Monophasic Pulsed Current Stimulation of Duty Cycle 10% Promotes Differentiation of Human Dermal Fibroblasts into Myofibroblasts

Mikiko Uemura, PT, Ph.D.1, Masaharu Sugimoto, PT, M.S.2, Yoshiyuki Yoshikawa, PT, Ph.D.3, Terutaka Hiramatsu, PT, M.S.4 and Taketo Inoue, Ph.D.5

1) Faculty of Health Science, Department of Rehabilitation, Kansai University of Welfare Sciences, Japan
2) Toyama Rehabilitation Medical Health & Welfare College, Japan
3) Faculty of Health Sciences, Department of Rehabilitation, Naragakuen University, Japan
4) Faculty of Rehabilitation, Housenka Hospital, Japan
5) Department of Emergency, Disaster and Critical Care Medicine, Hyogo College of Medicine, Japan

ABSTRACT. Objective: Many clinical trials have shown the therapeutic effects of electrical stimulation (ES) in various conditions. Our previous studies showed that ES (200 μA and 2 Hz) promotes migration and proliferation of human dermal fibroblasts (HDFs). However, the effective duty cycle and the effect of ES on myofibroblast differentiation are unclear. This study aimed to investigate the relationship between duty cycle and myofibroblast differentiation. Methods: HDFs were subjected to ES (200 μA and 2 Hz) for 24 h with the duty cycle adapted at 0% (control), 10%, 50%, or 90%. α-smooth muscle actin (SMA) and transforming growth factor (TGF)-β1 mRNA and α-SMA protein expressions were assessed. Collagen gel contraction was observed for 48 h after ES initiation and the gel area was measured. Cell viability and pH of culture medium were analyzed for cytotoxicity of the ES. Results: Cell viabilities were decreased in the 50% and the 90% groups but ES did not influence on pH of culture media. ES with a duty cycle of 10% significantly promoted the mRNA expression of α-SMA and TGF-β1. α-SMA protein expression in the 10% group was also significantly higher than that of the control group. Collagen gel subjected to ES with a duty cycle of 10% was contracted. Conclusion: Duty cycle can influence on myofibroblast differentiation and ES with a duty cycle 10% is the effective for wound healing.

Key words: Electrotherapy, Pressure ulcer, Microcurrent, Wound healing

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tion from fibroblasts to myofibroblasts results in granulation tissue contraction leading to wound closure. Myofibroblasts are characterized by the expression of α-smooth muscle actin (α-SMA), and transforming growth factor-β (TGF-β) influences myofibroblasts to promote collagen synthesis in granulation tissue. The effects of ES on the differentiation of HDFs have been demonstrated in vitro studies. Direct current upregulates TGF-β1 and collagen I/III expression in mouse fibroblasts and promotes α-SMA expression in HDFs. However, a pulse width of 300 ms within 600 ms promotes higher α-SMA expression in HDFs than a pulse width of 10 ms within 1200 ms. Thus, the effects of ES on HDFs are influenced by pulse width. The pulse width is a result of the duty cycle, which is the on-and-off ratio of one stimulation (Fig. 1); a duty cycle of 100% indicates direct current, and the effect of duty cycle on HDF differentiation with an ES of 200 μA and 2 Hz, which promotes migration and proliferation in HDFs, is unclear.

Moreover, safety is mandatory for medical instruments including the ES device. In our previous studies, we did not assess the effects of long-term stimulation in HDFs. Low-intensity or small-electric fields within 10 h showed good effects on HDFs, promoting migration and proliferation. Zhang et al. and Wang et al. showed that longer ES has good effects on the migration and secretion of pro-healing cytokines; however, the therapeutic effects and adverse effects of long-term micro current stimulation in HDFs are not clear. Therefore, in this study, we adopted >10-h stimulation and confirmed the effects of ES on cell viability. In clinical trials, it is necessary to decide the pulse width, as well as waveform, intensity, and frequency, to conduct ES. Thus, we hypothesized that the duty cycle might have an effect on promoting α-SMA expression in HDFs and their differentiation into myofibroblasts.

**Method**

**Cell culture and electrical stimulation**

Primary HDFs (CC-2511; Clonetics, San Diego, CA, USA) derived from a 33-year-old woman were used. HDFs were cultured in low-glucose Dulbecco’s modified eagle medium (DMEM; Wako, Osaka, Japan) supplemented with 10% fetal bovine serum (Nichirei, Tokyo, Japan) and 5% penicillin-streptomycin Solution (Wako) in a CO2 incubator at 37°C. HDFs that had undergone 3-7 passages were used in the experiments. HDFs were seeded in a 35-mm tissue culture dish (Wako) and cultured for 24 h. The culture medium was refreshed and platinum electrodes were set on both sides of a dish (Fig. 2). Thereafter, HDFs were subjected to monophasic-pulsed current stimulation (intensity, 200 μA; frequency, 2 Hz) for 24 h in a CO2 incubator. HDFs without ES were used as controls. Duty cycles of 10%, 50%, or 90% were adapted to confirm the influence of differences in duty cycles.

**Cell viability and cell number**

The effect of ES on cytotoxicity was assessed because the ES that induces cell death cannot be clinically applied even if it promotes pro-fibrotic factors and the contractile ability of HDFs. Cell viability and cell numbers were analyzed after ES by trypan blue staining. ES that induced cell death was not used to analyze gene and protein expression.

**TagMan real-time RT-PCR**

To detect whether ES promoted fibroblast differentiation into myofibroblasts, the mRNA expression of α-SMA and TGF-β1 were analyzed. After ES, the total RNA was extracted from HDFs using the High Pure RNA Isolation Kit (Roche, Basel, Switzerland). RNA was reverse-
Human dermal fibroblasts (HDFs) were seeded in a 35-mm tissue culture dish and cultured for 24 h in a CO_2 incubator at 37°C. Electrodes were set on both sides of a dish and connected to the electrical stimulation (ES) device. Then, HDFs were exposed to the ES for 24 h in a CO_2 incubator at 37°C.

transcribed into cDNA. The mRNA expression of α-SMA, TGF-β1, and GAPDH was then analyzed by TaqMan real time RT-PCR. Relative gene expression was calculated using the ΔΔCt method after normalization to GAPDH expression.

**Western blotting**

The expression of α-SMA is an indicator of myofibroblasts, and the expression of α-SMA was assessed by western blotting. After ES, protein was extracted from HDFs using pro-prep TM (Cosmo Bio, Tokyo, Japan) according to the manufacturer’s instruction. Pooled samples containing the same amount of protein were subjected to SDS-polyacrylamide gel electrophoresis (BIO-RAD, CA, USA) and transferred onto membranes (Thermo Fisher Scientific, MA, USA). After blocking with blocking reagents (GE Healthcare, Buckinghamshire, UK), the membranes were incubated overnight at 4°C with the following primary antibodies: anti-α-SMA antibody (ab5694; Abcam, Cambridge, UK) or anti-GAPDH antibody (G8795; Sigma-Aldrich, MO, USA). The membranes were then incubated with the appropriate secondary antibodies, anti-rabbit IgG antibody (NA934V; GE Healthcare) or anti-mouse IgG antibody (NA931V; GE Healthcare), for 1 h at room temperature. The membranes were incubated with ECL mix (GE Healthcare), and the blots were quantified by densitometry (Chemi Doc XRS; BIO-RAD). Western blotting was performed four times using pooled samples (n = 4 per group). Data were normalized using GAPDH and by relative expression and compared with levels in the control group with Image J (National Institutes of Health, Bethesda, MD, USA).

**Collagen gel contraction assay**

The collagen gel contraction assay was performed to analyze the contractile ability of HDFs using a collagen-based cell contraction assay kit (Cell Biolabs, CA, USA) according to the manufacturer’s protocol. First, 240 × 10^4 cells were mixed with collagen solution and incubated for 1 h at 37°C. The solution was then added to 1 mL of DMEM and incubated for 48 h at 37°C in a CO_2 incubator. Collagen gels were separated from the dishes with spatula and subjected to ES for 24 h. Gel contractions were observed at 24 and 48 h after ES initiation. The gel areas were measured using image J and the area change rates were calculated for each dish.

**Measurement of culture medium pH**

pH was measured in each culture medium after ES. A pH probe (Mettler Toledo, Zurich, Switzerland) coupled with a pH meter (Mettler Toledo) was used to measure pH. The pH probe was calibrated with a standard pH buffer solution (pH 4.01, 6.86, and 9.18; AS ONE, Osaka, Japan) before immersing the probe in culture medium to measure pH.

**Statistical analysis**

All data were tested using the Shapiro-Wilk test and F-test. Student’s t-tests were used to analyze the data that followed a normal distribution with equal variances, and the results with p < 0.05 were considered statistically significant. Bonferroni correction was used and p < 0.0125 was considered statistically significant when the data were compared among more than three groups. When the data did not follow a normal distribution, the Mann-Whitney U test was used to evaluate data. Results with p < 0.05 were considered statistically significant. All statistical analyses were performed using EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria).

**Ethical approval**

In this study, we used a primary cell culture sold by Clonetics, and therefore, the study did not require ethical approval.
Results

ES with high duty cycle has adverse effects on HDFs

To better understand the adverse effect of ES on fibroblasts, we examined the cell viability and cell numbers after ES. Cell viability was decreased in the 50% and 90% groups ($p < 0.0125$ vs. control) and the viability of the 90% group was less than 90% (Fig. 3). However, ES with a 10% duty cycle did not decrease cell viability. Cell number after ES in the 90% group was decreased to less than 70%, but there were no significant differences between control and stimulus groups (v.s. 10%; $p = 0.237$, v.s. 50%; $p = 0.339$, and v.s. 90%; $p = 0.021$, respectively). These results indicate that cell toxicity might occur as the duty cycle approaches 100%, and direct current and high duty cycle could suppress cell proliferation. Next, to confirm ionization by ES, we measured the pH of each culture medium. The pH values of culture media were 7.77 ± 0.14 (control), 7.93 ± 0.09 (duty cycle 10%), 7.91 ± 0.05 (duty cycle 50%), and 7.96 ± 0.08 (duty cycle 90%), with no significant differences between the control and stimulus groups.

ES promotes the expression of α-SMA and TGF-β1

The mRNA and protein expression levels were compared between the control and the 10% groups because ES with a duty cycle of 50% and 90% introduced cell death. ES with a 10% duty cycle significantly promoted the mRNA expression of α-SMA and TGF-β1 (Fig. 4, $p = 0.029$ and $p = 0.029$). The mRNA expression of α-SMA in the 10% group was more than twice that in the control group. The protein level of α-SMA in the 10% group was similarly significantly higher than that of the control group (Fig. 5, $p = 0.046$). Thus, ES with a duty cycle of 10% might promote the differentiation of fibroblasts into myofibroblasts.

ES promotes collagen gel contraction

To confirm the effect on wound closure, we investigated the contractile capacity of fibroblasts using the three-dimensional (3D) collagen gel contraction assay. Collagen gel contraction rates were compared between control and 10% groups. The areas of collagen gels were reduced after

![Fig. 3](image_url) Adverse effects of electrical stimulation on human dermal fibroblasts.

The cell viability of human dermal fibroblasts after electrical stimulation is shown. Data are presented as the mean ± SD; n = 4 per group. **, $p < 0.01$, significant difference between the electrical stimulation group and the control group. The statistical differences between the control and the treated groups were tested by Student’s t-tests with a Bonferroni correction.

![Fig. 4](image_url) The expression of α-SMA and TGF-β1 in human dermal fibroblasts after electrical stimulation.

Relative expression of (A) α-SMA and (B) TGF-β1 mRNA. Data are presented as box-whisker plots. The statistical differences between the control and the 10% group were tested by Mann-Whitney U test; n = 4 per group. *, $p < 0.05$, significant difference between the 10% group and the control group.
moted monophasic-pulsed microcurrent of a 10% duty cycle pro-
jures by analyzing the effects of duty cycle on HDFs. The
optimum duty cycle of ES therapy for healing pressure in-
24 and 48 h after ES initiation (with a 10% duty cycle contracted more than the control at
HDFs contracted for 24 and 48 h. The gel subjected to ES
in the gel areas since the beginning of ES in gels with
stimulation (Fig. 6A). Fig. 6B, 6C show the rate of change
of the wound surface is increased when pressure injury dete-
rion in both conditions ever, ES with 100 mV/mm promoted collagen gel contrac-
tion; however, duty cycle of 50% (pulse width of 300 ms within 600 ms) with 50 mV/mm did not promote
this. This is similar to the result of the present study. How-
ever, ES with 100 mV/mm promoted collagen gel contraction in both conditions. This could be due to the differ-
ence in intensity. Thus, duty cycle might be involved in the contractile ability of HDFs. ES with a duty cycle of 10%
also promoted α-SMA and TGF-β1 expression. As shown
previously, α-SMA expression is an indicator of myofibro-
blast, and collagen gel contractile ability reflects wound
contraction, which is necessary for wound closure. ES with
a duty cycle of 10% promoted both α-SMA expression and
collagen gel contraction. Thus, fibroblasts might differenti-
ate into myofibroblasts with ES. Moreover, TGF-β1 is a
key factor in wound contraction that functions by promot-
ing α-SMA expression, differentiation, and the secretion of
collagen. The present study results suggest that ES might
induce differentiation of myofibroblasts and wound con-
traction by autocrine secretion of TGF-β1 and α-SMA in
HDFs. However, this study did not assess temporal changes
in the expressions of mRNA and protein. α-SMA expres-
sion could be higher within 24 h after ES initiation, but the relationship between the expression of α-SMA and the du-
ration of ES is unclear in this study. Yoshikawa et al showed that 1-h ES with a duty cycle of 50% promotes cell
proliferation. Thus, the pro-fibrotic effects of ES on HDFs
might be influenced by stimulation duration. Moreover, it
is not clear which cell sensor received ES to promote TGF-β1
and α-SMA expression. TGF-β1-mediated signals are en-
hanced via integrin β1, which is a cell surface receptor.
Fig. 6  Collagen gel contraction.

(A) The collagen gel contraction in human dermal fibroblasts 24 and 48 h after electrical stimulation (ES) initiation. (B) Quantification of collagen gel contraction 24 h after ES initiation. (C) Quantification of collagen gel contraction 48 h after ES initiation. Data are presented as the mean ± SD; n = 4 per group. **, p < 0.01, significant difference between the ES with a duty cycle of 10% group and the control group. The statistical difference between the control and the 10% group was tested by Student’s t-test.

and contributes to some outside-in signals that regulate certain cellular functions including differentiation.

This study revealed that myofibroblast differentiation mediated by ES is influenced by duty cycle and that ES with a duty cycle of 10% promotes cell differentiation, whereas a duty cycle >50% induces cell death. Some studies revealed that difference in intensity or frequency affects cell behavior such as migration, proliferation, and the secretion of some cytokines. Thus, the effects of ES on cell behavior differed with these parameters. In this study, we examined the effects of differences in the duty cycle on HDF differentiation into myofibroblasts and viability. The results indicated that a duty cycle of 10% promotes wound contraction with myofibroblast differentiation. However, a higher duty cycle and long-term stimulation might have adverse effects. Therefore, it is necessary to set stimulation parameters, such as duty cycle, duration, intensity, frequency, and polarity, according to the purpose of ES for pressure injuries in clinical trials.

Limitation

The reason for the decrease in cell viability in the 50% and 90% groups was unclear. A 24-h ES with a duty cycle
of 10% promoted myofibroblast differentiation, but this study used only 24-h stimulation and did not reveal the relationship between ES duration and fibrotic effects. Therefore, 10% might not be the optimum duty cycle for HDFs with short duration. TGF-β1 mRNA expression was increased by ES with a duty cycle of 10%, but other cytokines and the signaling pathway that accelerates the secretion of fibrotic factors induced by ES are also unclear. Therefore, further study is needed to investigate the effect of duration on differentiation and which pathway is influenced by ES.

Conclusion

This study suggests that the duty cycle influences myofibroblast differentiation and HDF viability and shows that 10% is the effective duty cycle of monophasic-pulsed micro-currents for granulation tissue formation, which induces pressure injury healing.

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Conflict of Interest: The authors declare no conflicts of interest.

References


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