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Pulsed-Low Intensity Ultrasound Improved Wound Healing Process in Hyperglycemic Microenvironment

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ABSTRACT

Chronic wounds among diabetic patients have always been in a concerning state over the past years. The implementation of non-invasive procedures such as ultrasound techniques to accelerate the healing of diabetic wounds is believed to provide impressive benefits in terms of improving cells' physiology mechanistically. This current study focused on the effect of pulsed-low intensity ultrasound (PLIUS) as a mechanical stimulator for wound healing experiments under a hyperglycemic microenvironment. HSF 1184 cells were first optimized in 6-well plates using several seeding densities. The optimized seeding density, 4×10^5 cells/mL, was used for the scratch assay experiment. PLIUS probe was placed on the center of the scratched cells and treated with several doses for 5 minutes. The percentage of wound closure of HSF 1184 was analyzed using ImageJ software. The results revealed that the cells subjected to ultrasound treatment at a frequency of 1 MHz, intensity of 0.3 W/cm^2, and 50% of duty cycle exhibited the greatest extent of wound closure in comparison to the other two ultrasound dosages. In summary, it can be concluded that the utilization of low-intensity therapeutic ultrasound at a frequency of 1 MHz, an intensity of 0.3 W/cm^2, and a duty cycle of 50% holds promise for facilitating wound healing under a hyperglycemic microenvironment.

INTRODUCTION

Diabetes Mellitus (DM) is a common metabolic disease denoted by hyperglycemia, or high blood sugar resulting from a fault in either insulin production or the molecular mechanism of the secreted insulin (Akhtar et al., 2022; Saeedi et al., 2019). In 2019, the International Diabetes Federation (IDF) reported that 463 million individuals worldwide were diagnosed with T2DM, with 79% of cases originating from low and middle-income countries (Ganasegeran et al., 2021). The Southeast Asia region emerged with the highest prevalence, at 8.8%, while Malaysia led the Western Pacific region with 3.65 million cases, constituting 16.8% of 21.71 million individuals (Ganasegeran et al., 2021). Projection from IDF suggest that the global prevalence of diabetes will continue to escalate, reaching 578 million (10.2%) by 2030 and 700 million (10.9%) by 2045 (Saeedi et al., 2019).

Mansour et al. (2023) previously asserted that most diabetic patients face susceptibility to various complications, encompassing both macroangiopathy and microangiopathy. Macroangiopathy, involving damage to larger blood vessels. Complications such as coronary artery disease, peripheral vascular disease, and stroke are common manifestations of macroangiopathy in diabetes. Meanwhile, microangiopathy complications, such as retinopathy, nephropathy, and neuropathy, stem from damage to small blood vessels due to diabetes (Mansour et al., 2023). Among these, peripheral neuropathy, characterized by impaired blood vessels in the

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peripheral nervous system, predisposes individuals to a spectrum of foot issues, including deformities, infections, and ulceration. Notably, foot ulceration emerges as a paramount concern among diabetic patients, given its potential to impede wound healing, necessitate amputation, and even lead to fatalities (Raja et al., 2023). Commonly, normal wound will be healed within 14 days that involves 4 overlapping phases (Hemostasis, Inflammatory, Proliferation and Remodeling). While diabetic foot ulcers are chronic wounds that have a delay in healing due to complication involving in each wound healing phase and require stimulation from other chemical or physical factors to be able to undergo the healing process (Raja et al., 2023). The collective impact of macroangiopathy and microangiopathy highlights the multifaceted challenges faced by diabetic patients, emphasizing the importance of comprehensive management strategies.

Today, a range of biochemical-based therapies has been developed to address chronic wounds, specifically those associated with diabetes, encompassing both conventional wound dressings and pharmaceutical interventions. An optimal wound dressing should alleviate symptoms, hinder the exacerbation of wound infections, maintain a moist environment, facilitate tissue regeneration, and ultimately foster wound healing (Nguyen et al., 2023). However, conventional dressings such as non-adhesive dressings, foam and alginate dressings, and hydrogel wound care fail to meet all the criteria for an ideal wound dressing for chronic wounds, notably diabetic foot ulcers (Everett & Mathioudakis, 2018).

Therapeutic ultrasound emerges as a promising avenue for diabetic wound healing, serving as a mechanical stimulator, thereby offering an alternative approach to traditional biochemical-based treatments. Ultrasound, characterized by mechanical vibrations induced by ultrasound waves exceeding 20 kHz—beyond the human hearing range, has been a staple in clinical practice since the 1930s (Carovac et al., 2011; Conner-Kerr & Oesterle, 2017; Moyano et al., 2022). With over 60 years of widespread utilization, therapeutic ultrasound serves as a mechanical stimulator for wound healing (Conner-Kerr & Oesterle, 2017). It has been extensively employed in the treatment and facilitation of healing processes for various chronic wounds, including diabetic foot ulcers. The outcomes of such treatments have demonstrated remarkable therapeutic benefits, including enhanced cellular activity and accelerated wound closure favoring the formation of granulation tissue in the wound bed (Flores-Escobar et al., 2022). These effects contribute to heightened healing rates and shortened healing durations for challenging-to-treat wounds (Flores-Escobar et al., 2022).

Nonetheless, there are still many uncertainties surrounding the use of therapeutic ultrasound as a mechanical stimulator for healing diabetic wounds. Although previous research has highlighted the beneficial effects of therapeutic ultrasound in enhancing wound healing activity, there is a lack of focus on how ultrasound treatment affects cells in a hyperglycemic environment. This study seeks to investigate the reaction of human skin fibroblast (HSF) cells to therapeutic ultrasound, specifically low-intensity ultrasound, within a hyperglycemic microenvironment setup.

MATERIALS AND METHOD

Materials

Dulbecco's Modified Eagle Medium (DMEM) and Fetal Bovine Serum (FBS) were purchased from Capricorn Scientific whereas trypan blue solution was obtained from Sigma Aldrich (Malaysia). Trypsin and antibiotic-antimycotic were purchased from Gibco Company. Phosphate Buffered Saline (PBS) tablets pH 7.4 were obtained from Clontech Laboratories.

Cell Culture and Passage

HSF 1184 was cultured in a complete growth medium and maintained at 37°C in a humidified incubator with 5% CO2 (Chiu et al., 2020). The media was refreshed every three days to avoid nutrient depletion and toxic accumulation which can lead to cell death. Observation of the cells was done periodically using an inverted microscope (Nikon Eclipse Ti-S microscope with Q-imaging Retiga 2000R camera, Selangor).

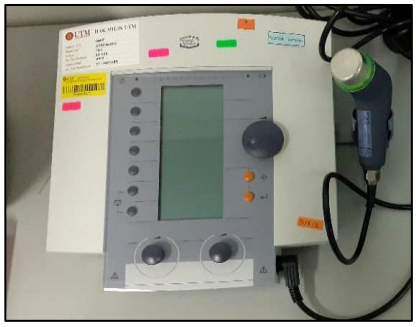

Fig. 1 The Sonopuls 492 ultrasound machine used in the study.

Fig. 2 The setup of the ultrasound transducer in a water bath.

Table 1 – Parameters of low-intensity therapeutic ultrasound.

Frequency (MHz)	Intensity (W/cm^2)	Duty Cycle (%)	Duration (Min)	Distance (cm)
1	0.2	20	5	0.5
	0.3	50		
3	0.3	50		

Optimization of Seeding Density of HSF 1184 in Hyperglycemic Microenvironment

HSF 1184 cells cultured in a modified growth medium with a glucose concentration of 150 mM were plated into 6-well plates at varying cell seeding densities: 2 × 105, 4 × 105, and 6 × 105 cells/mL, denoted as A, B, and C respectively. Each density had designated time points: A1 for 24 hours post-treatment, A2 for 48 hours post-treatment, and A3 for 72 hours post-treatment. The same labeling system applied to the other seeding densities. Cells then were left to grow at 37°C in a 5% CO2 incubator and observed after 72 hours using an inverted microscope. The observation and decision on optimized cell seeding density were made after 72 hours since the experiment duration lasted for only 3 days (Larsson et al., 2020). Cells that reached approximately 80% confluency after 72 hours were chosen as the optimized seeding density and proceeded to the next experiment (Phelan & May, 2016).

Low-Intensity Therapeutic Ultrasound Treatment

The Sonopuls 492 (JH Enraf Nonius, Petaling Jaya, Selangor) device was employed to administer ultrasound waves to the cells, as illustrated in Figure 1. The treatment group underwent exposure to specific ultrasound parameters, while the control group did not receive any ultrasound treatment.

Throughout the treatment process, HSF cells cultivated in a hyperglycemic microenvironment within 6-well plates were positioned atop the ultrasound transducer probe, maintaining a distance of 0.5 cm, at intervals of 5 minutes, as depicted in Figure 2. The ultrasound parameters utilized in the treatment were determined based on established studies, as detailed in Table 1 (Anwar & Mohd Bohari, 2019).

Scratch Assay

In vitro scratch assay has become one of the most economical, straightforward, and well-developed methods to study cell migration in vitro (Chen et al., 2023; Suarez-Arnedo et al., 2020). The basic principle of this assay is to create an artificial gap or scratch to study the migration of cells towards the artificial gap and later, the cells will gradually close the wound gap by establishing cells-to-cells interaction (Chen et al., 2023).

Cells with optimized seeding density which was 4 × 105 cells/mL were seeded into 6-well plates and grown into confluence by maintaining it at 37°C in a 5% CO2 incubator for 24 hours (Manstein et al., 2021). After 24 hours, the cells were then starved in a culture medium without FBS for 6 hours.

Then, a sterile 200 µl pipette tip was used to create the scratch with a consistent shape (Chen et al., 2023). Following that, the culture medium without serum was replaced with a hyperglycemic medium and the cells were treated with low-intensity therapeutic ultrasound afterwards. The studies for wound scratch assay were done in triplicate.

Image Analysis

The control and treated groups were observed at designated time intervals following treatment with PLIUS, specifically at 0, 0.5, 1, 18, 20, 22, and 24 hours. Wound closure images for both groups were captured using an inverted microscope at a magnification of 10×. Subsequently, the images were analyzed

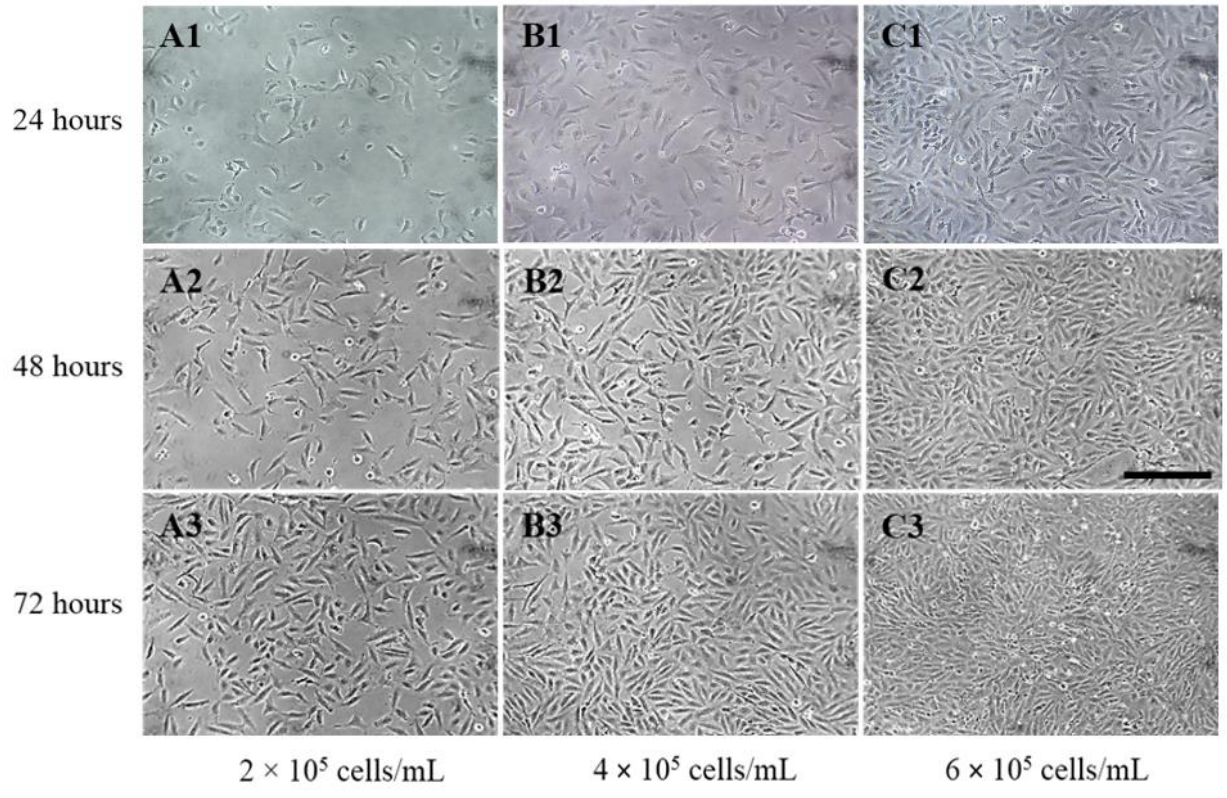

Fig. 3 Images of HSF 1184 captured at 10 × magnification after 24, 48 and 72 hours. Images labeled A, B and C represent the cells with seeding densities of 2×105, 4×105 and 6×105 cells/mL respectively. Scale bar: 100 µm.

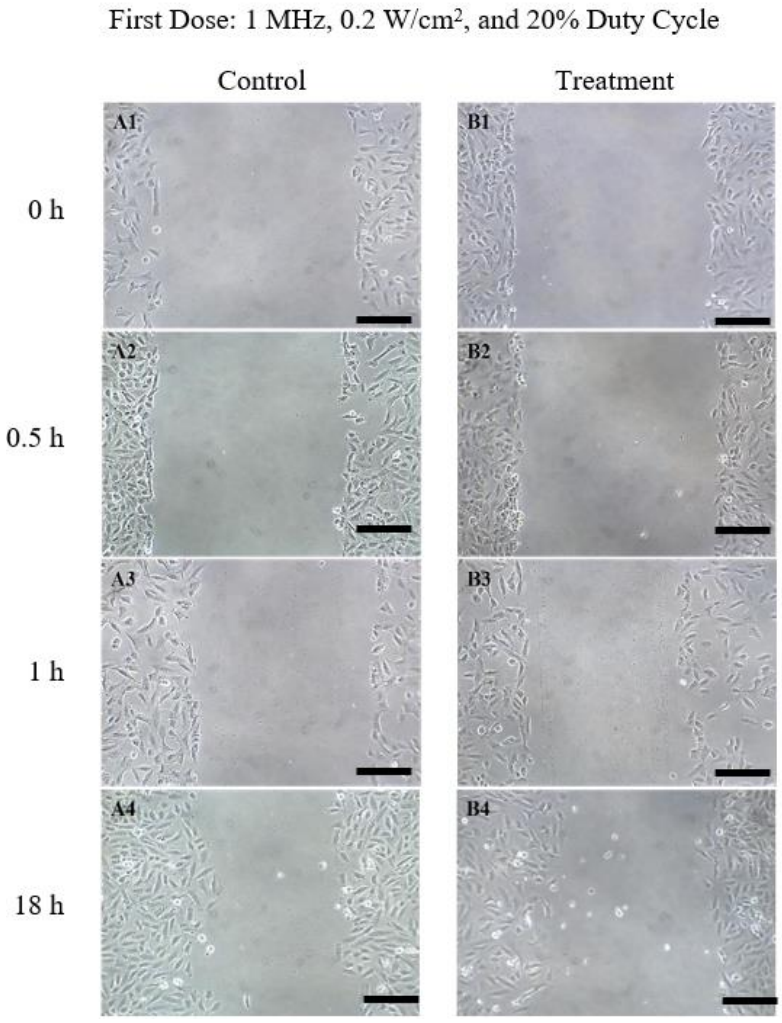

Fig. 4 Images of control and treatment group (1 MHz, 0.2 W/cm2, 20% duty cycle; 5 min; 0.5 cm) of HSF 1184 cultured in hyperglycemic condition labelled as A and B respectively at 10 × magnification at 0, 0.5, 1 and 18 hours. Scale bar: 80 µm.

utilizing ImageJ software (NIH, Bethesda, MD) (Suh et al., 2022). The formula for calculating wound closure is expressed as follows: Wound closure= [(Wound area at time 0 − Wound area at time x) × 100] (Suh et al., 2022).

Statistical Analysis

Results gathered from scratch assay were further analyzed using Microsoft Excel. The data from both treatment and control groups were compared and the doses of ultrasound that give the desired effect towards the cells would be selected.

RESULT AND DISCUSSION

Optimization of Seeding Density for HSF 1184 in Hyperglycemic Microenvironment

It was previously reported that cell seeding density can significantly influence the proliferation and differentiation activity of the cells (Wu et al., 2020). Optimum cell seeding density for types of cells can be determined by the confluency of cell monolayer. Confluency is defined as the percentage of coverage of cell monolayer on the surface of the culture vessel (Chiu et al., 2020). However, inadequately information in optimization of cells seeding density in hyperglycemic condition to mimic chronic condition in vitro needs further investigation. In this study, the seeding density of HSF 1184 was optimized in a hyperglycemic microenvironment. In this study, optimization was conducted to determine the optimum cell seeding density for an experiment that lasted for 72 hours. The optimum seeding density in this study is defined by cells reaching approximately 80% confluency after 72 hours of incubation (Phelan & May, 2016). In hyperglycemic conditions, fibroblast cells were able to proliferate, but at a restricted rate. The over-production of reactive oxygen species (ROS) at high glucose concentration may play roles in affecting its proliferative cycle (González et al., 2023).

In this study, different cell seeding densities were studied to determine the most optimized seeding density. The cells' confluency with seeding density ranging from 2×105, 4×105 and 6×105 cells/mL were observed at 24, 48 and 72 hour. Figure 3 depicts a consistent pattern of increasing cell confluency with higher cell seeding densities, indicating a corresponding rise in cell numbers preceding the escalation in seeding density. After 72 hours of incubation, all of three seeding densities labeled as A3, B3 and C3 showed different ranges of cell confluency. Cells labeled A3 and B3 showed approximately 70% and 80% confluency respectively. On the other hand, C3 showed the

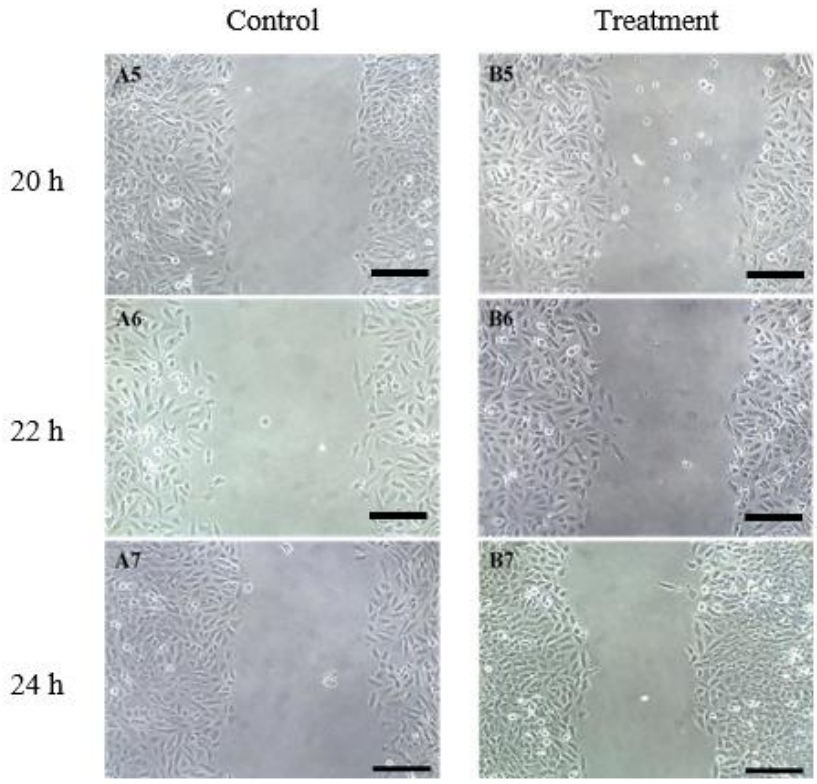

Fig. 5 Images of control and treatment group (1 MHz, 0.2 W/cm2, 20% duty cycle; 5 min; 0.5 cm) of HSF 1184 cultured in hyperglycemic condition labelled as A and B respectively at 10 × magnification at 20, 22 and 24 hours. Scale bar: 80 µm.

image of over-confluent cells after 72 hours. Over-confluency indicates that the surface of culture vessels is completely covered with cells, leaving no more space for the cells to form cell monolayer. Therefore, based on the observation recorded, the optimum seeding density for HSF 1184 under hyperglycemic microenvironment was 4×105 cells/mL and the optimized cell seeding density was used in the scratch assay experiment.

The over-confluent cells as seen in image C3 in Figure 3 could increase the possibility for the cells to be clumped together in the culture medium. When cells reach their confluency, the cells will either stop growing due to contact inhibition or continue proliferating until they clumped together in the suspension. The cells can also form a new layer of cells on top of the monolayer cells. Hence, they are not suitable for use as they can affect the accuracy of ultrasound treatment experiment (Chiu et al., 2020; Xue et al., 2023). Over-confluent cells are also often associated with impaired intracellular signaling networks that can give rise to aberrant and irreproducible results. Signaling network in cells refers to the capability of the cells to produce output and signaling each other to perform a physiological function in response to various stimuli including mechanical stimuli such as ultrasound waves (Yoo et al., 2022). On the other hand, seeding of a high count of cells can also lead to nutrient depletion which can result in cell death (Chiu et al., 2020; Xue et al., 2023).

Scratch Assay of HSF 1184 in Hyperglycemic Microenvironment

Cell migration can be characterized as the motion of cells either in the form of single cells or a collective of cells from one location to another (Chen et al., 2023; Pijuan et al., 2019). It is a vital step in many biological processes such as inflammatory and wound healing phases. The migration process requires the cells to change in terms of their motility behavior to enable them to move to proper location and perform their functions in a multicellular organism (Pijuan et al., 2019). In addition, cell migration can be influenced by many factors including physical, chemical, and biological factors (Pijuan et al., 2019).

In a previous study, the impact of various ultrasonic dosages on the migration of human skin fibroblast cells in normal microenvironment was investigated. The present work aimed to assess the impact of the effect of ultrasound on migration of fibroblast cells in hyperglycemic microenvironment. This study utilized three different dosages of ultrasound to investigate its potential in promoting wound healing in such a microenvironment. The list of ultrasound frequencies and its other parameters are shown in Table 1.

In the hyperglycemic environment, the study observed that cell migration towards the cell-free area occurred at a slower pace compared to the normal microenvironment (Nagy et al., 2019). According to Nagy et al. (2019), elevated glucose levels can disrupt normal cellular functions, including proliferation and migration. Additionally, the presence of protein O-GlcNAc, induced by high glucose concentrations, was identified as another contributing factor to the reduced migration rate of fibroblast and other cell types involved in the wound healing

process. This increase in O-GlcNAc levels ultimately leads to impaired wound closure due to the slower migration rate of fibroblast cells (Nagy et al., 2019).

group in which the control groups were not exposed to any ultrasound doses.

Figures 4 and 5 show the migration activity of HSF 1184

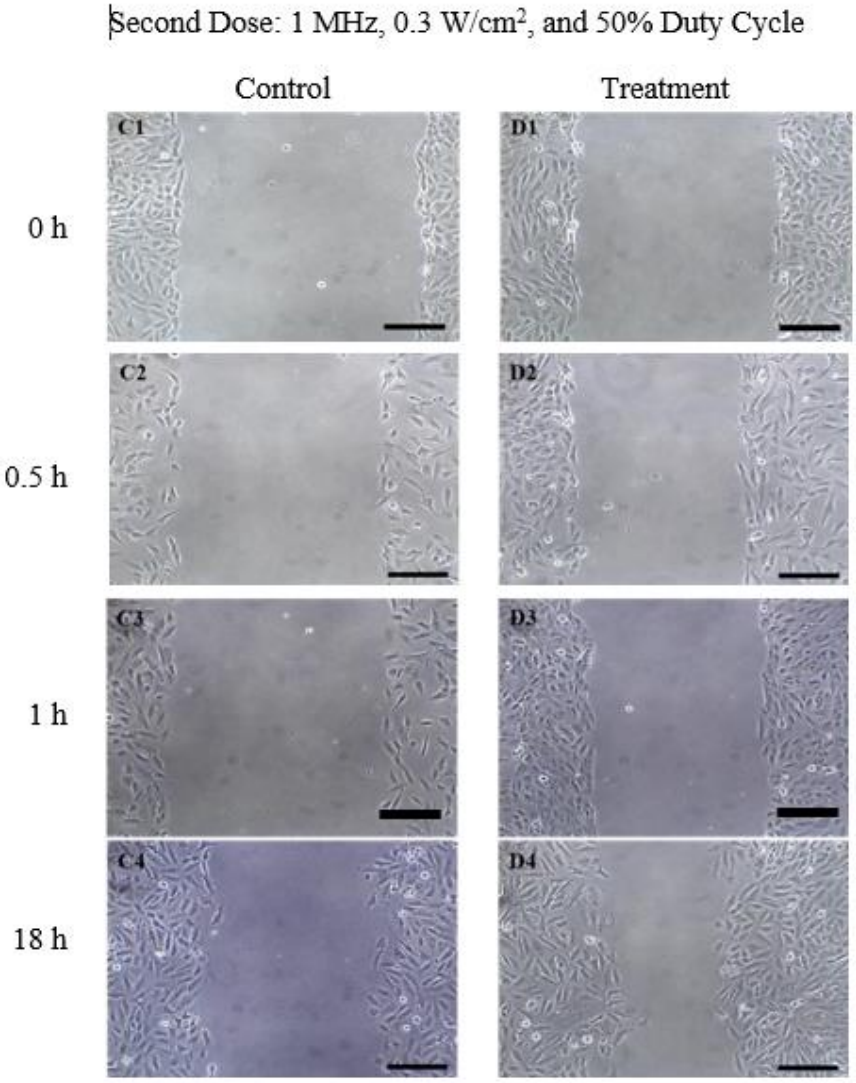

Fig. 6 Images of control and treatment group (1 MHz, 0.3 W/cm2, 50% duty cycle; 5 min; 0.5 cm) of HSF 1184 cultured in hyperglycemic condition labelled as C and D respectively at 10 × magnification at 0, 0.5, 1 and 18 hours. Scale bar: 80 µm

In comparison to the normal microenvironment, where cell migration occurs at a presumably faster rate, these findings suggest that the hyperglycemic environment adversely affects the cellular processes crucial for efficient wound healing. This underscores the importance of understanding and addressing the impact of hyperglycemia on wound healing mechanisms.

In this study, *in vitro* scratch assay is the method of choice to study the migration of HSF 1184 under a hyperglycemic microenvironment. The scratch assay is centered on the formation of an artificial gap or scratch on the cell monolayer and the migration of the cells toward the cell-free area in a controlled microenvironment will be studied (Bobadilla et al., 2019). In this experiment, the cells were treated with ultrasound for 5 minutes per treatment to avoid excessive heating that could further lead to cell damage (Anwar & Mohd Bohari, 2019). Meanwhile, the safe distance between the transducer and the bottom of 6-well plates containing cells was kept constant at 0.5 cm. Additionally, each treated group of cells had its own control

treated with the first dose of ultrasound which was 1 MHz, 0.2 W/cm^2, and 20% of duty cycle at intervals of 0, 0.5, 1, 18, 20, 22 and 24 hours. The cells treated with the first dose of ultrasound did not show a tremendous increase in terms of percentage of wound closure even after 18 to 24 hours of observation. However, the percentage of wound closure for the cells treated with the first ultrasound dose increased steadily over the desired time intervals. Overall, the ultrasound dose yielded a positive effect on cell migration. Nevertheless, in comparison to the other two doses, the first dose of ultrasound (1 MHz, 0.2 W/cm^2, and 20% of duty cycle) recorded a significantly slower cell migration.

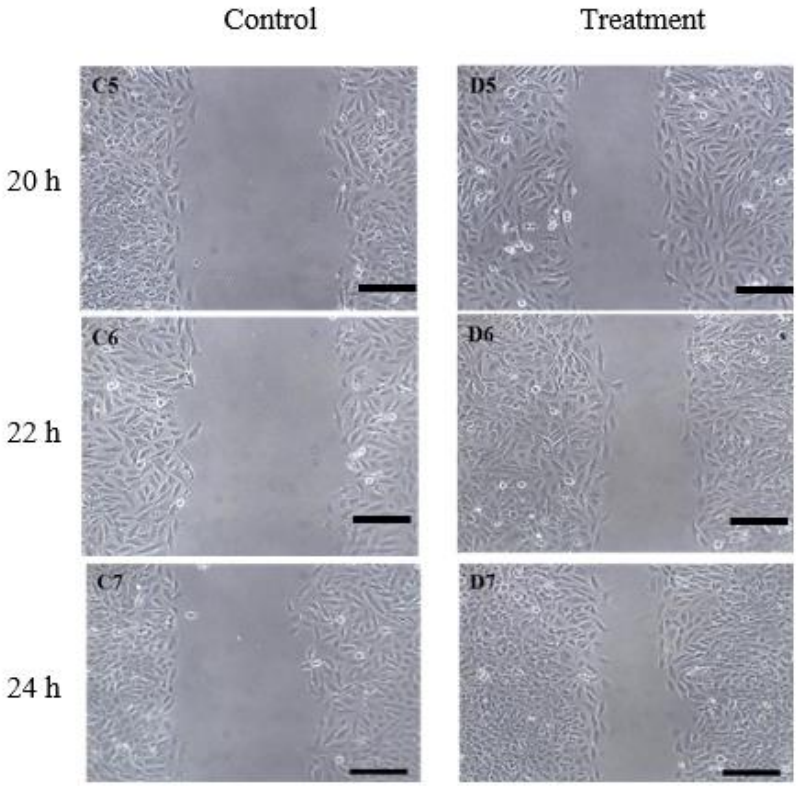

Fig. 7 Images of control and treatment group (1 MHz, 0.3 W/cm2, 50% duty cycle; 5 min; 0.5 cm) of HSF 1184 cultured in hyperglycaemic condition labelled as C and D respectively at 10 × magnification at 20, 22 and 24 hours. Scale bar: 80 µm.

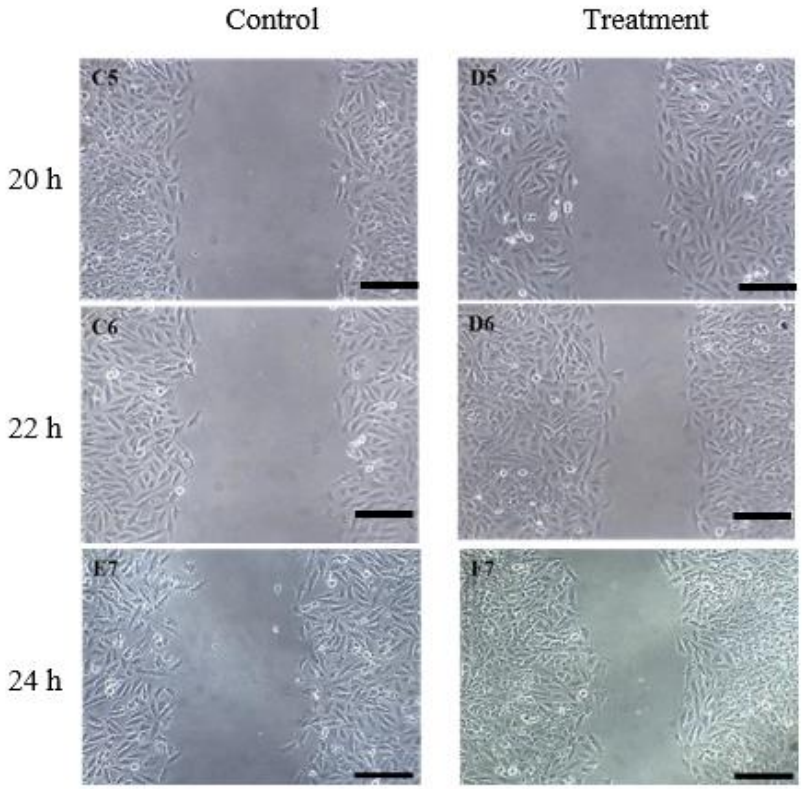

Fig. 9 Images of control and treatment group (3 MHz, 0.3 W/cm2, 50% duty cycle; 5 min; 0.5 cm) of HSF 1184 cultured in hyperglycemic condition labelled as E and F respectively at 10 × magnification at 20, 22 and 24 hours. Scale bar: 80 µm.

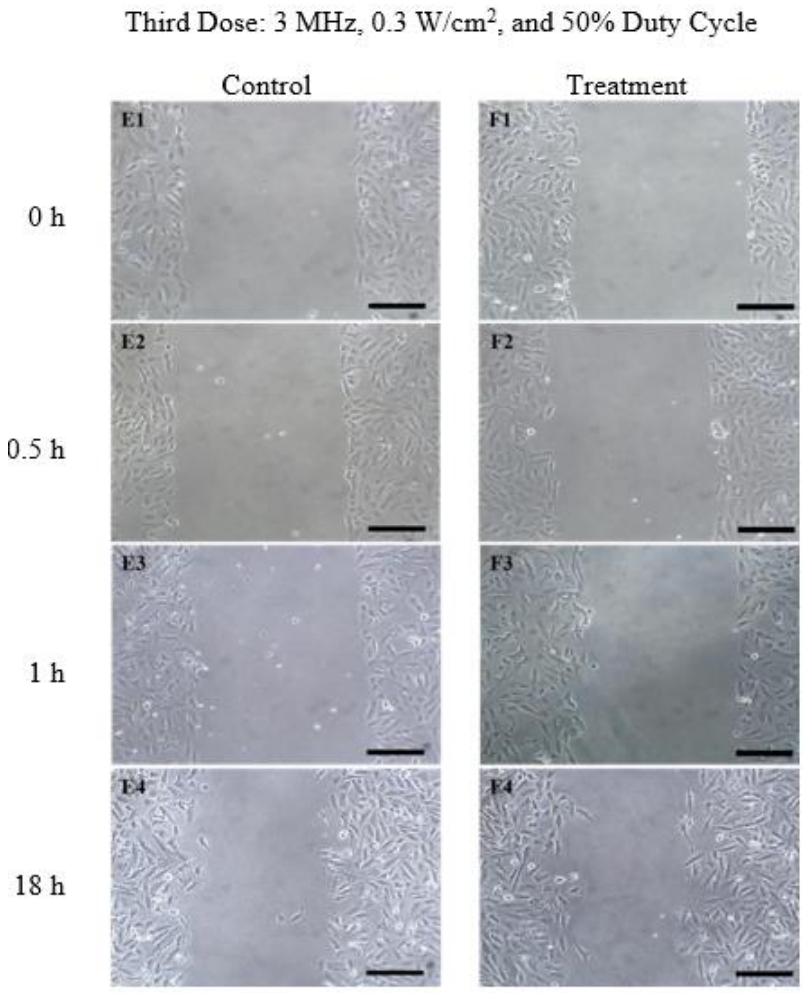

Fig. 8 Images of control and treatment group (3 MHz, 0.3 W/cm2, 50% duty cycle; 5 min; 0.5 cm) of HSF 1184 cultured in hyperglycemic condition labelled as E and F respectively at 10 × magnification at 0, 0.5, 1 and 18 hours. Scale bar: 80 µm

On the other hand, Figures 6 and 7 show the migration of cells treated with the second dose of ultrasound, 1 MHz, 0.3 W/cm2, and 50% of duty cycle at intervals of 0, 0.5, 1, 18, 20, 22 and 24 hours. There was a significant increase in the percentage of wound closure for treated cells after 18 hours of observation in comparison to control group. In like manner, the cells treated with the second dose of ultrasound were presenting the most significant increase in the percentage of wound closure compared to the other two ultrasound doses. Thus, 1 MHz, 0.3 W/cm2, and 50% of duty cycle is one of the potential candidates for the most optimum ultrasound doses for the cell migration activity under the hyperglycemic microenvironment.

Figures 8 and 9 show the cells treated with the third dose of ultrasound, which was 3 MHz, 0.3 W/cm2, and 50% of duty cycle at intervals of 0, 0.5, 1, 18, 20, 22 and 24 hours. At the first two intervals which were 0.5 and 1 hour, the cells treated with the third ultrasound dose (3 MHz, 0.3 W/cm2, and 50% of duty cycle) did not exhibit a notable increase in wound closure rate compared to its control group. However, after 18 hours, the cells started to make a remarkable movement towards the cell-free area, revealing a quite significant difference in terms of percentage of wound closure compared to its control group.

Figure 10 shows the graph of the percentage of wound closure for all the treatment and control groups of HSFs 1184. According to the observation for all the experiments for the three ultrasound doses, the motion of cells towards the cell-free area did not show an impressive migration activity from 0.5 to 1 hour. However, cell migration started to show a significant migration trend from 18 to 24 hours for all the ultrasound doses. The cells treated with ultrasound showed a higher percentage of wound closure compared to their respective control groups. This indicates that the ultrasound treatment has a positive effect on stimulating the cells' motility to move towards the cell-free area under the hyperglycemic microenvironment. Likewise, a higher percentage of wound closure is parallel to the higher cell migration activity. The comparison of the cell migration for all three ultrasound doses showed that the second dose which was 1 MHz, 0.3 W/cm2, and 50% of duty cycle exhibited the highest percentage of wound closure. This was followed by the third ultrasound dose with frequency of 3 MHz, intensity of 0.3 W/cm2, and 50% of duty cycle. Finally, the first dose which was 1 MHz, 0.2 W/cm2, and 20% of duty cycle demonstrated the lowest percentage of wound closure compared to the other two ultrasound doses.

The difference in frequency of an ultrasound treatment can give rise to different cellular and molecular effects. A study suggested that 1 MHz ultrasound which was used in the first and second doses can enhance cell proliferation which in turn will encourage the wound closure rate (Man et al., 2012). However, a further study should be conducted to understand the role of cell

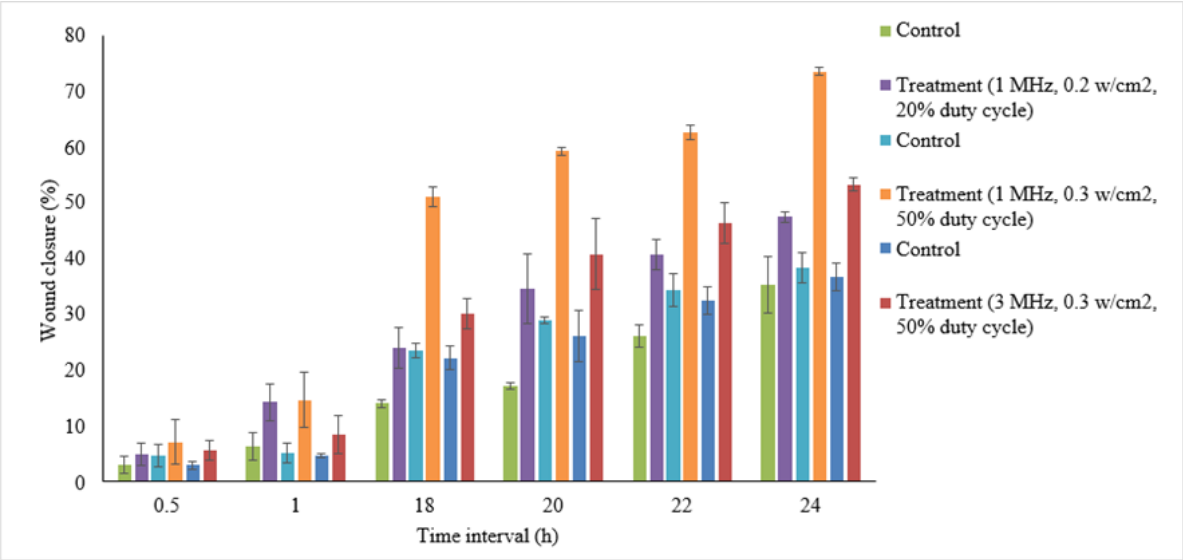

Fig. 10 Percentage of wound closure for all of control and treatment groups of HSFs 1184 cultured in hyperglycemic condition at specific time intervals.

proliferation in wound closure experiments. From the graph in Figure 10, the percentage of wound closure for cells treated with the second and third ultrasound doses showed a significant difference between each other despite having similar intensity and duty cycles for the treatments. Hence, the difference in frequency used in the treatments was believed to contribute to the difference in wound closure rate between the two treatments. In addition, another study highlighted that the location of fibroblast cells in the dermal layer of skin has hugely influenced the selection of frequency in ultrasound treatment for wound healing activity. According to Alkahtani et al. (2017), a lower ultrasound frequency which is 1 MHz has a higher penetration rate which is 3 to 5 cm compared to 3 MHz with a penetration ability ranging from 1 to 2 cm. Thus, 1 MHz used in the first and second ultrasound doses is proven to be able to penetrate to dermal layer in which the fibroblast cells are located at (Alkahtani et al., 2017).

In the other context, a previous study affirmed that 50% of the duty cycle can produce desired mechanical effects towards the cells which in return will increase the desired cellular behavior including cell migration activity (Mason & Rathmell, 2011). In contrast, a possible reason that contributed to the lowest percentage of wound closure for the cells treated with the first ultrasound dose was its duty cycle which was 20%. The duty cycle of the ultrasound which was lower than 50% might not be able to give a similar effect as ultrasound with 50% duty cycle, especially in terms of cell migration activity. In the meantime, a study also agreed that 50% of the duty cycle is vital for the cells to cool in between the pulses and reduce the chance of cell damage due to the thermal effect generated from the ultrasound treatment (Mason & Rathmell, 2011).

The second ultrasound dose in this experiment, which was 1 MHz, 0.3 W/cm2, and 50% of duty cycle exerted the most desired dose-response result. To recap, the second dose used frequency of 1 MHz which was proven to have a deeper penetration ability that allowed it to penetrate fibroblast cells located in the dermal layer of the skin (Alkahtani et al., 2017). Furthermore, the duty cycle for the second ultrasound dose was 50%. A great deal of studies have agreed that a 50% duty cycle was appropriate to avoid the intense heating from the ultrasound waves and promote cell migration (Mason & Rathmell, 2011). From the scratch assay analysis, the study concluded that the optimum ultrasound dose for wound healing activity in the hyperglycemic microenvironment was 1 MHz, 0.3 W/cm2, and 50% of duty cycle. The results from this study are hoped can provide a greater understanding of creating a therapeutic regimen for wound repair under a hyperglycemic microenvironment in vitro.

CONCLUSION

Based on the study, the optimized seeding density of HSF 1184 under a hyperglycemic microenvironment was 4×10^5 cells/mL. The optimized seeding density was used for scratch assay to study the effect of low-intensity therapeutic ultrasound on wound closure under a hyperglycemic microenvironment. The low-intensity therapeutic ultrasound treatment with a frequency of 1 MHz, intensity of 0.3 W/cm^2, and 50% of the duty cycle was proven to be the most potent ultrasound dose to accelerate wound healing under hyperglycemic microenvironment in vitro.

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