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Bioeffects of low-intensity continuous ultrasound (LICUS) on wound healing in corneal stromal cells *in vitro*

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Abstract—Corneal wound induced by traumatic injury to the cornea has developed as a clinical problem. Corneal stromal cells (keratocytes) play an important role in corneal injury occurred. Low-intensity ultrasound (US) is a non-invasive and safe technique therapeutic application. Low-intensity continuous ultrasound (LICUS), a form of low-intensity US is a fast-growing topic in tissue regeneration and treatment for diseases. However, the underlying cellular and molecular mechanisms of biological effects of LICUS on corneal wound healing, which are potentially related to the upregulation of cell proliferation and migration have rarely been reported. The current study aimed to determine whether LICUS had effective bioeffects on cellular wound healing and to determine the optimal LICUS intensity for accelerating the wound healing process in keratocytes *in vitro*. In the experiment, a linear scratch was introduced on the monolayer of keratocytes. The scratched regions are parallelly treated with several LICUS spatial-average intensities (no power, 15, 30, 50 mW/cm²) for 5 min per day. The wound healing assays were based on microscopic images (100 ×) after scratch introduced and following 24 h, 48 h, 72h. The area of the scratched region was quantitatively measured. The results showed that the initial area of scratch in all groups was 1.77 ± 0.04 mm². LICUS intensity of 30 mW/cm² displayed the most effective rate of treatment than other groups in the scratched regions, with the area of 1.2609 ± 0.08 mm² at 24 h and the wound closed up at 48h. The intensity of 15 mW/cm² and 50 mW/cm² had comparable bioeffects on keratocytes, which totally healed at 72 h. These findings indicate that LICUS at 30 mW/cm² intensity has the potential to be a treatment technique for traumatic corneal injuries.

Keywords— cellular wound healing, Low-intensity continuous ultrasound (LICUS), linear scratch, keratocytes

I. INTRODUCTION

The cornea as a transparent avascular tissue, is a structural barrier and protects the eye from infections [1]. It also contributes to two-thirds optical power of the eye, through refracting and focusing incident light on the retina [2]. Ocular trauma is one of the causes of corneal blindness. One and a half to two million patients undergo corneal blindness worldwide annually due to corneal trauma [3]. Untreated corneal injuries or inappropriate treatment result in disrupted vision, and even permanent blindness due to bacterial infections [4]. Thus, it is important to investigate

medical therapies to accelerate corneal wound healing after traumatic injuries.

The stroma which contributes about 90% of the total thickness of the entire cornea, takes the main role in response to an occurred corneal injury [5]. Keratocytes, as known as fibroblasts, are the major cell type in the stroma. Corneal wound healing is a complex process. Upon injury, keratocytes undergo either cell death or transition into activated phenotypes. Cell regeneration or fibrotic scar construction is caused by these phenotypes [6] during corneal wound healing.

Ultrasound (US) is defined as a series of mechanical waves with a frequency above the maximum human hearing (20 kHz). When the waves propagate in a medium, mechanical forces are generated. Medical ultrasound has been widely used as a non-invasive tool in diagnosis, therapy, and operation [7, 8]. The intensity of US is a decisive parameter for the applications. Low-intensity US in the therapeutic applications, controlling the intensity at the range of 0.03-1.0 W/cm² [9]. Depending on duty cycle, low-intensity US includes two modes, low-intensity continuous ultrasound (LICUS) and low-intensity pulsed ultrasound (LIPUS). Different from the fitful cell oscillatory with specific temporal gaps induced by LIPUS, LICUS keeps up the cell vibration during the whole treatment. Although in the past few decades, a fast-growing interest in the effects of low-intensity therapeutic US, there is no certain conclusion on which mode is more effective in tissue regeneration and treatment for diseases.

A previous study found that LICUS improves nitric oxide (NO) levels, resulting in raised vasodilation, blood flow, and an enhancement in the nutrient supply [10]. Because of the increased blood flow, macrophage is enhanced to migrate into the injury area to reduce acute inflammation [11]. Another paper reported in a rat muscle wound model, LICUS exhibited an ability to boost fibroblast proliferation, capillarization [12]. However, the underlying cellular and molecular mechanisms of the biological effects of LICUS on corneal wound healing, which are potentially related to the upregulation of cell proliferation and migration have never been reported.

In this study, the bioeffects of LICUS at several intensities on wound healing in keratocytes *in vitro* were explored. In addition, an optimal LICUS intensity dose for

accelerating cell migration and proliferation was reported. Specifically, a linear scratch was introduced on the monolayer of keratocytes. Then, LICUS at no power (control), 15, 30, 50 mW/cm² intensities were treated for the scratched regions. The wound healing assays of the scratched regions were conducted in this study.

II. METHOD

A. Cell culture

Human keratocytes (P10872; Innoprot) up to passage three were cultured in cell culture flasks with Dulbecco's modified eagle medium in an incubator with 5 % CO₂ at 37 °C. When the confluency of keratocytes achieved 80% in the culture flask, these cells were trypsinised and seeded at 50,000 per well into six-well plates. After two days, keratocytes formed an adherent monolayer at the surface of each well. The culture medium was refreshed every day.

B. LICUS treatment Specifications

A schematic diagram of LICUS treatment apparatus is shown in Fig. 1A. A lab-made single-element focused transducer (central frequency of 1.47 MHz, 9 mm focal length) was submerged into the culture medium and positioned 9 mm from the culture surface in each well (Fig. 1B). Before the transducer was submerged into the culture medium, it was wiped with 70% ethanol and sterilised by an ultraviolet (UV) lamp. The six-well culture plate was supported by a silicone rubber to minimize reflections [13]. The transducer was driven by a function generator and a radio frequency (RF) power amplifier. The driving signal was a sin sinusoidal wave at 1.47 MHz for LICUS treatment. LICUS at four spatial-average intensities (no power (control), 15, 30, 50 mW/cm²) were examined. Each intensity was parallelly examined three times and each well was treated for 5 min every 24 h.

C. Wound healing assay

The keratocytes proliferation and migration were accessed through wound healing assays *in vitro*. A linear scratch was introduced in each well with a 20 uL pipette tip. The monolayer of keratocytes was washed three times with phosphate-buffered saline (PBS) to remove the floating cells. Then, keratocytes were incubated with the culture media and treated with LICUS. The images of the scratched regions for each intensity group were recorded with a light microscope (100 × magnification) after scratch introduced (0 h) and following 24 h, 48 h, and 72h, then analysed with Image J v1.8 software.

Fig. 1 (A) Schematic diagram of LICUS experimental setup. (B) Treating keratocytes with LICUS in a six-well culture plate. The transducer was clamped in position that the transducer was submerged in culture media in a flow hood. The plate was supported by silicone.

III. RESULTS

The confluency and the morphology of keratocytes after LICUS treatment at different time points are shown in Fig.2. Among the four LICUS intensity groups, the scratched regions treated with LICUS intensity of 30 mW/cm² displayed the fastest rate of cell migration and proliferation to cover the wound within 24 h. At 48 h, the morphology of keratocytes became spindle-shaped in appearance. LICUS at 15 mW/cm² and 50 mW/cm² exhibited a similar speed of cell migration, with scratched regions closed up at 72 h. The LICUS control group demonstrated the lowest recovery speed that the scratched region was not fully healed within 72 h.

Fig. 2 Scratched regions treated with LICUS at different intensities imaged by a microscope (x100) at 0h, 24h, 48h, 72h after scratch introduced. The wound edge was denoted by red lines. The scale bar represents 500 μm .

The area of scratched region treated with different LICUS intensities was quantitatively measured based on each microscopic image at 0h, 24h, 48h, and 72h, presented in Table 1. The initial scratched area (0h) was around $1.77 \pm 0.08 \text{ mm}^2$ for all the LICUS intensity groups. LICUS intensity at 30 mW/cm^2 showed the most effective rate of treatment than other groups, with the area of $1.26 \pm 0.09 \text{ mm}^2$ at 24 h and the wound recovered at 48h. LICUS intensity of 15 mW/cm^2 and 50 mW/cm^2 had comparable bioeffects on the keratocytes, with $1.08 \pm 0.10 \text{ mm}^2$ and $0.87 \pm 0.11 \text{ mm}^2$ at 48 h, respectively, then total healing at 72 h. By contrast, the rate of wound healing in the control group was apparently slower than LICUS treated groups. The scratched regions were not recovered at 72 h, with $0.52 \pm 0.05 \text{ mm}^2$.

Table 1 Size of scratched areas after treated with intensity of 15 mW/cm^2 , 30 mW/cm^2 , 50 mW/cm^2 and control at 0h, 24h, 48h and 72h after scratch introduced.

	15mW/cm^2 (mm^2)	30mW/cm^2 (mm^2)	50mW/cm^2 (mm^2)	Control (mm^2)
0h	1.77 ± 0.10	1.75 ± 0.10	1.77 ± 0.08	1.79 ± 0.04
24h	1.45 ± 0.10	1.26 ± 0.09	1.47 ± 0.10	1.56 ± 0.09
48h	1.08 ± 0.10	-	0.87 ± 0.11	1.08 ± 0.12
72h	-	-	-	0.52 ± 0.05

IV. DISCUSSION

In this study, the bioeffects of LICUS at the intensities of no power, 15, 30, 50 mW/cm^2 on wound healing in keratocytes *in vitro* were studied. LICUS showed the ability to enhance wound healing in keratocytes by promoting cell proliferation and migration. The optimal LICUS intensity dose to accelerate wound healing in keratocytes among the applied intensities was 30 mW/cm^2 .

Since the last two decades, LICUS has been proved that it is useful in pain relief [14] and soft-tissue regeneration[15]. Also, the studies showed that LICUS has huge promise for cancer treatment [16] and drug delivery [17]. In this study, we found that LICUS can promote keratocytes' migration and proliferation into the injury regions, which provides a potential therapeutic method for traumatic injury in the cornea.

With regard to the LICUS intensity, 30 mW/cm^2 has been frequently used not only in LIPUS studies [18, 19], but in LICUS research, for example, LICUS at 30 mW/cm^2 resulted in distinct therapeutic effect in bone fracture healing [20]. Our results showed the agreement with these publications in the intensity at 30 mW/cm^2 had the most effective bioeffects in treatment for the wound. A study [21] reported that 30 and 90 mW/cm^2 of LIPUS were adequate intensities for cell stimulation *in vitro*. However, the biological effects of LICUS at 90 mW/cm^2 on cellular wound healing have not been reported yet. In the next step, we will examine the effectiveness of LICUS intensities with a large span from 15 mW/cm^2 to 100 mW/cm^2 .

The cornea consists of epithelium, Bowman's layer, stroma, Descemet's membrane, and endothelium [1]. The density and the attenuation property of the layers are different, leading to energy loss when the LICUS waves propagate through these tissue layers. Therefore, the optimal LICUS parameters including frequency, power, and intensity need to be further investigated in a three-dimensional (3D) environment that contains multiple layers with similar properties to the human cornea. In the future, a 3D corneal model will be constructed *in vitro* with a collagen-based hydrogel by tissue engineering techniques to study the effectiveness of LICUS on wound healing.

V. CONCLUSION

This study showed that LICUS was capable to enhance wound healing in keratocytes through promoting cell proliferation and migration. In addition, the optimal LICUS intensity dose to accelerate wound healing in keratocytes among the applied intensities was 30 mW/cm^2 . These findings provide a potential therapeutic method for traumatic corneal injuries.

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