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Low intensity pulsed ultrasound exposure increases prostaglandin E_2 release in human dermal fibroblasts

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Though ultrasound is applied to the treatment of pressure ulcers, there was little evidence of benefit associated with the use of it in the treatment of pressure ulcers. Therefore, in order to augment the therapeutic evidence of ultrasound exposure in wound healing, the effect of low intensity pulsed-ultrasound on the release of prostaglandin E2 (PGE2) in human dermal fibroblasts was investigated. Human dermal fibroblasts obtained from skin samples were exposed to a low intensity pulsed-ultrasound by specifically designed apparatus. An enzyme-linked immunosorbent assay determined the release of PGE2 in the medium. A low intensity pulsed-ultrasound increases the PGE_2 release of dermal fibroblasts in a time-dependent fashion. PGE_2 release by 30 mW/cm^2 ultrasound exposure reached maximum 1.24-fold at 1 hour. In terms of the intensities of ultrasound, the weaker intensity of ultrasound was exposed, the more effect of PGE_2 release was observed. Finally, a specific inhibitor of cyclooxygenase-1, resveratrol partly reduced PGE2 release of dermal fibroblasts by ultrasound exposure. Thus, our results identify the effect of low intensity pulsed ultrasound to explain the potential mechanism by which it may augment the healing of skin ulcer. Further studies are required to ascertain the functional relevance of the production of PGE_2 in angiogenesis, which is required for the tissue development and regeneration.

Key Words

Ultrasound,
Pressure ulcers,
Prostaglandin E_2 (PGE_2),
Dermal fibroblasts,
Angiogenesis.

Introduction

Pressure ulcers are common in both acute and community health care settings.[1)] They represent a major burden of sickness and reduced quality of life (QOL) for elderly patients. The prevention and treatment of them involves many strategies. Recently, the pressure-relieving mattresses are commonly used as measures for the prevention and treatment of pressure ulcers.[2)] Physical therapies such as an ultrasound are also applied to their treatment. In clinical, however, meta-analysis of the randomized controlled trials (RCTs) shown that there was little evidence of benefit associated with the use of ultrasound in the treatment of pressure ulcers, while the possibility of a beneficial effect can not be ruled out due to the small number of trials.[2)]

Ultrasound, a physical modality with high-frequency sound waves to penetrate to the soft tissue, helps augment the healing process of tissue injuries.[3)] For example, the effect of low intensity

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pulsed-ultrasound on a fracture healing is known well.[4] The acceleration of fracture repair by pulsed-ultrasound was shown in both animal models and clinical studies. As one of the molecular mechanisms of the promotion of fracture repair evoked by an ultrasound, it seems to induce the activity of cyclooxygenase-2 (COX - 2) transcriptionally lead to prostaglandin E2 (PGE_2) synthesis in osteoblasts.[5]

PGE_2 is considered to be an important regulator of angiogenesis.[6] Some findings indicate that PGE_2 stimulates the synthesis of angiogenic growth factors such as vascular endothelial cell growth factor in cancer cells[7] and basic fibroblast growth factor (b-FGF) in fibroblasts.[8] On the contrary, PGE_2 is a mediator of these angiogenic agents-induced neovessel assembly through the release of proteases and the augmentation of endothelial cell migration and tubular formation depending on the expression of various cell surface molecules induced by PGE_2.[9] The exact mechanism by which ultrasound accelerates a wound healing remains still unknown, however, if PGE_2 is induced by ultrasound exposure in dermal fibroblasts, it may augment the healing of skin ulcer through an induction of angiogenesis required for tissue regeneration.

In order to augment the therapeutic evidence of ultrasound exposure in wound healing process, we hypothesized that ultrasound renders its effect through the induction of PGE_2 from dermal. Therefore, the present study was designed to investigate the effect of low intensity pulsed ultrasound on the release of PGE_2 in conjunction with the role of its cognate enzyme, cyclooxygenase in human dermal fibroblasts.

Materials and Methods

1. Cell culture

Human skin sample was surgically obtained, after consent, from the patients with a pressure ulcer at the time of wound debridement. Human dermal fibroblasts were isolated from these samples as described previously with some modification.[10] Briefly, minced tissue was dissociated using 0.2% of collagenase (Sigma Chemical Co., St. Louis, MO) and 0.25% of trypsin-EDTA (DIFCO LABORATORIES, Detroit, MI) at 37℃ for 2 hours, with gentle stirring. The dissociated cells were then suspended in culture medium, consisting of Dulbecco's modified Eagle's medium (DMEM ; Gibco BRL, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS ; Biowhittaker, Walkersville, ML) and antibiotics. Then, they were cultured in 75-cm^2 tissue culture flask (Corning Glass Works, Corning, NY) at a cell density of 10^4 cell/cm^2.

When this primary culture reached confluence, cells were reseeded into the other flasks at the same cell density for further passages. For the experiment, cells were detached and resuspended finally in DMEM supplemented with 2% FBS and plated in 6-well culture plates, 34 mm in diameter (FALCON, Becton Dickinson Labware, Franklin Lakes, NJ, USA) at a density of 5×10^5 cells/well. After overnight incubation, the attachment of cell was confirmed and the medium was replaced with 4 ml of fresh DMEM containing 2% FBS and antibiotics. This monolayer culture of human dermal fibroblast was subjected to the experiments described below.

2. *Ultrasound exposure systems.*

The ultrasound exposure system consists of an array of six transducers specifically designed for a 6-well tissue culture plate. This array is at the bottom of a water tank and the culture plate is located at a distance of 20 mm from the surface of the transducers. Unidirectional propagation of the ultrasound signal is accomplished by using an absorption chamber on top of the culture plate directly coupled to the culture medium. The ultrasound input signal was a 200-μ second-burst sine wave of 1.5 MHz repeating at a frequency of 1 kHz. The ultrasound intensity was varied from 7.5 mW/cm^2 spatial average and temporal average (SATA) up to 60 mW/cm^2 (SATA) depending on the experimental design. Exposure period was 20 minutes in all experiments otherwise stated. The transducers were powered by a power amplifier, which was connected to a variable-signal generator (15 MHz Function/Arbitrary waveform generator, Hewlett Packard, Palo Alto, USA). All experiments were performed in a water tank at 37℃. At varying time interval after ultrasound exposure, medium was collected for the following PGE_2 assay. Control samples were also subjected to the same operations without ultrasound exposure in the same conditions.

3. *PGE_2 assay.*

In order to evaluate PGE_2 release by human dermal fibroblasts, the concentration of PGE_2 in culture medium of cells exposed with or without ultrasound was determined by an enzyme-linked immunosorbent assay (ELISA) kit (NEOGEN Corporation, Lexington, KY, USA). After cells were treated with test procedure, the conditioned medium was collected and stored at -80℃ until assay. PGE_2 concentrations were determined from a standard curve of serial dilutions of control PGE_2 provided.

4. *Statistical analysis.*

Statistical analysis was carried out on all data points with regard to control by two-factor ANOVA after confirming the equality of distribution by Bartlett test. Each data point represented the mean of PGE_2 concentration obtained from the experiment performed in quadruplicate using identical cells originated from the same skin sample with corresponding standard error of the mean (SEM). Reproducibility of the results in experiment was confirmed by a repetition of same experiment done on the other day. P values under 0.05 were considered statistically significant.

Results

A low intensity pulsed ultrasound is reported to interact with the pathway of PGE_2 synthesis in MC 3 T 3-E 1[5], therefore, to investigate whether ultrasound exposure could promote PGE_2 synthesis in human dermal fibroblasts, cells were cultured up to 24 hours after 20-minute ultrasound exposure with a intensity of 30 mW/cm^2 (SATA) which is paralleled to clinical setting for a treatment of fracture repair.

Results of this experiment are presented in Fig. 1. Ultrasound increased the release of PGE_2 of human dermal fibroblasts in a time-dependent fashion. The release of PGE_2 of unexposed samples was also stimulated in a same manner, but the increase is much lower compared to the samples that received ultra-

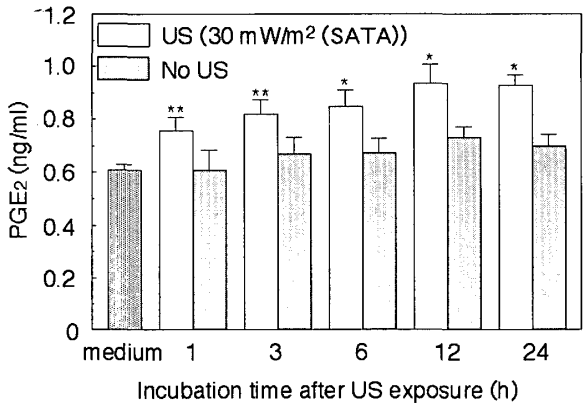


Figure 1. Effect of low-intensity pulsed ultrasound on PGE_2 release of human dermal fibroblasts (Time kinetic study)

30 mW/cm^2 of ultrasound increased the release of PGE_2 in human dermal fibroblasts in a time-dependent fashion. As compared to untreated samples in each incubation period, net increase of PGE_2 release by ultrasound exposure reached 1.24-fold at 1 hour with statistically significance and continued up to 24 hours in this series. (** : $p<0.01$, * : $p<0.05$ vs. untreated samples).

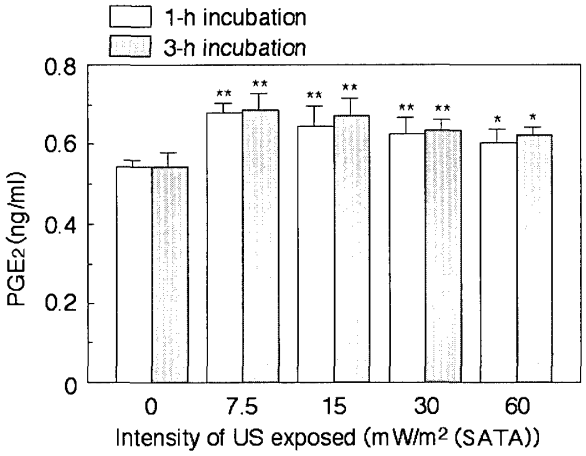


Figure 2. Effect of low-intensity pulsed ultrasound on PGE_2 release of human dermal fibroblasts (Dose kinetic study)

All intensities of ultrasound increased the concentration of PGE_2 in conditioned medium comparing with unexposed samples in both 1-h and 3-h incubation significantly (** : $p<0.01$, * : $p<0.05$ vs. untreated samples in each incubation period). In this experimental condition, the weaker intensity of ultrasound was exposed, the more effect of PGE_2 release was observed in fibroblasts. As compared to untreated samples, the PGE_2 release was attained maximum 1.25-fold at 1-hour and 1.26-fold at 3-hour with statistically significance respectively by 7.5 mW/cm^2 (SATA) of ultrasound exposure and these phenomena were attenuated in depending on the ultrasound intensity exposed up to 60 mW/cm^2 (SATA).

sound. As compared to untreated samples, the induction of PGE_2 release by ultrasound exposure reached 1.24-fold at 1 hour with statistically significance and continued up to 24 hours in this series.

Also, whether a lower or higher intensity of ultrasound exposure affects PGE_2 release in fibroblasts was investigated. Cells were cultured for continuously up to 3 hours after exposing of the ultrasound with a varying intensity up to 60 mW/cm^2 (SATA). As shown in Fig 2, all intensities of ultrasound significantly increased the concentration of PGE_2 in conditioned medium comparing with unexposed samples in both 1-h and 3-h incubation. In the clinical setting, 20-minute exposure with 30 mW/cm^2 (SATA) of ultrasound is approved for promoting the new bone formation. However, in this experimental condition, the weaker intensity of ultrasound was exposed, the more effect of PGE_2 release was observed in fibroblasts. As compared to untreated samples, the PGE_2 release was attained maximum 1.25-fold at 1-hour and 1.26-fold at 3-hour with statistically significance respectively by 7.5 mW / cm^2 (SATA) of ultrasound exposure and these phenomena were attenuated in depending on the ultrasound intensity exposed up to 60 mW/cm^2 (SATA).

The production of PGE_2 is mediated by either COX-1 or COX-2. COX-1 is a constitutive form of cyclooxygenase and catalyses PGE_2 synthesis, which is required for physiological reactions such

Table Effect of COX-1 or COX-2 inhibitor onPGE_2 release of dermal fibroblasts by ultrasound

PGE_2 release induced by 7.5 mW/cm^2 of ultrasound exposed was reduced by a supplement of resveratrol, a specific inhibitor of COX-1. At 60-min incubation, net release of PGE_2 by ultrasound was abolished partly by not NS-398 (COX-2 inhibitor) but resveratrol (COX-1 inhibitor), suggesting something of an association between the release of PGE_2 by ultrasound exposure and the activity of COX-1.

	US (-)	US (+) 7.5 mW/cm^2	*P*
0	0.51 ± 0.032	0.63 ± 0.066	----
Resveratrol (20 μM)	0.51 ± 0.037	0.55 ± 0.026	P = 0.032
NS-398 (10 μM)	ND	0.66 ± 0.030	NS

(NS: no significance vs. ultrasound exposed without NS-398)
(ND: no detected)

as platelet aggregation and modulation of renal blood flow.[11] On the other hands, activities of COX-2, another isoform of cyclooxygenase, is induced by various stimulations such as inflammatory cytokines in every kind of cells including osteoblasts, fibroblasts and macrophages. Therefore, to determine whether there is any direct link between PGE_2 release and these isoforms of COX by ultrasound-exposed cells, activity of either COX-1 or COX-2 was inhibited by the addition of selective COX inhibitors. It is reported that resveratrol specifically inhibits COX-1 activity with an ED_{50} of 15 μM[12] and 10 μM of NS-398 demonstrates a sufficient inhibitory effect through selective attenuation of COX-2 activity on PGE_2 production in vitro.[13]

The data presented in table indicate that PGE_2 release of dermal fibroblast induced by 7.5 mW/cm^2 (SATA) of ultrasound exposed was significantly reduced by a supplement of 20 μM resveratrol. Contrary, little effect of 10 μM NS-398 was observed on the PGE_2 release by ultrasound, suggesting something of an association between the release of PGE_2 by ultrasound exposure and the activity of not COX-2, but COX-1 in dermal fibroblasts.

Discussion

A low intensity pulsed ultrasound is clinically used as a treatment modality for fracture repair.[14] As the molecular pathway that mediates these phenomena, it is recently made apparent that the ultrasound exposure induces anabolic responses of osteoblastic cells such as an expression of immediate-early genes in-

cluding *c–fos* and COX–2, and an up–regulation of messenger RNA level of osteocalcin and insulin–like growth factor I (IGF–1).[15]

Therapeutic ultrasound is also historically used in the management of soft tissue injuries such as a pressure ulcer. However, a limited clinical research is available and no consensus exists regarding the efficacy of an ultrasound for treating pressure ulcers.[16] In meta–analysis of the RCTs, it is reported that there was an increase in the weekly healing rates associated with the ultrasound/ultraviolet combination therapy compared with a laser but no statistical significant difference between ultrasound/ultraviolet and control group.[17] A double–blinded, single –case, baseline–AB study was also conducted to assess the efficacy of pulsed low–intensity ultrasound on wound healing.[16] As a result, the healing rate was significantly faster during the pulsed low –intensity ultrasound period compared to the placebo. But there was no predominance in the efficacy of pulsed low–intensity ultrasound on wound healing.

Thus, the apparent effect of an ultrasound intervention could not be determined. However, pulsed low–intensity ultrasound likely has some appreciable effects on the healing of pressure ulcer because some authors has reported the effect of low–intensity pulsed ultrasound enhanced an early healing of soft tissue injuries in vivo animal model and induced cell proliferation, collagen synthesis[18] and production of angiogenesis–related cytokines such as interleukin–8 and b–FGF by human fibroblasts in vitro.[20]

In this report, we have demonstrated that ultrasound exposure increased the release of PGE_2 in human dermal fibroblasts. In terms of the physical mechanism, ultrasound provides a mechanical force to the cellular system. Several kinds of mechanical stresses induce the production of PGE_2 through the induction of COX–2. Fluid shear stress and tensile force stretching the cell membranes stimulate PGE_2 release in bone cells. In this context, ultrasound appears to exert its action in ways similar to fluid shear stress and tensile force stretch in dermal fibroblasts, though any mechanical forces must interact with one or more cellular pathways to elicit a biological effect.

The time kinetics of PGE_2 release induced by ultrasound exposure is similar to that of other cell types such as osteoblasts previously reported.[5] However, the maximum effect of PGE_2 release has been achieved in the weakest intensity of ultrasound exposure available in this experiment. This is a quarter of the intensity of ultrasound applied in clinical setting. Treatment parameters to use clinically in physiotherapy have been often proposed that the relatively longer treatment with higher outputs are beneficial based on a literature review. The reason why the weaker energy is suitable for the PGE_2 release of dermal fibroblasts by ultrasound exposure is unclear. The peak effect of pulsed short–wave diathermy on human fibroblast proliferation is estimated to be 13.8 W (mean power) because the effect of over 12 W (mean power) of pulsed short–wave diathermy on cell proliferation was attenuated in a dose dependent fashion.[20] Furthermore, cell proliferation was highest with the 5–minute treatment duration compared with longer treatment. In terms of the induction of trans–membrane voltage, sufficient voltage is detectable by a bone cell in gap junction contact with other bone cells preferably in the lower frequency ranges of ultrasound in contrast to the pulsed electromagnetic fields.[21]

The dose of PGE_2 released by ultrasound exposure is relatively small compared with the PGE_2 in ultrasound unexposed dermal fibroblasts. Only 25% increase of PGE_2 release was observed with a single stimulation, however, repetitive treatment applied in clinical case may augment the biological effect induced by ultrasound exposure in vivo. The effect of repetitive exposure of ultrasound on PGE_2 release of dermal fibroblasts should be studied in further investigation.

The relationship between the up-regulation of PGE_2 release by ultrasound exposure and the induction of COX-2 was not further established at present. The release of PGE_2 by ultrasound exposure was partly suppressed to an unexposed level by not NS-398 but resveratrol in comparison with cells in the absence of these inhibitors. Based on these data, a direct link between PGE_2 release by the effect of ultrasound exposure and the induction of COX-2 in human dermal fibroblasts was not proposed though an inducible form of enzyme, COX-2, is a critical enzyme for converting an arachidonic acid into PGE_2 in osteoblasts. As COX-1 exists constitutively in the endoplasmic reticulum or around the nucleus in all types of cells, it is unlikely that COX-1 activity is directly induced by ultrasound exposure in dermal fibroblasts. However, COX-1 is partly induced in endothelial cells by stimulation of estrogen.[22] Thus, COX-1 might induced and be partly responsible for PGE_2 release by ultrasound exposure in human dermal fibroblasts. In order to identify the role of COX in ultrasound induced PGE_2 release, quantitative RT-PCR for evaluating mRNA expression of both COX-1 and COX-2 in human dermal fibroblasts is prepared.

In conclusion, our results identify the possible cell that mediate the effect of ultrasound in wound healing and help to explain the potential mechanism, at the cellular level, by which ultrasound may augment the healing of skin ulcer. Our results suggest that the production of PGE_2 in human dermal fibroblasts by ultrasound exposure may play an important role in the acceleration of wound healing through angiogenesis. Further studies are required to ascertain the functional relevance of the production of PGE_2 in angiogenesis in vitro and in vivo.

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