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Angiogenic effects of low-intensity cathodal direct current on ischemic diabetic foot ulcers: A randomized controlled trial

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ABSTRACT

Aims: This study investigated the effect of low-intensity cathodal direct current (CDC) of electrical stimulation (ES) on the release of hypoxic inducible factor-1α (HIF-1α), nitric oxide (NO), vascular endothelial growth factor (VEGF), and soluble VEGF receptor-2 (sVEGFR-2) in the wound fluid of ischemic diabetic foot ulcers (DFUs).

Methods: This study was a randomized, single-blind, placebo-controlled trial. Thirty type 2 diabetes patients with ischemic foot ulcerations were randomly assigned to receive either low-intensity CDC at sensory threshold (ES group, $n = 15$) or placebo treatment (control group, $n = 15$) for 1 h/day, 3 days/week, for 4 weeks (12 sessions). After debridement during the first and twelfth treatment sessions, wound fluid was collected before and after ES application to determine the levels of HIF-1α, NO, VEGF, and sVEGFR-2. Wound surface area (WSA) was measured at the first, sixth, and twelfth sessions.

Results: At the first session, after ES application, wound-fluid levels of HIF-1α were significantly increased (+61.98 pg/mL) compared to the control group (−3.85 pg/mL, $P = 0.01$). After ES application at the first and twelfth sessions, wound-fluid levels of VEGF were also significantly increased (+36.77 and +39.57 pg/mL, respectively) compared to the control group (+4.15 and +0.15 pg/mL, $P = 0.007$ and $P = 0.019$, respectively). There was no significant effect on NO and sVEGFR-2 levels between the groups.

Conclusions: Low-intensity CDC has positive effects on the release of HIF-1α and VEGF in the wound area of ischemic DFUs. Furthermore, our results suggest that applying ES to ischemic DFUs can be a promising way to promote angiogenesis and to achieve better outcomes in diabetic wound healing.

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1. Introduction

In recent decades, the increased incidence of diabetes mellitus has been associated with a higher rate of clinically significant and challenging complications [1]. Diabetic foot ulcer (DFU) is a major complication of diabetes mellitus and is considered the major cause of non-traumatic lower extremity amputations (up to 88%) [2].

Wounds in diabetic patients can take longer to heal than similar wounds in non-diabetics [3]. In a DFU, decreased peripheral blood flow and impaired angiogenesis and vasculogenesis reduces circulation to the wound site, so that the healing process cannot occur as it does in non-diabetic wounds [4].

Angiogenesis is a critical component of the proliferative phase of repair. In the wound bed, angiogenesis is primarily controlled by factors such as vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF-2), and nitric oxide (NO) [5]. VEGF stimulates the formation of new vessels by binding to VEGF receptor-2 (VEGFR-2) to induce angiogenesis [6]. VEGFR-2 has two isoforms: soluble VEGFR-2 (sVEGFR-2) and membranous VEGFR-2. VEGF functions predominantly through binding to membranous VEGFR-2 to induce angiogenesis [7]. Binding VEGF to sVEGFR-2 within the wound fluid decreases the availability of VEGF for binding to membranous VEGFR-2 and results in diminished angiogenic effects of this factor [8,9]. NO is a critical mediator of normal wound healing, required for wound-related upregulation and activation of growth factors such as VEGF [10]. However, several animal studies have reported that levels of VEGF and NO are downregulated in diabetic wounds [11–15]. Hypoxic inducible factor-1α (HIF-1α) acts as a crucial stimulator of several angiogenic factors, such as VEGF, FGF-2, and NO, in wound healing [16]. Numerous studies have reported that the expression of HIF-1α is impaired in diabetic wounds [16–19]. Decreased HIF-1α in diabetic wounds leads to impaired production of prime angiogenic factors, such as VEGF and NO, in response to hypoxia, which results in reduced neovascularization and an impaired wound healing process in diabetic animal models [16]. It is suggested that over-expression of HIF-1α in a high-glucose environment is the key target in the treatment of diabetic wounds [18]. Therefore, the abnormal expression of angiogenic factors and their receptors, including HIF-1α, VEGF, NO, and sVEGFR-2, may be a possible mechanism for impaired angiogenesis, resulting in poor healing of the DFU. In such situations, stimulation of angiogenesis by increased expression of angiogenic factors may be necessary to promote adequate healing.

From a review of the literature, we identified numerous studies, including *in vitro*, *in vivo*, and clinical trials, that reported that electrical stimulation (ES) has potential benefits for promoting healing in wounds of various etiologies [20]. ES therapy has been shown to increase skin perfusion [21,22], which may be further related to increased expression of VEGF, FGF-2, and NO, resulting in enhanced angiogenesis [23]. Bevilacqua and colleagues [24], in a clinical study, reported that frequency-modulated ES enhanced the plasma levels of VEGF in both diabetic and non-diabetic subjects. In our recent study, we reported that cathodal direct current (CDC) increased the plasma levels of VEGF and NO in patients with DFUs [25]. To our knowledge, however, no study has investigated the effect of ES on the expression of HIF-1α, NO, VEGF, and VEGFR-2 within wound fluids extracted from DFUs.

To understand the mechanisms underlying the therapeutic effects of ES on wound healing, it is mandatory to examine the cellular and molecular changes that occur in wound sites treated with this physical energy. Therefore, the present study was designed to investigate the release of HIF-1α, NO, VEGF, and sVEGFR-2 in wound fluid after the application of low-intensity CDC in ischemic DFUs. We also studied the effect of this type of ES on decreasing WSA.

2. Methods

2.1. Design overview

All type 2 diabetic patients with foot ulcers distal to the ankle joint who were treated at Hajar Hospital in Tehran, Iran, between November 2013 and September 2014 were eligible to participate in this single-blind, randomized controlled trial. The study was approved by the medical ethics committee of Tarbiat Modares University, and was registered with the US National Institutes of Health (ClinicalTrials.gov) as #NCT02432859.

2.2. Study population

Study eligibility was determined by the patients' physicians. The inclusion criteria were type 2 diabetes, ischemic DFU (ischemia was diagnosed by an ankle-brachial index of 0.5–0.9 and absent or decreased pulse rate in the dorsalispedis and tibialis posterior arteries), wound size of >2 cm^2, light neuropathy (based on the UK scale), and a Wagner foot classification of 2. Subjects were excluded if they had osteomyelitis, a cardiac pacemaker, angioplasty, severe infection, cancer, kidney failure, other skin diseases, or any medical conditions for which ES is contraindicated, such as pemphigoid. All participants provided written informed consent before entering the study.

2.3. Randomization and intervention

Thirty volunteer type 2 diabetic patients with ischemic foot ulcers were enrolled in the study. Eligible participants were randomly assigned in an allocation ratio of 1:1 to either the ES group ($n = 15$) or the control group ($n = 15$), using permuted blocks with a block size of 4. The randomization was performed by research investigators. Concealment of allocation was performed using sequentially numbered, sealed, opaque, stapled envelopes. The patients and the angiogenic-outcome assessors were blinded to the treatment allocation.

In the ES group, patients received CDC with sensory threshold intensity (3.36 ± 0.58 mA) for one hour, 3 days/week (every other day) for 4 weeks (12 sessions). Due to the high

likelihood of sensory nerve impairment in diabetic patients, we used a sensory threshold ratio from the forearm to the leg and from the thigh to the leg in order to determine the sensory threshold intensity of patients in the ES group and to prevent skin burns. These ratios in normal matched participants were obtained in our previous study. This procedure was performed to prevent electrochemical burns in diabetic patients [25]. This sensory threshold was rechecked again at the first session each week. In this study, the active electrode was the negative pole (cathode) of the direct current. Electrical stimulation with CDC was applied to the wound site through the active electrode (carbon rubber electrode, $2 \times 3\ cm^2$) placed near the proximal edge of the ulcer, over intact skin. The passive electrode (positive pole, $3 \times 4\ cm^2$) was placed 20 cm proximal to the cathode electrode and secured with a leg cuff. In the control group, the study protocol was the same as in the ES group, but the intensity current was zero. The stimulator used in the present study was the BTL-5000 series (BTL Industries, Ltd., Staffordshire, United Kingdom), and the parameters were determined based on previous works [25–27]. All patients (ES and control) received similar conventional therapy, including debridement, cleaning of the wound with saline, and dressings.

2.4. Study outcomes

The primary outcome measures were the changes across groups (ES and control) and time-points (pre-intervention and immediately post-intervention at the first and twelfth sessions) to elicit the amount of VEGF, sVEGFR-2, HIF-1α, and NO within wound fluids. The secondary outcome measure was the change in WSA across groups (ES and control) and time-points (at the first, sixth, and twelfth sessions).

At the first and twelfth sessions, after debridement, wound fluid was collected before and immediately after the intervention. For this purpose, the ulcer was covered with a sterile filter paper for 15 min, until wound fluid absorbed through the paper. Each filter paper was incubated for 30 min in 2 mL of phosphate-buffered saline containing antiprotease enzyme. Next, the paper was centrifuged (15 min at 3500 rpm and 4 °C), and the obtained supernatant solution was frozen at −80 °C until use.

Concentrations of VEGF, sVEGFR-2, and HIF-1α in the wound fluids were measured with commercial ELISA kits according to the manufacturer's protocols (human VEGF, sVEGFR-2, and HIF-1α ELISA, Cusabio Biotech Inc., Wuhan, China). The concentration of NO in wound fluids was determined using an NO assay kit (Colorimetric Assay, Zellbio, Ulm, Germany).

The WSA measurement was made after debridement at the first, sixth, and twelfth sessions. In order to measure WSA, a photograph was taken with a digital camera (Casio Exilim EX-H5, Casio Computer Co., Ltd., Japan) with a metric ruler placed next to the ulcer. WSA was calculated using Design CAD software, version 23.0 (IMSI/Design, LLC, Novato, CA, USA). This software allows the user to place the cursor on the edge of the ulcer and to trace a non-geometric outline. Once the tracing is complete, the software calculates the area of the ulcer in cm^2. This method for measuring WSA has been found to yield reliable results [28].

2.5. Sample size and statistical analysis

Using G*Power software, we estimated that 10 patients with DFUs in each group would be needed to detect a difference between groups, based on α of 0.05, β of 0.2, and the VEGF concentration of the previous study [25].

The Shapiro-Wilk test demonstrated that the data were normally distributed in both groups ($P > 0.05$). Test of homogeneity was shown that variances were equal ($P > 0.05$). Baseline demographic data between groups were compared with the t-test and the χ^2 test. Repeated-measure ANOVA was used to assess the changes of VEGF, sVEGFR-2, HIF-1α, NO, and WSA. The pairwise comparison was performed by using Bonferroni test. An independent t-test was used for comparison of outcome measures between groups. The level of significance for all statistical tests was set at $\alpha = 0.05$. Analyses were conducted using SPSS statistical software, version 16.0 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Study population

As shown in Fig. 1, a total of 30 patients with DFU were initially enrolled in the study, and 24 participants completed the trial (ES, $n = 13$; control, $n = 11$). One patient in each group withdrew for medical reasons and hospitalization, while four patients (three in the control group and one in the ES group) opted to leave the study for personal reasons. We conducted the sensitivity analysis to assess the robustness of the conclusions under different assumptions made to account for the missing data. The results of the sensitivity analyses were consistent with the primary analysis. Thus, the results remain robust.

The baseline demographic characteristics were similar in both groups and no significant difference was found between the groups ($P > 0.05$) (Table 1).

3.2. VEGF concentration in wound fluid

There was not a significant effect for VEGF levels ($F = 2.89$, $P = 0.08$, $r = 0.15$) and interaction between VEGF level and group ($F = 2.04$, $P = 0.15$, $r = 0.11$), reflecting similar changes in VEGF levels over time for both the ES and the control groups (Fig. 2(a)).Only a significant between-subjects effect was seen in VEGF levels ($F = 8.56$, $P = 0.009$, $r = 0.35$). An independent t-test showed significantly higher VEGF levels post-intervention at the first and twelfth sessions in the ES group compared to the control group ($t = 3.09$, $P = 0.007$ and $t = 2.67$, $P = 0.019$, respectively). There was no significant difference between the two groups pre-intervention at the first ($t = 2.06$, $P = 0.056$) and twelfth sessions ($t = 1.15$, $P = 0.27$) (Table 2).

3.3. sVEGFR-2 concentration in wound fluid

The results for sVEGFR-2 concentration indicated no significant main effects for sVEGF-2 levels ($F = 0.69$, $P = 0.52$, $r = 0.04$) and interaction between sVEGFR-2 level and group ($F = 0.81$, $P = 0.46$, $r = 0.05$), (Fig. 2(b), Table 2).

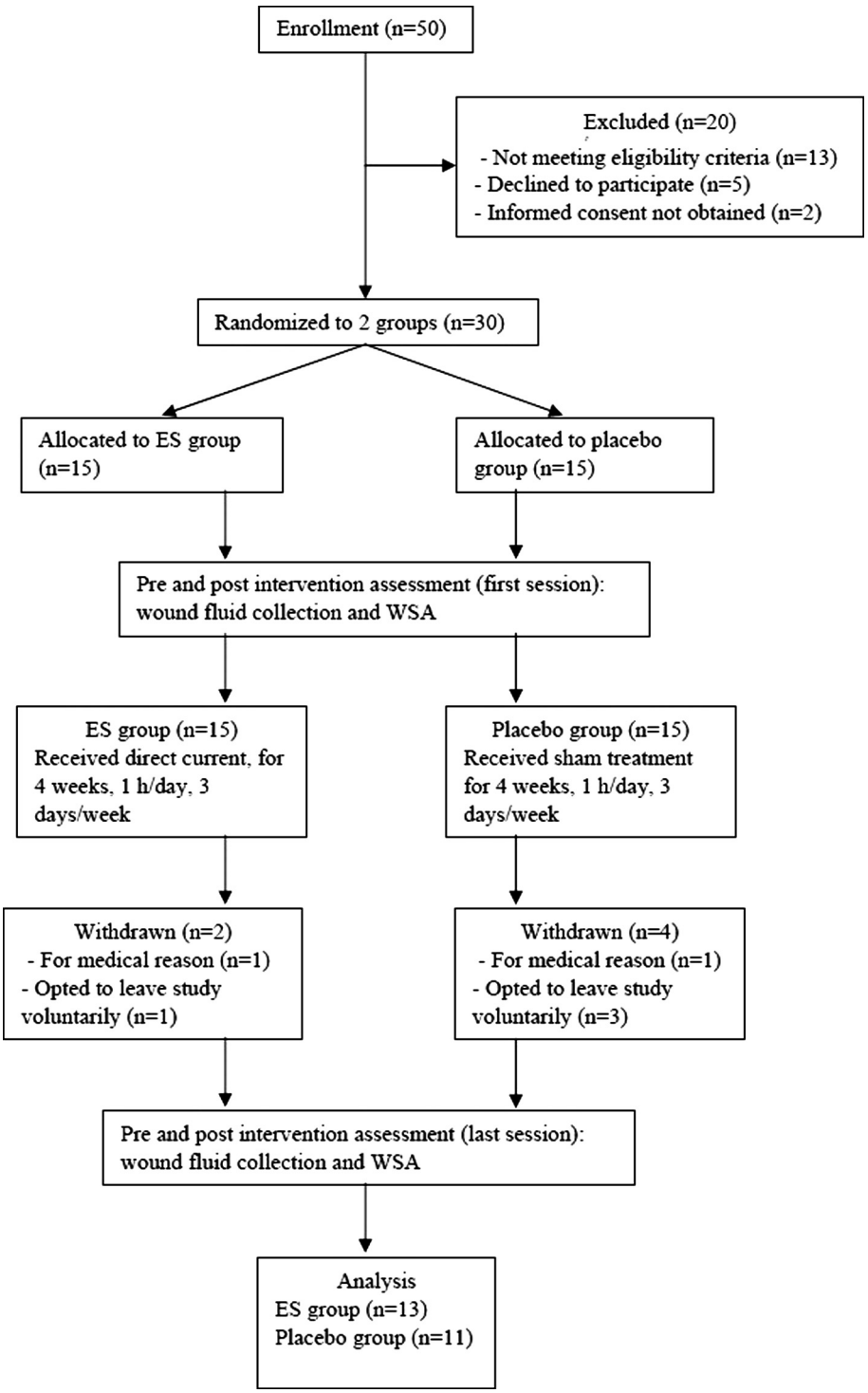

Fig. 1 – Flowchart of the study design.

3.4. *HIF-1α concentration in wound fluid*

The results showed a significant effect for HIF-1α levels ($F = 3.05$, $P = 0.03$, $r = 0.15$). There was a significant interaction effect between HIF-1α level and group ($F = 4.17$, $P = 0.01$, $r = 0.20$), reflecting different changes in HIF-1α levels over time for the ES group compared with the control group (Fig. 2(c)). A pairwise comparison revealed that HIF-1α concentrations increased significantly after ES application at the first and twelfth sessions ($P = 0.009$ and $P = 0.03$, respectively). At first session this increase was significant compared to the control group ($t = 2.71$, $P = 0.01$), (Table 2).

Table 1 – Demographic characteristic of participants.

	Electrical stimulation group ($n = 13$)	Control group ($n = 11$)	P value
Age (year)	60.8 (5.5)	60.1 (6.4)	$P = 0.7$
BMI (kg/m^2)	24.8 (3.2)	23.6 (2.7)	$P = 0.3$
Sex (M/F), n	8 (63%)/5 (37%)	6 (55%)/5 (45%)	$P = 0.1$
Duration of diabetes (year)	9.5 (3.3)	10.3 (2.4)	$P = 0.5$
Duration of DFU (month)	3.3 (1)	2.3 (1.1)	$P = 0.07$
History of DFU (%)	20%	10%	$P = 0.2$
Initial WSA (cm^2)	4.19 (2.2)	3.82 (1.7)	$P = 0.7$
Fasting blood glucose (mg/dL)	137.9 (35.6)	136.6 (31.4)	$P = 0.9$
HbA_{1c} (%)	8.1 (1.1)	7.5 (1)	$P = 0.3$
Creatinine (mg/dL)	1.2 (0.28)	1.1 (.21)	$P = 0.7$
ABI	.88 (0.06)	0.89 (.14)	$P = 0.4$

Data are means (SD), unless otherwise indicated. P values were calculated for the difference among groups using independent t test and χ^2 test. $P < 0.05$ was considered significant. BMI, Body Mass Index. ABI, Ankle-Brachial Index.

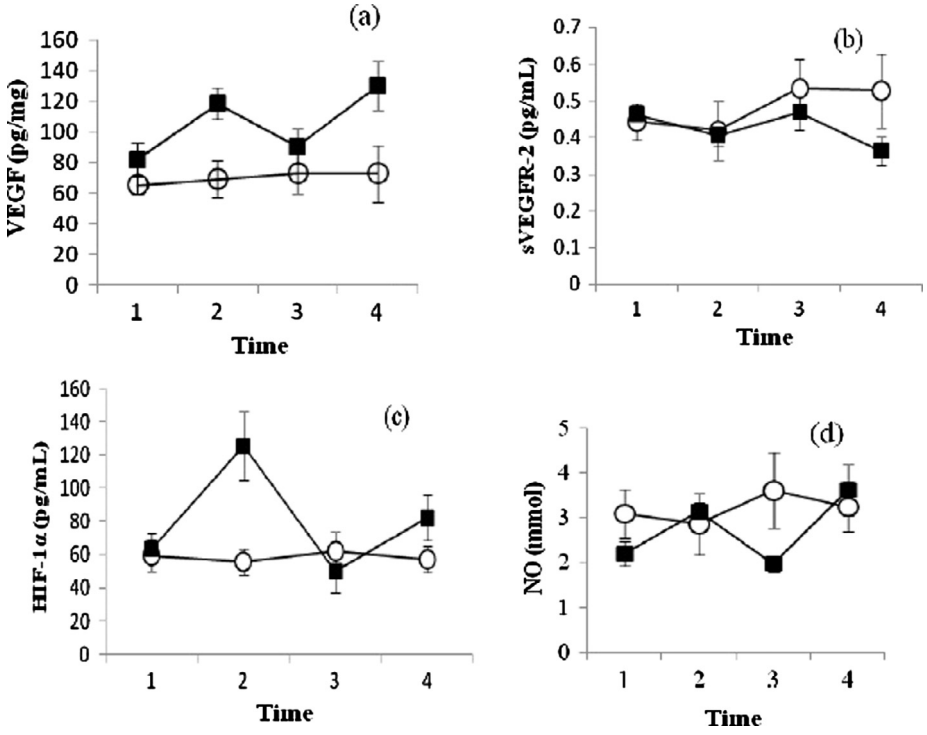

Fig. 2 – (a) VEGF changes over time for the ES and control groups. Interaction between group and time was not significant ($P = 0.15$). (b) sVEGF changes over time for ES and control groups. Interaction between group and time was not significant ($P = 0.4$). (c) HIF-1α changes over time for ES and control groups. Interaction between group and time was significant ($P = 0.01$). (d) NO changes over time for ES and control groups. Interaction between group and time was not significant ($P = 0.1$). ES group: black squares; control group: white circles. (1) First session, before intervention; (2) first session, after intervention; (3) last session, before intervention; (4) last session, after intervention. Values are mean ± SE.

3.5. *NO concentration in wound fluid*

Levels of NO showed an increase after ES application at the first and twelfth sessions, but the effects for NO levels ($F = 0.95$, $P = 0.42$, $r = 0.05$) and the interaction between NO and group ($F = 1.9$, $P = 0.14$, $r = 0.10$) were not significant (Fig. 2(d), Table 2).

3.6. *Wound surface area*

A significant effect was observed for WSA ($F = 55.29$, $P = 0.000$, $r = 0.82$) and for interaction between WSA and group ($F = 11.1$, $P = 0.003$, $r = 0.34$). A pairwise comparison indicated that in both groups, WSA was significantly decreased from the first to the sixth sessions ($P = 0.000$) and from the sixth to the

Table 2 – Descriptive data of primary outcome measures in both groups at each time point.

	First session		Last session	
	Pre intervention	Post intervention	Pre intervention	Post intervention
VEGF (pg/mL)				
ES group ($n = 13$)	81.93 (19.89)	118.7 (39.09)	90.59 (48.6)	130.16 (65.8)
Control group ($n = 11$)	65.17 (13.14)	69.32 (26.26)	73.07 (11.63)	73.22 (21.9)
P value	0.056	0.009	0.2	0.019
VEGFR-2 (pg/mL)				
ES group ($n = 13$)	0.46 (0.12)	0.40 (0.13)	0.46 (0.19)	0.36 (0.15)
Control group ($n = 11$)	0.44 (0.13)	0.41 (0.22)	0.53 (0.24)	0.52 (0.36)
P value	0.77	0.8	0.52	0.19
HIF-1α (pg/mL)				
ES group ($n = 13$)	63.65 (31.65)	125.63 (24.47)	50.08 (42.98)	82.24 (46.09)
Control group ($n = 11$)	59.67 (26.74)	55.82 (22.24)	62.2 (32.1)	57.32 (22.45)
P value	0.7	0.019	0.51	0.14
NO (μM)				
ES group ($n = 13$)	2.18 (0.91)	3.13 (0.7)	1.97 (0.58)	3.61 (1.95)
Control group ($n = 11$)	3.09 (1.57)	2.86 (1.96)	3.61 (2.41)	3.24 (1.59)
P value	0.17	0.7	0.09	0.6

Data are means (SD). *P* value refers to between-group differences in each time point by independent t test. $P < 0.05$ was considered significant.

twelfth sessions ($P = 0.000$). Although the results did not indicate a statistically significant effect on WSA between the two groups ($F = 0.05$ and $F = 0.81$, respectively), the percentage decrease of WSA (percentage decrease compared to WSA at the first session, Fig. 3) at the twelfth session was significantly higher in the ES group than in the control group (59.49% versus 27.07%, $P = 0.02$).

4. Discussion

In this study, we showed that ES can effectively enhance the release of HIF-1α in the wound fluid of ischemic DFUs. WSA in both the ES and the control groups significantly decreased by the sixth and twelfth sessions compared to the first and sixth sessions, respectively. This decrease may be due to the similar conventional therapy used in both groups, including debridement, cleaning of the wound with saline, and dressings, whereas the percentage decrease in WSA at the twelfth session was significantly greater in the ES group compared to the control group.

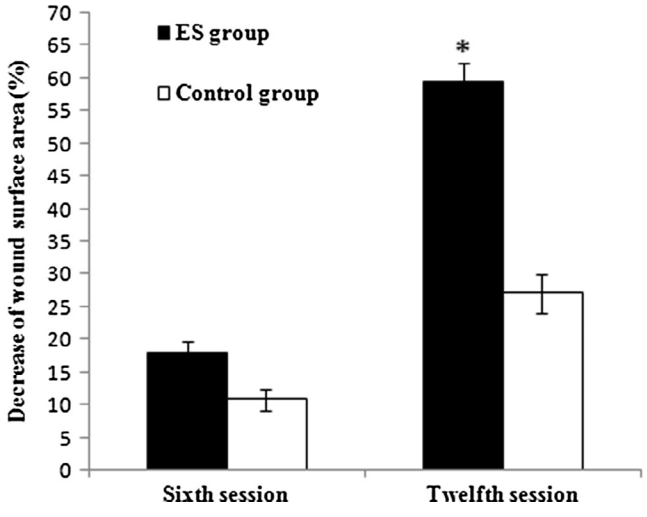

Fig. 3 – Percentage decrease of WSA at the sixth and twelfth sessions in the ES and control groups. *Significant difference compared with control group at the twelfth session.

Previous evidence showed that ES stimulates an angiogenic effect by increasing the expression of VEGF and FGF-2 [24–27,29–32]. Mohajeri and colleagues [25] investigated the effect of cathodal current with sensory intensity on the release of VEGF and NO in patients with DFU. They reported a significant enhancement of plasma levels of VEGF and NO following the application of ES. Bevilacqua et al. [24] showed a significant enhancement in plasma concentrations of VEGF in both non-diabetic and diabetic subjects during the application of ES; however, these subjects did not have DFUs. Sebastian et al. [30] and Ud-Din et al. [33] applied ES to cutaneous wounds caused by punch biopsy in healthy volunteers. They showed significant upregulation of VEGF in cutaneous wounds receiving ES. The present study appears to be the first to show the release of angiogenic factors, such as VEGF, sVEGFR-2, HIF-1α, and NO, in the wound fluid of DFUs following the application of ES. The obtained results, including the significant upregulation in HIF-1α expression in the wound fluid observed immediately after one hour of ES, may suggest probable enhancement of wound healing after application of ES. Immediately after ES application at first and twelfth sessions, VEGF expression was increased compared to control group. This increase may be having clinical significance.

In the wound healing process, HIF-1α directly regulates the expression of VEGF, NO, and other hypoxia-response genes [16]. There is evidence that expression of HIF-1α, VEGF, and NO is impaired in wound sites in diabetic animal models [11–15,17,19]. Liu et al. [34] have suggested that impaired expression of HIF-1α plays an important pathogenic role in the impaired expression of angiogenic factors, such as VEGF, FGF-2, and NO, in diabetic animal wounds. These researchers have demonstrated that correction of HIF-1α expression in

diabetic mice induced increased VEGF expression and improved angiogenesis and wound healing. Mace et al. [18] demonstrated that sustained expression of HIF-1α in diabetic animal wounds can restore HIF-1α function, reactivate HIF-1α target genes, promote angiogenesis, and accelerate cutaneous healing. According to the present study, expression of HIF-1α and VEGF is substantially increased in the wound fluids of DFUs immediately after one hour of ES application. Although not significant, a slight rise in NO expression in wound fluids was observed following application of ES. Interestingly, as seen in Fig. 2, there were similar trends for HIF-1α, VEGF, and NO following the application of ES. Therefore, it is speculated that increased HIF-1α immediately after ES application may target some signaling pathways to increase angiogenic gene expression, such as VEGF, in DFUs.

ES was demonstrated to increase the proliferation and migration of fibroblasts, keratinocytes, endothelial cells, and macrophages as main sources of expression of angiogenic factors [35] into wound sites [20,36,37]. It has been previously reported that ES leads to the release of VEGF by a direct effect on endothelial cells [31]. Also, Roubhia et al. [37] and Wang et al. [38] reported that ES increased the expression of FGF-2 and TGF by human skin fibroblasts.

The levels of circulating growth factors may increase in patients with DFUs [39]. In addition, it is proposed that ES influences skin perfusion via vasodilation of the skin [40,41]. Thus, it is possible that low-intensity ES is able to enhance skin perfusion around ulcers and may therefore assist in the mobilization of circulating growth factors, such as HIF-1α, VEGF, and NO, toward the wound site. However, the exact mechanism of elevation of growth factors by ES is not fully understood.

In this study, we observed that ES significantly increased the percentage decrease in WSA at the twelfth session compared to the control group. Our results reinforced previous findings on the beneficial effect of ES in promoting wound closure in patients with DFUs. Yarboro and Smith [42], in a case study, reported that the application of transcutaneous electrical nerve stimulation to ischemic DFUs induced complete healing of wounds after 12 weeks. Petrofsky et al. [40–42] reported that the healing rate in DFU subjects increased using a biphasic pulsed current for four weeks. Baker et al. [43] also observed an enhanced healing rate in DFU patients following the application of ES (with asymmetrical biphasic current) for four weeks. Mohajeri-Tehrani et al. [25] showed that DFU patients who received CDC for 12 sessions had faster healing of ulcers than did the placebo group. One key difference between our study and the others is that all of our participants had the same wound etiology, ischemic DFU. Certainly, different DFU etiologies may affect the therapeutic effects of ES, the interpretation of results, and the generalizability to DFU patients [44].

It was proposed that a low-intensity direct current, mimicking the natural electrical field that is created following an injury, may promote wound healing in chronic wounds such as DFUs by restoring or enhancing the endogenous current of the injury [45]. At the cellular level, diabetic wounds display less migration and proliferation of fibroblasts, keratinocytes, and endothelial cells, as well as decreased collagen synthesis and delayed re-epithelialization [6]. Thus, it appears that ES used within the parameters of this study, by restoring or enhancing the endogenous current at the injury site [45] and the galvanotaxis effects [20], can affect the migration and proliferation of these cells and therefore promote the healing of DFUs. In addition, our results demonstrated that the amounts of HIF-1α and VEGF within wound fluids were significantly higher in patients receiving ES. However, further investigations are required to determine the related cellular and molecular signaling pathways that trigger the angiogenesis and healing processes following the application of ES.

There are some limitations to this study that need to be addressed in future research. First, the skin blood flow was not directly measured in this study, and we suggest that measurement of skin blood flow and/or transcutaneous oxygen pressure should be performed in future experiments. Second, measuring angiogenic factors in both plasma and wound fluids may provide more detailed information about the effects of ES. In addition, our study was conducted with a small sample size, and additional subjects would improve the statistical power of our results.

5. Conclusion

According to the results of the present study, application of ES has an immediate positive effect on the release of HIF-1α and VEGF in wound fluids and on the reduction of WSA in ischemic DFUs. Furthermore, the results suggest that applying ES to ischemic DFUs could be a promising way to promote angiogenesis and to achieve better outcomes. However, more studies are needed to identify the related cellular and molecular signaling pathways that trigger angiogenesis and the healing process following the application of ES.

Conflict of interest

None of the authors has any conflicts of interest to declare.

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