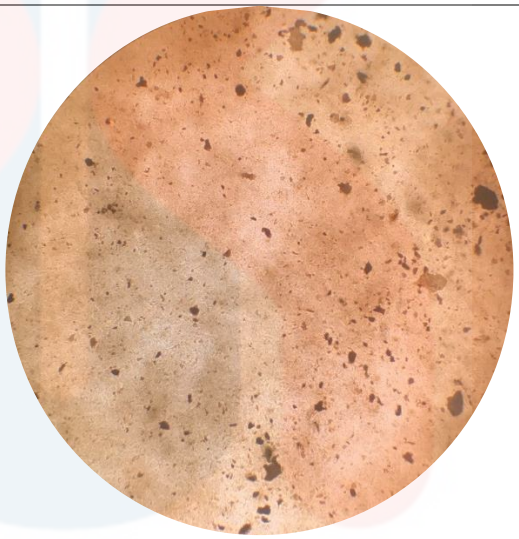
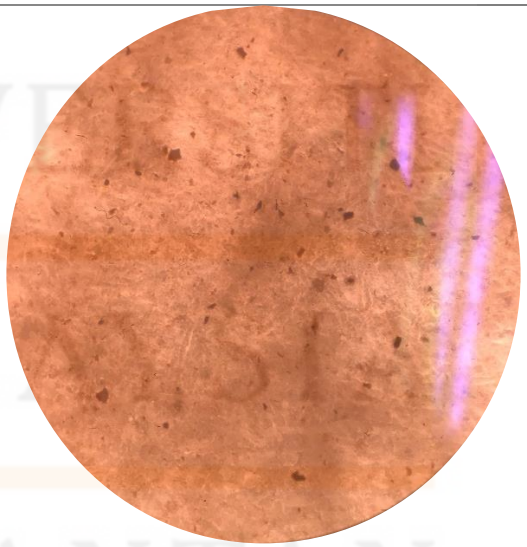
450	
500	

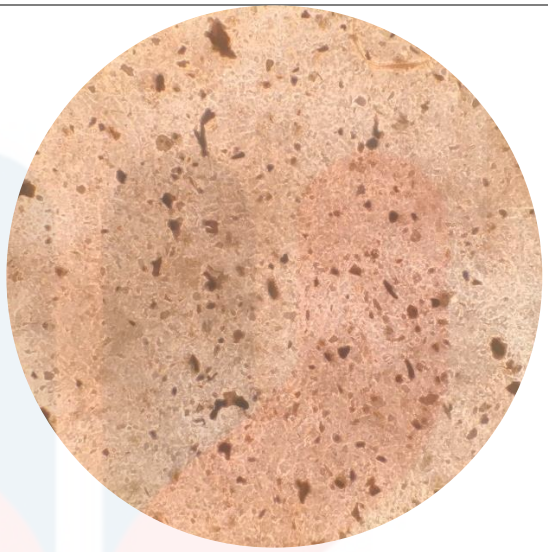
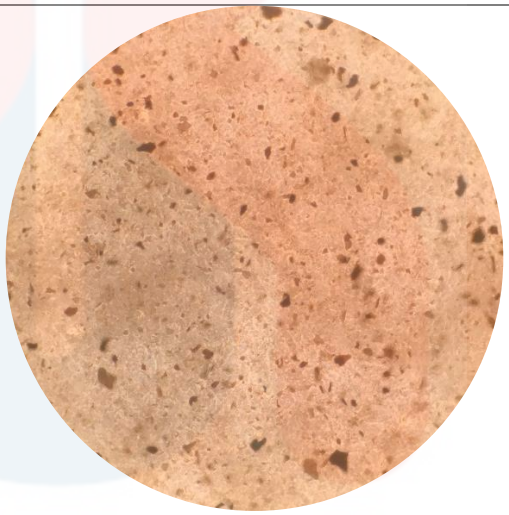
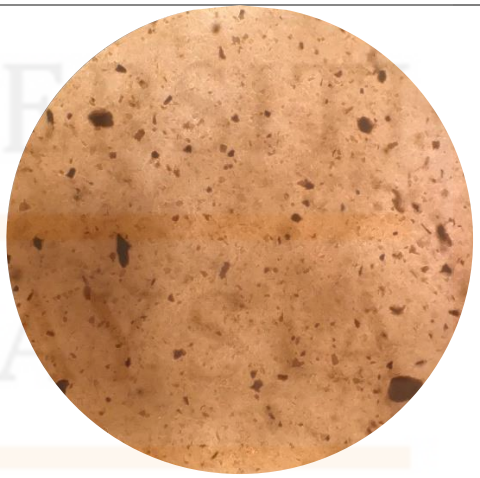
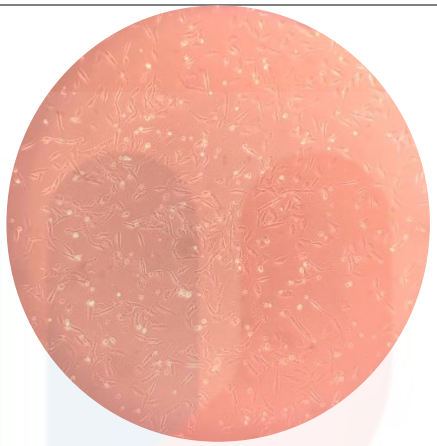
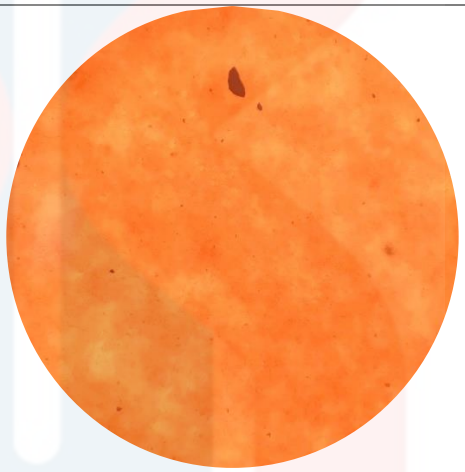
550	
600	
650	

Table 8.3: Microscopic evaluation of Vero cells treated with Graphene Oxide on Day 3.

The Volume of Graphene Oxide (µL)	Microscopic Evaluation

NT	
700	
750	

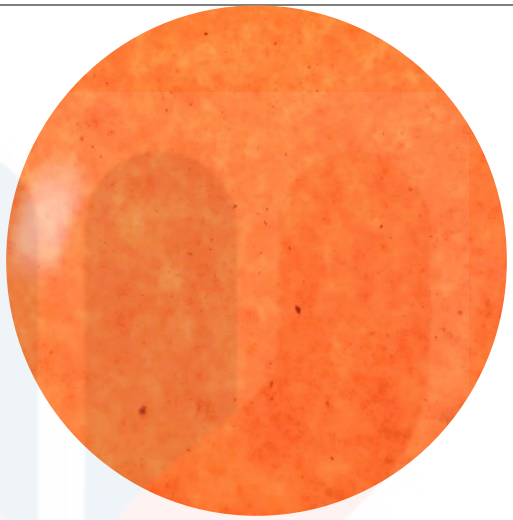
800	

Table 8.1 until Table 8.3 shows the microscopic evaluation of Vero Cells on day 1 and 3 respectively. From the observation, several cells were counted from all three wells treated with different volumes of Graphene Oxide which is tabulated in Table 8.4 until Table 8.6. This is to obtain an average number of Vero cells for each volume of Graphene Oxide used in treatment.

Table 8.4: Number of cells upon microscopic evaluation of Vero cells treated with Graphene Oxide on Day 1.

Volume Of Graphene Oxide (µL)	**Number of cells**		
	Well 1	**Well 2**	**Well 3**
NT	514	980	165
25	1838	1600	1907
50	2114	2064	2165
100	2513	3522	3713

Table 8.5: Number of cells upon microscopic evaluation of Vero cells treated with Graphene Oxide on Day 2.

	Number of cells

The volume of Graphene Oxide (µL)	Well 1	Well 2	Well 3
NT	425	222	442
150	260	417	573
200	107	424	891
250	556	489	1044
300	412	462	984
350	440	304	873
400	232	312	551
450	300	322	426
500	104	341	297
550	100	354	290
600	380	375	362
650	140	210	170

Table 8.6: Number of cells upon microscopic evaluation of Vero cells treated with Graphene Oxide on Day 3.

The volume of Graphene Oxide (µL)	Number of cells		
	Well 1	Well 2	Well 3
NT	13	14	17
700	1	6	8
750	2	3	6
800	0	2	4

Table 8.7: Impact of Graphene Oxide towards Vero cells based on different parameters.

The volume of Graphene Oxide (µL)	Mean No. of the cells	Cell counting (x 10^5)/ ml	Confluency of the cells (%)
NT	553	276.5	100
25	1781	890.5	322
50	2114	1057	382
100	3249	1624.5	587
150	416	208	75
200	474	237	86
250	696	348	126
300	619	309.5	112
350	539	269.5	97
400	365	182.5	66
450	349	174.5	63
500	247	123.5	45
550	246	123	44
600	372	186	67
650	173	86.5	31
700	5	2.5	0.9
750	4	2	0.7
800	2	1	0.36

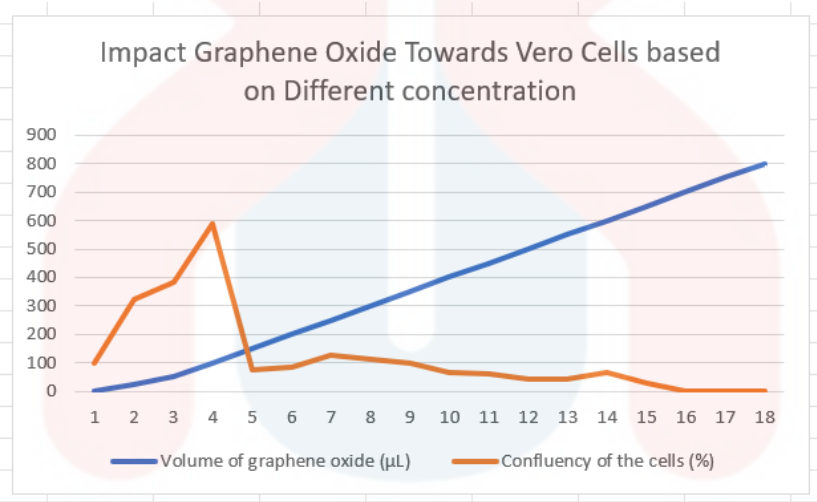
Table 8.7 shows the impact of graphene oxide towards Vero Cells based on different parameters. The volume of graphene oxide which is the most effective in promoting cell growth is 100 µL which increases the percentage of the confluency of the cell to 587%.

While on the volume of graphene oxide 800 µL, the percentage of the confluency of the cells drops to 0.36% which is the lowest number of cells. Thus, the confluency of the

cells increases from the non-treatment when the volume of the graphene oxide was in the range of 25µL until 300µL and slowly drop from 100% to the volume of 350µL to 800µL.

9.0 DISCUSSION

This experiment was done to investigate the impact of Graphene Oxide on the cell culture. The experiment shows the treated cells with graphene oxide were the most effective in increasing the number of cells along with the high concentrations of graphene oxide, but the number of cells becomes decreasing once it reaches certain concentrations.



Based on the results, the number of cells increased compared to non-treatment cells on the volume of graphene oxide was 25µL to 300µL. This is because Graphene oxide consists of the integration of graphene-based flakes that could support cellular proliferation leading to skin regeneration, thus providing wound healing. Furthermore, due to its structure, graphene, through the covalent linking of amino groups of chitosan with carboxyl groups of graphene oxide, can act as the cell scaffold for new tissue formation, as demonstrated.

The hydrophilic property of Graphene oxide allows it to avoid aggregation in cell culture media due to its oxygen concentration. The aggregation phenomenon reduces nutrition delivery, causing oxidative stress and triggering the apoptotic pathway.

Furthermore, the oxygen functional groups of Graphene Oxide may be able to modulate protein adsorption in the extracellular matrix (ECM), resulting in improved cell adhesion and proliferation.

However, the number of cells decreases starting from the volume of 350µL until 800µL. This is because, at certain concentrations, the graphene oxide can cause damage to the plasma membrane structural integrity by penetrating through the plasma membrane and probably disrupting the phospholipid bilayer. Other than that, the structure of the graphene oxide which is larger and consists of spike-like on the membrane surface can lead to injury to cells when in high concentration.

Graphene oxide also can cause toxicity at a high-volume concentration and based on the journal which mostly believes that one of the processes driving nanomaterial toxicity is the production of cellular oxidative stress. Overproduction of oxidative-free radicals causes oxidative stress, which is caused by an imbalance between the oxidative and antioxidative defense mechanisms of cells and tissues. This leads to cellular impairment such as deficiencies in energy metabolism, cell signalling and cell cycle regulation, cell transport pathways, and general biological dysfunction, as well as immunological activation and inflammation.

10.0 CONCLUSION

In conclusion, Graphene Oxide promotes cell growth as supported by the result based on the increase in the percentage of confluency of the Vero cells. Therefore, Graphene oxide can improve the growth of selected cell lining of the Vero Cells. However, graphene oxide in certain concertation deemed too high a concentration can lead to cell death. Hypothesis accepted.

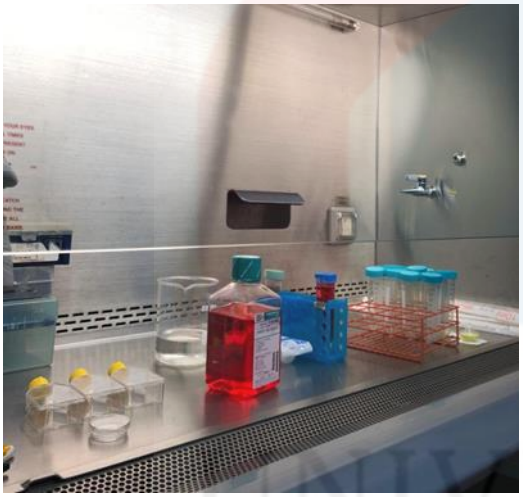
11.0 RECOMMENDATIONS AND FUTURE WORK

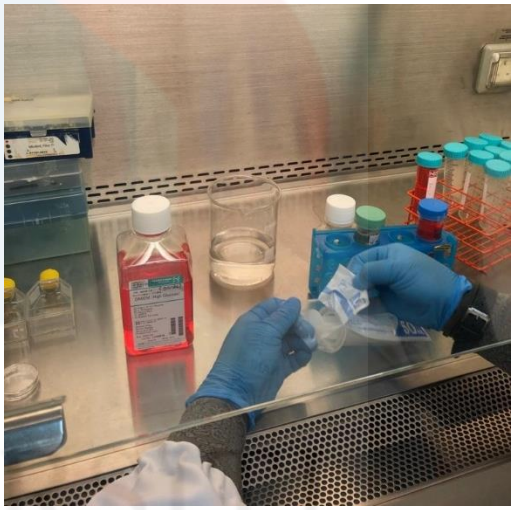
The impact of graphene oxide on cell culture growth can be enhanced by expanding the type of cells, for example, HeLa cells, skin cells, and other cells to help identity which cells react the most towards graphene oxide. Other than that, the cytotoxicity test must be add on in the method to ensure the graphene oxide's potential on the cells by determining the number of living cells. The resazurin assay can be add on into the method.

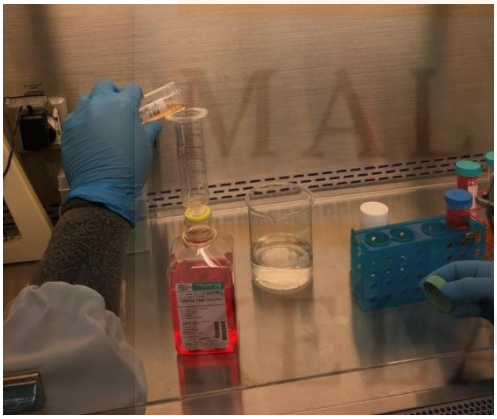
In addition, the scope of research can be increased to learn more about the impact of graphene oxide on cells.

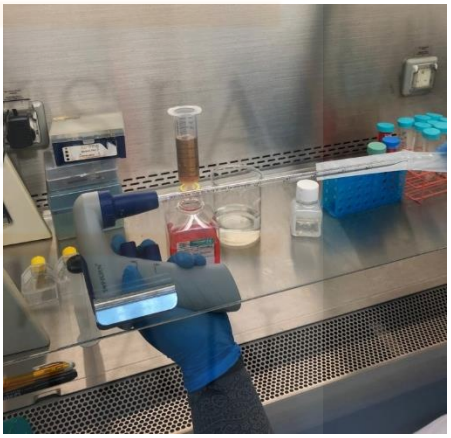
Furthermore, the cell culture methods and treatment of cells can be improved to preserve the results of the outcomes and increase the likelihood of future better outcomes.

APPENDICES









REFERENCES

Zare, P., Aleemardani, M., Seifalian, A., Bagher, Z. and Seifalian, A., 2021. Graphene Oxide: Opportunities and Challenges in Biomedicine. *Nanomaterials*, 11(5), p.1083.

Verre, A., Faroni, A., Elliot, M., Silva, C., Muryn, C., Reid, A. and Vijayaraghavan, A., 2022. Biochemical functionalization of graphene oxide for directing stem cell differentiation. *Journal of Molecular Structure*, 1249, p.131578.

Garcia-Alegria, E., Eliot, M., Stefanska, M., Silva, C., Heeg, S., Kimber, S., Kouskoff, V., Lacaud, G., Vijayaraghavan, A. and Batta, K., 2016. Graphene Oxide promotes embryonic stem cell differentiation to hematopoietic lineage. *Scientific Reports*, 6(1).

Hussein, K., Abdelhamid, H., Zou, X. and Woo, H., 2019. Ultrasonicated graphene oxide enhances bone and skin wound regeneration. *Materials Science and Engineering: C*, 94, pp.484-492.

Delavary, B., van der Veer, W., van Egmond, M., Niessen, F. and Beelen, R., 2011. Macrophages in skin injury and repair. *Immunobiology*, 216(7), pp.753-762.

Zhang, K., Zheng, H., Liang, S. and Gao, C., n.d. *Aligned PLLA nanofibrous scaffolds coated with graphene oxide for promoting neural cell growth*.