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Original research article

The effect of short duration of electrical stimulation on wound healing in acute wound in a rat model

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ABSTRACT

Background: Prolonged application of electrical stimulation (ES) will cause skin discomfort and burns, and therefore may be harmful in the elderly population with morbid conditions. Therefore, it is needed to apply ES in a short duration. However, currently, no studies have been conducted that investigated the effect of short duration of ES on wound healing. This study purpose was to investigate the effect of short duration of ES on wound healing of acute wounds in rats.

Methods: In this study, a wound was created on the back of rats. Rats were assigned to one of the following 4 groups; ES treatment 5 minutes (5-min group), ES treatment 10 minutes group (10-min group), ES treatment 15 minutes (15-min group), and a control group (film dressing). ES (20 Hz, 320 µs, 50 µA) was delivered for 11 days.

Results: From day 4 onward, wound sizes were significantly lower in the 10-min group when compared to the 15-min group, and control group on day 10 and 11. In the 10-min group, inflammation was the lowest when compared to other groups, while the intensity of fibroblast was significantly higher than in the 15-min and control groups. Reepithelialization was most advanced in the 10-min group compared with other groups. The number of MMP-9 positive cells was not significantly different among groups, whereas the number of VEGF-positive cells was significantly higher in the 10-min group when compared with other groups.

Conclusion: ES application for 10 minutes significantly reduced inflammation, improved reepithelialization and angiogenesis when compared to shorter and longer applications. ES for 10 minutes also significantly improved wound healing when compared to the use of modern dressing alone. Therefore, it is recommended to apply ES for 10 minutes a day to improve wound healing.

1. Background

The presence of wounds will cause an increase in costs for both patients and the health economy [1]. Unhealed wound also will have a high impact on depression, a low quality of life (QOL), and even mortality [1]. In a previous study, it was estimated that 6.5 million patients in the USA suffer from unhealed wounds and that more than US $25 billion is spent to treat such wounds [2]. In addition, a study in the UK showed that the cost to treat wounds was around was roughly 3% of the total expenditure on healthcare, or around US$ 4.6 billion per year [2]. The prevalence of wounds might increase in the aging population as a main sufferer of wounds increases worldwide [3]. Considering the impact of wounds, many clinicians and wound care experts aim to identify novel therapies for improving wound healing.

Many therapies have been used to treat wounds, such as modern dressing [4], topical therapies [5], ultrasound etc [6]. However, wounds are still a huge problem both economically and clinically. Recently, electrical stimulation (ES) has gained significant attention for many purposes, including improving blood flow and reducing pain [7–9]. ES is a therapy that delivers a low electrical current through an electrode to the target tissue. The epidermis of intact skin has electrical potential, resulting from the uneven distribution of sodium ions, thereby causing a potential difference at the range of 10–60 mV [10]. Damaged to the epidermis will cause a change in the electrical current of the body, resulting in an electrical field [11]. During wound healing, this electrical field will improve the migration of epithelial cells [12].

Previous studies showed that ES has beneficial effects on wound healing [13–21]. It has been shown that ES accelerated the healing of

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acute wounds [16], diabetic ulcers [20,21], pressure ulcers [18], and venous ulcers [22]. However, most previous studies have focused on the intensity (frequency) of application [16,19,22–26]. In addition, ES has been applied at the range of 30 minutes – 8 hours a day [17,22,24–26]. However, it has been shown that prolonged application of ES can cause discomfort and even skin burns [27,28]. The effect of skin burns and discomfort is harmful to patients, especially the elderly. Moreover, most patients with unhealed wounds frequently also suffer from other disease and morbid conditions, and therefore long-term application of ES may be harmful to the body. Therefore, it is needed to apply ES for a short-term duration in one day. However, currently, no studies have been conducted that investigated the effect of short duration of ES on wound healing. Therefore, in this study, we aimed to investigate the effect of short duration of ES on wound healing.

In a previous study, it was shown that the most important phase during wound healing is proliferation phase [16]. Angiogenesis is a critical component in the proliferation phase of wound healing [16]. In addition, MMP-9 expression is one of most important factors during the process of wound healing [29]. Previously, it has been showed that the prolonged and excessive production of MMP-9 will lead to delayed wound healing, whereas low levels or no MMP-9 expression were associated with healed wounds [30]. High MMP-9 expression also correlated with the deterioration of wounds into chronic wounds [29]. Currently, the role of ES on MMP-9 expression is still unclear. Therefore, in this study we investigated MMP-9 and angiogenesis following the treatment with short duration of ES.

2. Methods

In this study, male Wistar rats (180–200 g; 12–14 weeks of age) were used. Rats were obtained from the Pharmaceutical Laboratory Animal, Universitas Muhammadiyah Purwokerto, and had access to food and water ad libitum. Before wounding, animals were acclimatized for seven days. This study protocol was approved by the ethical committee, Faculty of Medicine, Jenderal Soedirman University, Purwokerto (1208/KEPK/III/2017)

2.1. Procedure

To create a wound, rat hair was removed by shaving. Removal of hair was conducted 1 day before the wounding procedure. The wounding procedure was based on previous studies [31]. In brief, one full-thickness wound, 1 cm long, was created on the back of rats. After wounding, the wound was cleansed with NaCl, then the wound was covered with modern dressing (film dressing) (BSN medical, Hamburg, Germany). Next, ES was applied to the left and right side of the wound. The application of ES was based on our previous studies [20,21]. In brief, the electrodes of the ES device were attached to the right and left side of the wound areas. ES was applied for 5 minutes (5-min group), 10 minutes (10-min group), 15 minutes (15 min-group) for 11 eleven days (20 Hz, 320 µs, 50 µA). Rats in the control group received modern dressing (film dressing), and did not receive ES. After finishing the application of ES, wounds were covered with gauze to fix the position of the dressing. All rats survived and lived until the end of the study.

2.2. Wound size

From day 0 to day 11, the sizes of the wounds were measured by ImageJ software as based on previous studies [32]. Relative wound size were calculated based on a previous study [32]. The formula used was as follows: Relative wound size = size at day n – size at day 0)/(size at day 0) [32]. Rats were sacrificed and skin tissue was harvested on day 11 post wounding.

2.3. Histological examination

The wound tissue and normal skin surrounding of wound were obtained for histological analysis. After sacrificing the rats, a sample of skin was immersed in 10% formalin. The skin tissue was then dehydrated in a series of ethanol and xylene. The tissue samples were finally embedded in paraffin. Next, skin tissue was cut at 4 µm thickness. The sectioned skin was then deparaffinized and rehydrated in xylene and ethanol. Subsequently, the sectioned skin was stained with Hematoxyllin and eosin staining and Van Giesson staining, and sections were observed under a light microscope.

2.4. Immunohistochemistry

The preparation of the skin sections was similar as described above. For immunohistochemistry, the method used in this study was based on the approach used in a previous study [31]. Paraffin sections were mounted onto Polylysine-coated slides. To inactivate the activity of endogenous peroxidase, sections were incubated in 3% H_20_2 for 30 min at room temperature. For immunostaining of MMP-9, antigen retrieval was performed by immersion in citrate buffer (pH 6.0) for 15 min and for immunostaining with VEGF, antigen retrieval was carried out by autoclaving the slides in Tris EDTA pH 9 for 15 min. Sections were then incubated with primary antibodies. The primary antibody used in this study included a mouse anti- rat MMP-9 (Abcam, Cambridge, UK) at 1:100 or a mouse anti-rat VEGF antibody (Abcam, Cambridge, UK) at 1:100. The sections were washed with PBS and incubated with a biotin-conjugated secondary antibody (1:100). Streptavidin-peroxidase was used to visualize the staining, and diaminobenzidine substrate as a chromogenic agent (Sigma Aldrich Co, St. Louis, USA). Lastly, hematoxylin was used as a counterstaining agent. The sections were observed under a light microscope (Olympus, Tokyo, Japan) and images were taken. MMP-9-positive cells and VEGF-positive cells were calculated by counting the number of positive cells in eight randomly selected fields using a magnification of 400 X.

2.5. Statistical analysis

The analysis was performed using SPSS version 20 software (IBM Corp., Armonk, NY, USA). The data of wound size was presented as the mean ± standard deviation. Wound size, intensity of inflammation, and the level of fibroblasts among the four groups were assessed by the Kruskal Wallis test, followed by the Man-Whitney U test. Moreover, data regarding MMP-9-positive cells and VEGF-positive cells were analyzed by the ANOVA test, followed by Tukey's test. $P < 0.05$ was considered significant.

3. Result

3.1. Wound size

The macroscopic appearance of each wound is presented in Fig. 1. On day 0 to day 1, the appearance of the wound was similar between groups. On day 1 the necrotic tissue started to appear in the control group, while in other groups, this was not observed. On day 5, the necrotic tissue in the control group was thicker when compared to the other groups. On day 7, the necrotic tissue was still thick in the 15-min group and the control group, while in the 5-min and 10-min group, the necrotic tissue was reduced when compared with other groups. In addition, the wound size in the 10-min and 5-min groups were smaller when compared to that of other groups. When comparing the control group and the 15-min group, the wound size in the 15-min group was larger when compared to the control group. On day 11, the necrotic tissue disappeared in the 10-min group and the wound was almost healed. The wound size in the 5-min group was almost similar to that in the 10-min group, however, wounds in the 5-min group were still

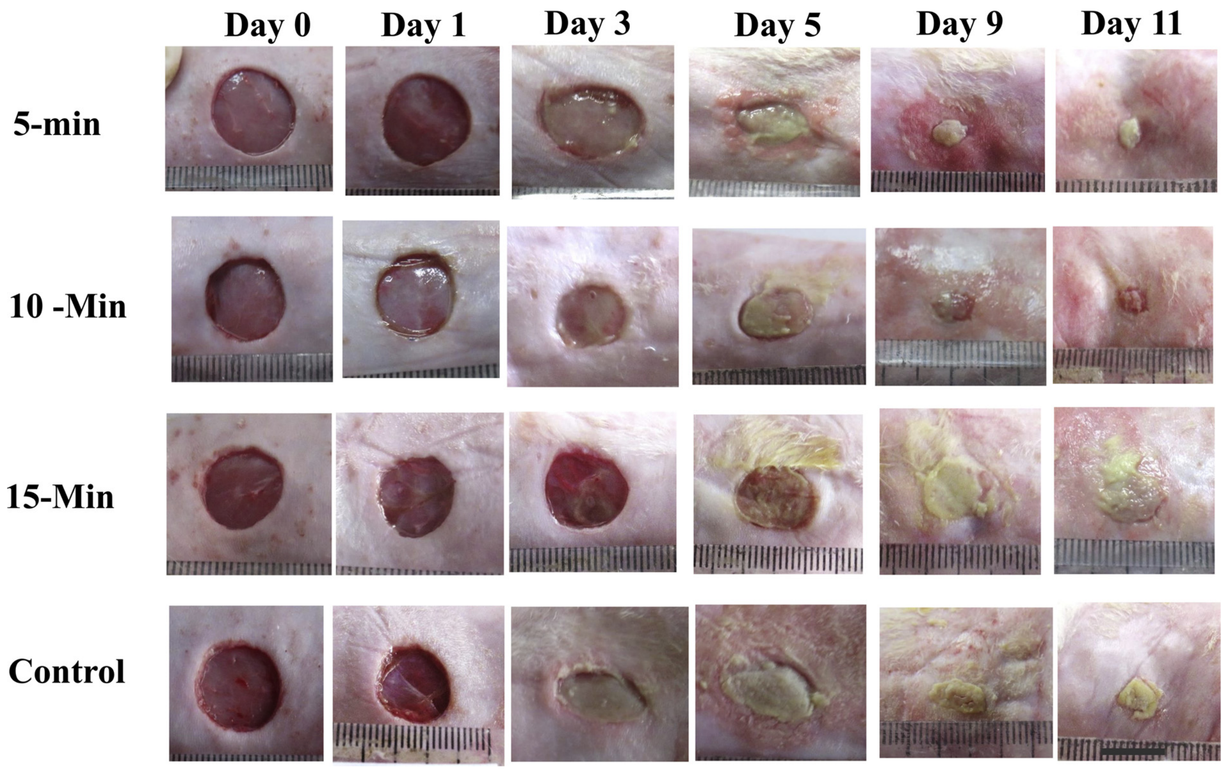

Fig. 1. Appearance of wounds in the 5-min group, 10-min group, 15-min group, and the control group (bar indicates 1 cm). Wound beds on day 11 in the 5-min, 15-min, and control groups were fully covered by necrotic tissue.

covered with necrotic tissue. In the 15 min-group and the control group, wounds were still larger compared to the 5-min and 10-min groups.

The mean wound sizes and the differences between wound sizes per group are presented in Table 1 and Fig. 2. In the 5-min group, there was significant difference in wound size between 5-min duration and 15-min duration during the late inflammation phase (P = 0.043), and early proliferative phase (day 7, P = 0.043; day 8, P = 0.043, and day 9, P = 0.02). However, in the 5-min group, the data was not significantly different compared with other groups on day 10 to day 11. In the 10-min group, the wounds gradually decreased over the healing period. From day 4 onward, the wound size in the 10-min group was significantly smaller when compared with the 15-min group (day 4 = 0.021, day 5, P = 0.021, day 6, P = 0.021, day 7, P = 0.043, day 8, P = 0.043, day 9, P = 0.021, day 10, P = 0.020, day 11, P = 0.021). From day 10–11, wounds in the 10-min group was also significantly smaller compared with control group (day 10, P = 0.021, day 11, P = 0.021). In the control group, the wound size was significantly smaller when compared with the 15-min group on day 8 and 9 (day 8, P = 0.029, day 9, P = 0.043).

Table 1
The ratio of the wound size and initial size per group on day 0.

Days	5-min	10-min	15-min	Control
0	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
1	0.93 ± 0.11	0.89 ± 0.16	0.92 ± 0.13	0.91 ± 0.05
2	0.88 ± 0.10	0.83 ± 0.24	0.74 ± 0.20	0.84 ± 0.04
3	0.83 ± 0.00	0.69 ± 0.10	0.83 ± 0.22	0.68 ± 0.03
4	0.71 ± 0.18	0.63 ± .0.08*	0.93 ± 0.21	0.66 ± 0.05
5	0.66 ± 0.04*	0.61 ± 0.05*	0.85 ± 0.15	0.67 ± 0.01
6	0.66 ± 0.30	0.56 ± 0.03*	0.82 ± 0.14	0.60 ± 0.10
7	0.53 ± 0.20*	0.51 ± 0.10*	0.88 ± 0.23	0.58 ± 0.04
8	0.39 ± 0.26*	0.35 ± 0.16*	0.75 ± 0.13	0.42 ± 0.05*
9	0.30 ± 0.28*	0.18 ± 0.08*	0.71 ± 0.19	0.35 ± 0.14*
10	0.23 ± 0.20	0.15 ± 0.03*,†	0.58 ± 0.28	0.34 ± 0.12
11	0.22 ± 0.19	0.07 ± 0.04*,†	0.47 ± 0.27	0.28 ± 0.11

Values indicate mean ± standard deviation.
* = P < 0.05, 5-min/10-min/control group vs 15-min group.
† = P < 0.05, 5-min/10-min vs control group.

3.2. Tissue damage, inflammation, fibroblasts, and reepithelialization

The results of the hematoxylin and eosin (H&E staining) in the epidermis and dermis are presented in Fig. 3 and Table 2. The number of inflammatory cells in the 10-min group was significantly lower when compared to the 5-min (P = 0.04), 15-min (P = 0.015), and control groups (P = 0.011), while inflammation in the 5-min group was significantly lower when compared with the control group (P = 0.04) and 15-min group (P = 0.011). When comparing the inflammatory cells in the control group to that in the 15-min group, the number of inflammatory cells in the control group was significantly lower compared to that in the 15-min group (P = 0.04)

The fibroblast intensity is presented in Table 2. The intensity of fibroblast in the 10-min group was increased when compared with the 5-min group. However, there was no significant difference between these two groups (P = 0.490). The intensity of fibroblast was higher in the 5-min and 10-min groups when compared with the control and 15-min groups (5-min vs control; P = 0.04, 5-min vs 15-min; P = 0.01, 10-min vs control; P = 0.032, 10-min vs 15-min, P = 0.013). The appearance of reepithelialization is shown in Fig. 4, which shows that reepithelialization was more advanced in the 5-min and 10-min groups when compared with the control and 15-min group. The most advanced reepithelialization occurred in the 10-min group. The 15-min and control groups showed minimal reepithelialization from the wound edge toward the center of the wound. The center of these groups was still uncovered with epithelial cells.

3.3. Collagen alignment

The result of Van Gieson staining in the dermis layer are shown in Fig. 5, which show that the density of collagen fibers was more obvious

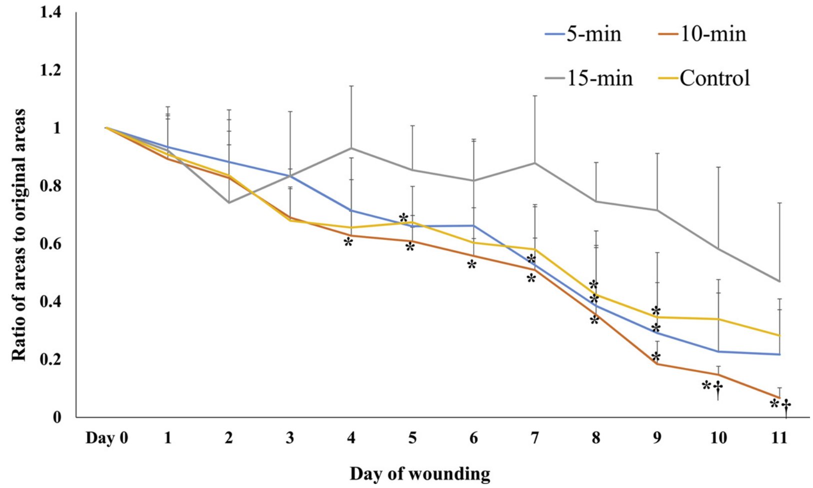

Fig. 2. Changes in wound size in each group from day 0 onwards (n = 5).

in the 5-min and 10-min groups than in the control and 15-min groups. Moreover, collagen alignment in the 5-min and 10-min groups revealed a proper alignment of collagen deposition, while in the other groups less and irregular arranged collagen fibers were observed. The 15-min group and the control group showed little deposition of collagen fibers. When comparing the 5-min and 10 min groups, the collagen deposition in the 10-min group was more increased, well aligned, and denser when compared with 5-min group.

3.4. Immunohistochemistry

Fig. 6 shows the MMP-9 positive cells in the dermis layer for each group. Positive MMP-9 cells were indicated by brown colored fibroblast cells. Most positive cells were located in the dermis layer. The comparison of the number of MMP-9-positive cells is shown in Fig. 7. In general, the number of MMP-9 positive cells in the 10-min group was lower when compared to that in other groups, however, no statistical differences in the number of MMP-9-positive cells were observed among the four groups.

Data regarding VEGF-positive cells are shown in Fig. 8. VEGF-positive cells were indicated by brown colored fibroblast cells. The differences in number of VEGF-positive cells are presented in Fig. 9. Fig. 9 shows that the intensity of VEGF-positive cells in the 10-min group was significantly higher compared to that in the 5-min ($P < 0.001$), 15-min group ($P < 0.001$) and control groups ($P < 0.001$). The number of VEGF-positive cells in the 5-min group was significantly higher when compared to that in the 15-min group ($P < 0.001$) and control groups ($P = 0.047$). Moreover, the 15-min group was more likely to have a lower number of VEGF-positive cells when compared to the control group, however no statistically significant differences were observed in the number of VEGF-positive cells between the 15-min and control groups.

Table 2
The level of inflammation and fibroblasts per group.

Groups	Polymorphonuclear neutrophils (PMNs)	Fibroblast
5-min	$2^{†,≠}$	$3^{†,≠}$
10-min	$1^{*,†,≠}$	$3.5^{†,≠}$
15-min	4	2
Control	$3^{†}$	2

Values represent the median score.
Rating scale: 0 = absent, 1 = occasional, 2 = moderate, 3 = abundant, 4 = very abundant.
* = $p < 0.05$ (10-min vs 5-min).
† = $p < 0.05$ (5-min/10-min/control vs 15-min).
≠ = $p < 0.05$ (5-min/10-min vs control).

4. Discussion

To our knowledge, this is the first study in which short duration of ES on wound healing was investigated. In this study, we compared the

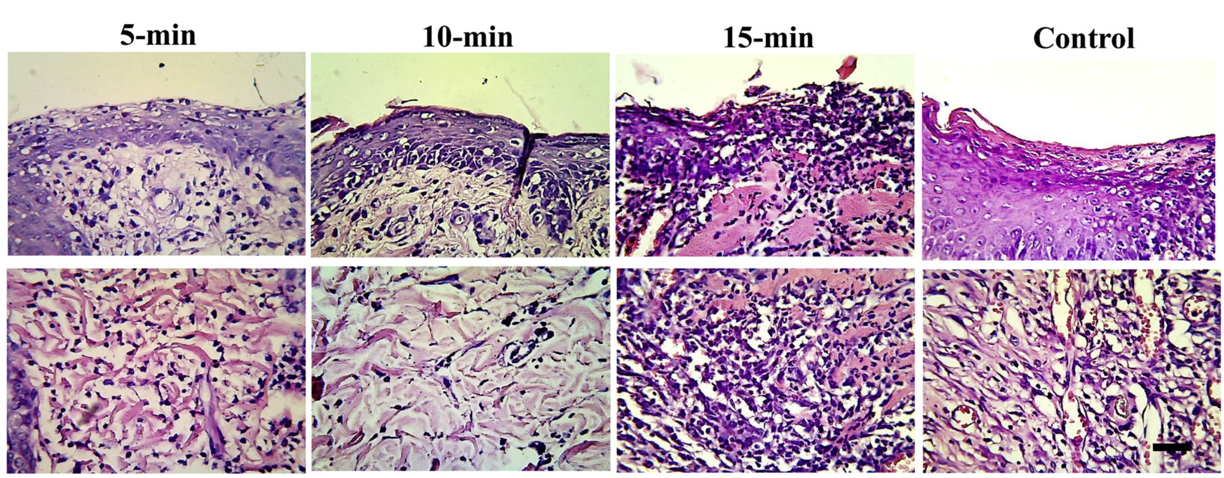

Fig. 3. Hematoxylin and eosin staining of the 5-min, 10-min, 15-min, and control groups on day 11 in the epidermis (upper picture) and dermis (lower picture) (bar = 100 μm).

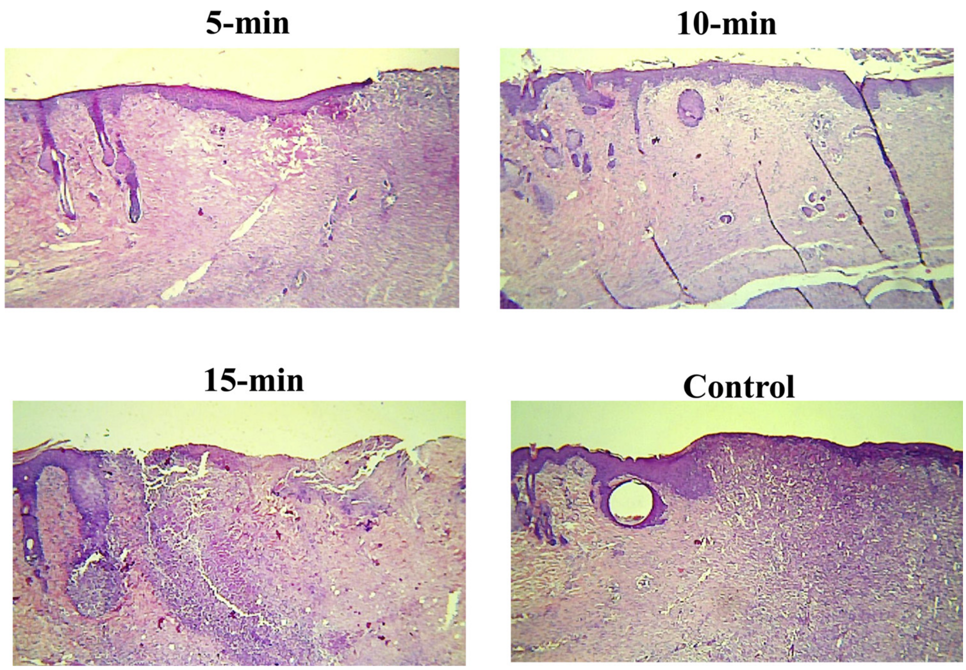

Fig. 4. The appearance of reepithelialization in the 5-min, 10-min, 15-min, and control groups on day 11 at (magnification 200×).

application of short duration (5-min, 10-min, and 15-min) of ES on wound healing of acute wounds. Wound healing was more improved in the application of ES 10-min when compared to the 5-min and 15-min groups.

Excessive inflammation will impede wound healing [29]. Our histological results showed that the application of 5-min and 10-min ES decreased inflammation when compared with the control group. The results of our study corresponds with the findings of our previous study and that of other studies that showed that ES reduced inflammation [20,21,33,34]. The mechanism by which ES reduced inflammation might involve the ability of ES to reduce the migration of excessive neutrophils, monocytes, and macrophages to the sites of injury [34]. In

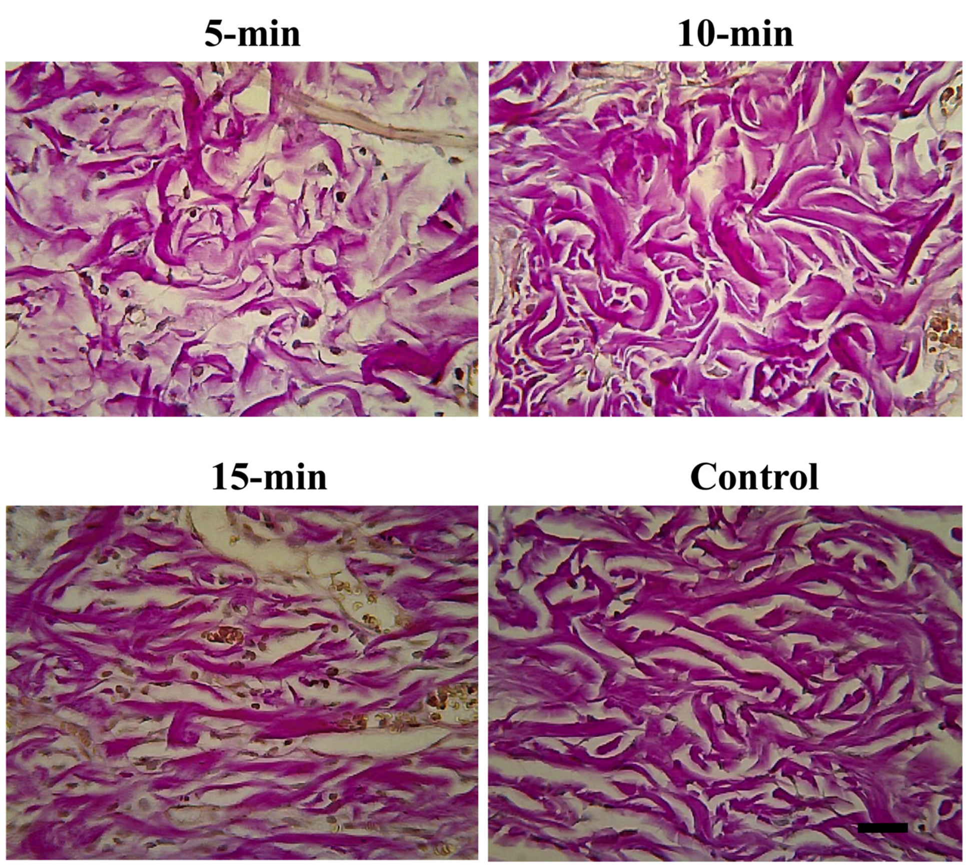

Fig. 5. Van Giesson staining in the 5-min, 10-min, 15-min and control groups on day 11 (bar = 100 µm).

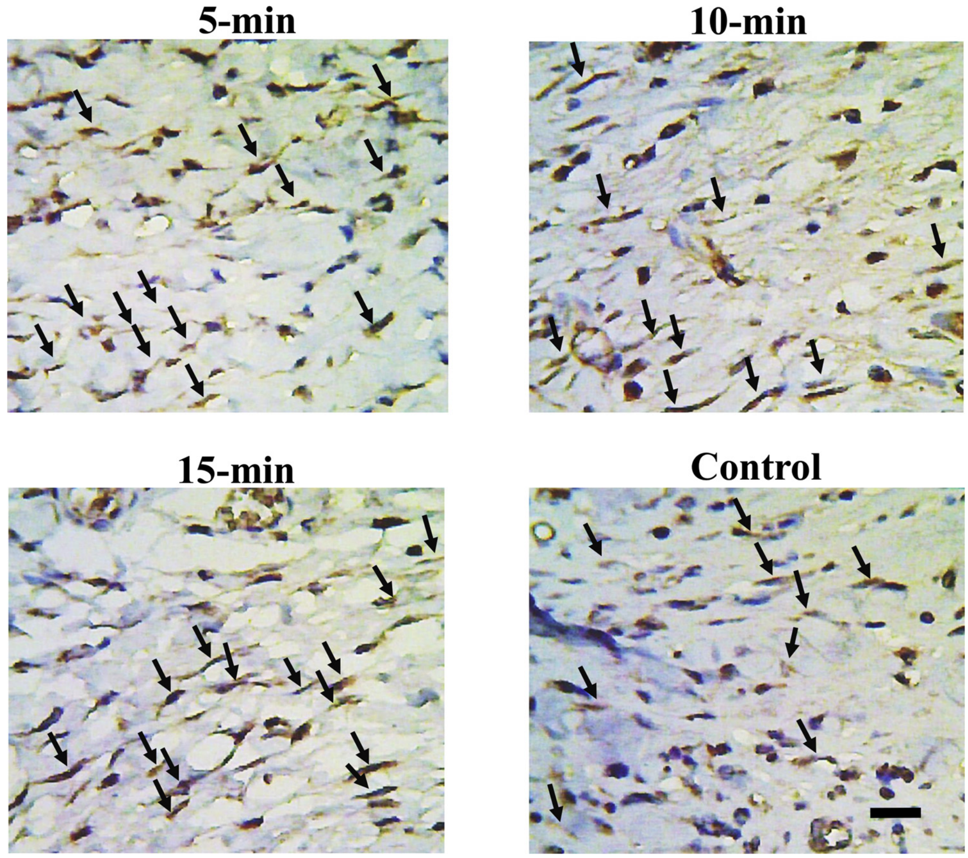

Fig. 6. MMP-9-positive cells in the dermis in the 5-min, 10-min, 15-min, and control groups on day 11 (n = 5) (bar = 100 μm). (For interpretation of the references to colour in the text, the reader is referred to the web version of this article.)

a previous study, it was shown that ES shortened the longer inflammation phase by decreasing the number of CD3+ cells [34]. In our study, the level of inflammation seen in the 15-min group was higher when compared with that of the control group. It may be suggested that the ES application of 15-min causes vasoconstriction of blood vessels, resulting in the reduction of oxygen. In a previous study by Morton et al. (1994), it was revealed that the reduction of oxygen during ES will cause an increase of reactive oxygen species (ROS) [35]. The increase in ROS will lead to the increase of inflammatory cells to the site of injury, leading to more excessive inflammation and tissue damage [35].

In our study, we showed that ES improved the reepithelialization of wounds. Wounds treated for 5 minutes and 10 minutes showed improvement of reepithelialization as was shown by the H&E staining when compared with the control group. The data obtained in our study corresponds with the data presented in a previous study that revealed that ES improved reepithelialization [36]. The improvement of reepithelialization might be due to the ability of ES to induce keratinocyte migration [12]. In addition, in a previous study, it was shown that ES increased levels of Col IV, which is a connective tissue promoter of keratinocytes, in the wound areas [34]. The increase in Col IV will

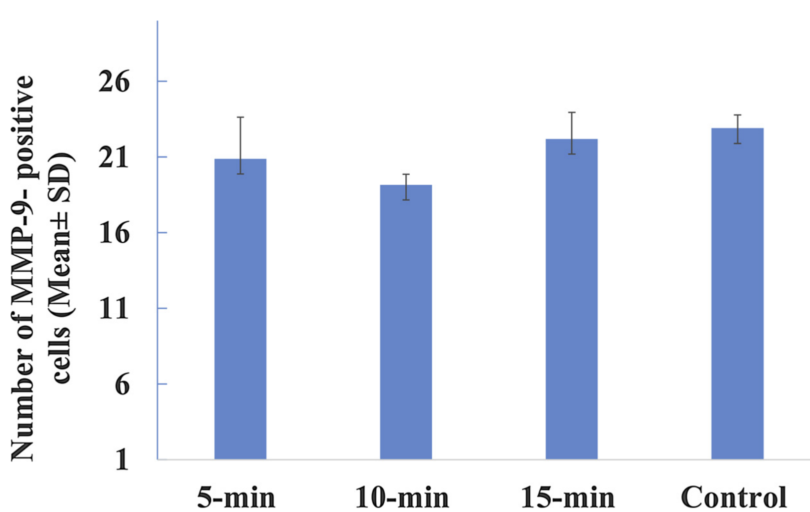

Fig. 7. Level of MMP-9-positive cells among the 5-min, 10-min, 15-min, and control groups on day 11. No significant differences were observed among the four groups.

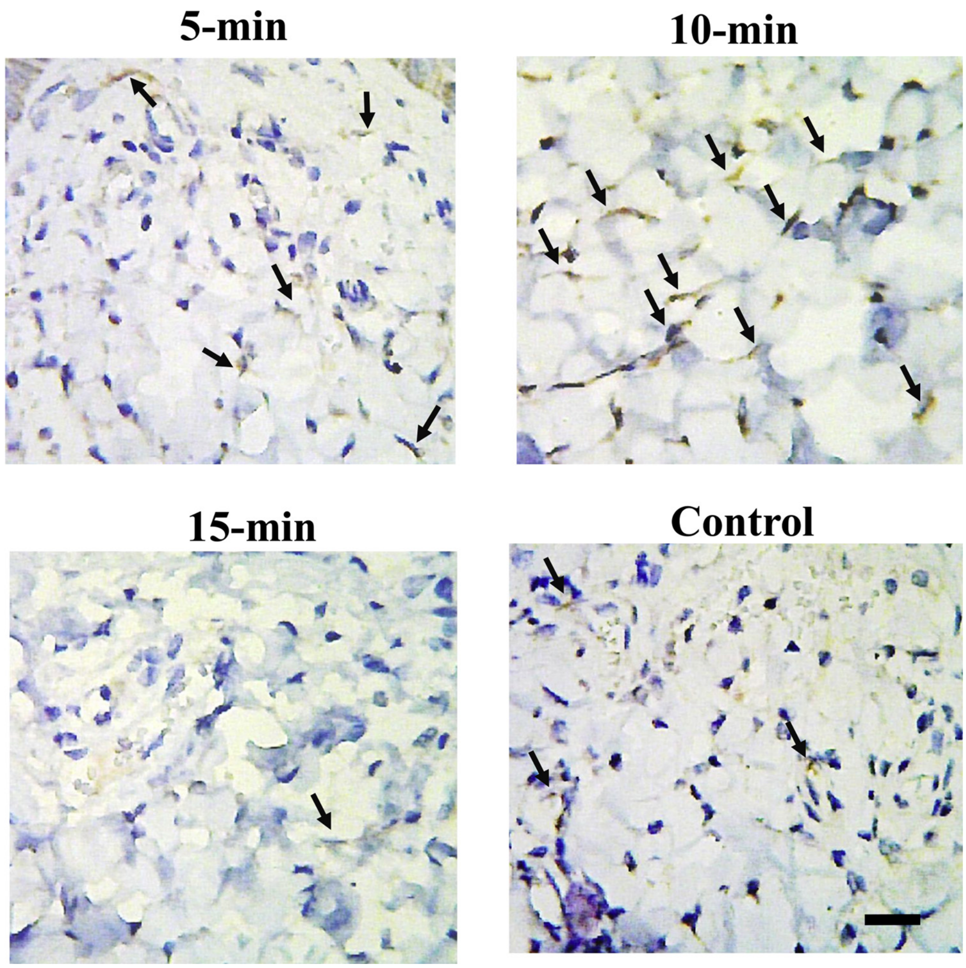

Fig. 8. VEGF-positive cells the dermis in the 5-min, 10-min, 15-min, and control groups on day 11 (n = 5) (bar = 100 μm). (For interpretation of the references to colour in the text, the reader is referred to the web version of this article.)

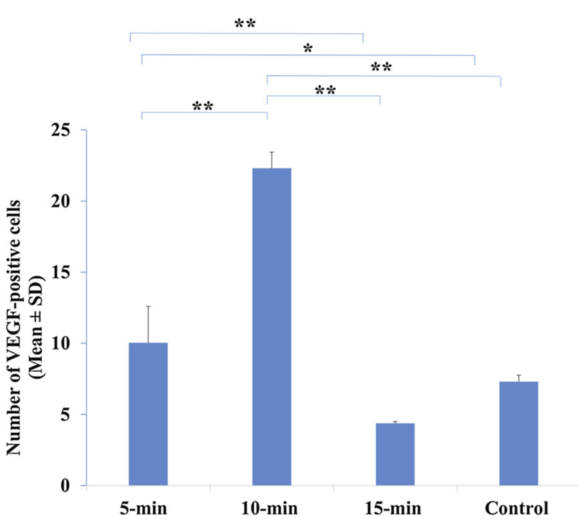

Fig. 9. Differences in the level VEGF-expressing cells among the 5-min, 10-min, 15-min, and control groups on day 11. * = P < 0.05, ** = P < 0.01 (Tukey's test) (n = 5).

enhance keratinocyte proliferation and reconstitution of the basement membrane [34]. In this study, we observed that in the 15-min group, there was disruption of new epidermis by excessive inflammatory cells, resulting in delayed reepithelialization process. Thus, the influx of inflammatory cells might be due to the increase in ROS due to vasoconstriction of the blood vessel. In our study, we also found that the application of short duration of ES improved granulation tissue. On day 11, in the 10-min group was covered with granulation, while the control group was still covered with slough. Based on the previous study, ES improved the blood flow in wounded areas [37]. The improvement of granulation tissue might be due to the increase of blood flow in the wound bed when compared with the control group. The improved blood flow will cause a reduction of necrotic tissue in the wound bed. In our study, the improved blood flow in the 10-min group was observed by an increase in VEGF positive cells when compared with the control group. In this study, the application of ES for 5 minutes might not improve blood flow as much as in the 10-min group, indicating that VEGF positive cells were increased in the 10-min group when compared with the 5-min group. Therefore, the 5-min group was still covered with necrotic tissue on day 11. Further studies involving measurement of blood flow are needed to elucidate these findings.

Prolonged and excessive production of MMP-9 will lead to disordered wound healing [29]. In a previous study, it was shown that chronic wounds, such as diabetic ulcers and pressure ulcers had higher levels of MMP-9 [30]. In our study, the number of MMP-9 positive cells were not different among the four groups. This is a surprising result given that in previous study it was shown that the application of ES caused downregulation of MMP-9 [38]. This is also a new finding in this study that the improvement of wound healing following treatment with short duration of ES might not due to reduction of MMP-9. Taken together, further studies are needed to confirm the above-mentioned findings.

Angiogenesis is the process in which new blood vessel (capillaries) forms in the wound site [39,40]. Insufficient angiogenesis causes a delay in wound healing and formation of a chronic wound [37]. In our study, application of ES for 5 minutes and 0 minutes increased the number of VEGF-positive cells in wounds. Our data corresponded with the findings presented in a previous study about the effect of ES on angiogenesis [37]. In previous studies it was shown that ES induced angiogenesis through enhanced VEGF production by endothelial cells [34,37]. In this study, the number of VEGF-positive cells in the 15-min group was lower when compared with that in the control group, which did not receive ES. Based on these results, it might be suggested that the application of 15 min induced vasoconstriction of blood vessels, rather than improved blood flow into wounded areas.

This study has some limitations. In our study, we only investigated a single time point. The effect of short ES application might be different on other phases of wound healing, such as inflammation and the maturation phase. Next studies in which different times of ES application are needed. In our study, we used one condition of stimulation (20 Hz, 320 μs, 50 μA). Therefore, the conclusion is limited to the present stimulation condition. However, this study also had strength that is worth to note. This study has high originality since it is the first study to investigate the effect of short duration of ES on wound healing. Moreover, this study has an impact on the use of ES in the clinical setting, especially for elderly who suffer from other disease and morbid conditions, and cannot use long application of ES.

5. Conclusion

This is the first study to investigate the effect of short duration of ES on wound healing. Our study showed that the application of ES for 10-min significantly reduced inflammation, improved reepithelialization and angiogenesis when compared to shorter and longer applications. ES for 10 minutes also significantly improved wound healing when compared to the use of modern dressing alone. It is recommended to apply ES for 10 minutes a day to improve wound healing.

Conflict of interest

The authors declared no potential conflicts of interest with respect to publication of this article.

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Ethical approval

The Institutional Ethics Committee of Medical faculty, Universitas Jenderal Soedirman, Indonesia approved this study (1208/KEPK/III/2017)

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