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Effect of High Voltage Pulsed Current on the integration of total skin grafts in rats submitted to nicotine action

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ABSTRACT

Objective: The aim of this study was to evaluate the impact of High Voltage Pulsed Current (HVPC) on the integration of total skin grafts in rats submitted to nicotine action.
Materials and methods: For this purpose, 60 adult Wistar rats randomly distributed in 6 groups of 10 animals were analyzed. The electrical stimulation (anodic and cathodic stimulation, motor level, 30 min at 10 Hz; minimum voltage 20 μs and 100 μs pulse interval) was applied for seven days, starting on the third day after surgery and after the dressing was removed from the graft.
Results: Anodic HVPC promoted greater graft integration, demonstrating a lower percentage of tissue contraction, a lower number of inflammatory infiltrates and a greater amount of vascular endothelial growth factor (VEGF), as well as a higher number of newly formed blood vessels.
Conclusions: HVPC can positively influence the integration of skin grafts in nicotine-treated rats. anodic HVPC is shown to promote greater integration in relation to a lower percentage of tissue contraction, a lower number of inflammatory infiltrates and a greater amount of vascular endothelial growth factor and newformed blood vessels. Whereas, the cathodic polarity has presented smaller amount of tissue gap.

1. Introduction

Skin grafting is an important operative procedure in plastic surgery and is also considered a valuable weapon in the arsenal of reconstructive surgery. It is indicated in cases of reconstruction from trauma or wounds, tumour removal, burns and infections [1,2].

Wound healing is a complex pathophysiological process, and chronic lesions do not respond well to an isolated treatment. Nicotine released from the smoke causes a limited vasoactive effect on the skin and subcutaneous tissue, which clearly reduces tissue blood flow thus compromising aerobic metabolism. Furthermore, it is recognised as the main cause of postoperative wound complications in smokers, which may interfere with the synthesis of collagen and prejudice the normal healing process [3–5].

The electrical stimulation can interfere with the healing of wounds, directing cell migration during the process [5], reducing ischemia [6] and increasing microcirculation. It can be an effective and safe complement and, as a consequence, potentiates repair processes as well as increases the viability of flaps [7]. The application of High Voltage Pulsed Current (HVPC) in the treatment of different cutaneous lesions has been investigated with positive results in regeneration [8–12]. However, the influence of this resource on grafts under the influence of nicotine has not yet been established, and its role in healing in smoking patients is also unknown. In view of the large number of smokers, the importance of a study that evaluates the effect of the resource as an alternative to minimise the deleterious effects of nicotine in the integration of the total skin graft is justified.

2. Methods

Sixty male adult rats weighing between 280 and 310 g (Wistar), by the Central Biotherium of the Medical School of Ribeirão Preto, University of São Paulo (FMRP-USP), were evaluated. The experimental procedures were performed in the laboratory of the Postgraduate Program in Rehabilitation and Functional Performance. The animals were kept in a biotherium, with a light-dark cycle of 12 h, and housed

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in individual cages where they received commercial feed and water *ad libitum*.

The animals were weighed and randomly assigned to 6 groups of 10 animals:

Sham: animals submitted to the surgical procedure of skin grafting, following the simulated application of the HVPC.

HVPC-Anodic: animals submitted to the surgical procedure of skin grafting, and later submitted to anodic HVPC.

HVPC-Cathodic: animals submitted to the surgical procedure of skin graft, and later submitted to cathodic HVPC.

Nicotine: animals submitted to nicotine injection into the subcutaneous tissue at a dose of 2 mg/kg, twice a day for a period of 28 days until the surgical procedure, and thereafter submitted to the graft surgical procedure of skin.

Nicotine/HVPC-Anodic: animals submitted to nicotine injection and subsequently submitted to the graft of skin. Then, anodic HVPC was applied.

Nicotine/HVPC-Cathodic: animals submitted to nicotine injection and subsequently submitted to the skin graft. Then, cathodic HVPC was applied.

2.1. Nicotine

The nicotine used in the experiment (nicotine sulphate, L-1-methyl-2-(3-pyridyl)-pyrrolidine sulphate, grade II, PM 422-6; Sigma) was diluted with saline solution to the concentration of 1 mg/ml [13], and injected into the subcutaneous tissue of the rats for 28 days until the surgical procedure.

2.2. Surgical procedure

The grafts were delineated with dimensions 2 cm long by 2 cm wide performed on the back of each animal [14]. The graft arrangement was established between the scapulae, below the lower angle of the scapula and 3 cm below the first graft (Fig. 1).

In order to perform the grafting surgery, the animals were anesthetised with intraperitoneal injection of ketamine hydrochloride (Agener União®, Ribeirão Preto, SP, Brazil) at 0.1 mL per 100 g, and associated with xylazine hydrochloride (Dopaser®, Marília, SP, Brazil) at 0.07 mL per 100 g, according to the criteria established in a previous study [15]. The animals were immobilised on surgical boards for manual traction depilation with 70% alcohol antisepsis. The surgical sites in the dorsal region of the animal, located below the inferior angles of the scapulae, and the other 3 cm below the scapula [14] were demarcated by a stainless-steel mould (2 × 2 cm), which is specially made for standardisation of grafts. Subsequently, the skin was resected to the limit of the muscular fascia with scalpel blade [15]. The application of the grafts in the recipient areas was carried out by transposing the skins of the respective areas. The fixation of the graft was done with a single point of 6.0 nylon thread in the corners and 5.0 along the edges for better fixation of the dressing.

After the surgical procedure, a blade of rayon-like tissue (Curatec®, São Paulo, SP, Brazil) and four layers of gauze were applied to the graft, followed by tying of the 5.0 gauge threads on the dressings (brown dressing or type tie-over) and removal on the third postoperative day. Then, the animals were identified and allocated in individual cages. After skin grafting surgery, the animals received dipyrone (Biovet®, São Paulo, SP, Brazil) as analgesic at 1 drop per 150 ml.

2.3. High Voltage Pulsed Current (HVPC)

HVPC was performed with the Neurodyn High Volt equipment (ANVISA 10360310008, IBRAMED®, Amparo, SP, Brazil). The application of HVPC was started on the third day after surgery, when the dressing was removed from the graft, sessions were performed with anodic and cathodic stimulation, motor level, 10 Hz, minimum voltage to produce contraction was 20 μs and 100 μs pulse interval. Daily sessions of 30 min each were performed and repeated for seven

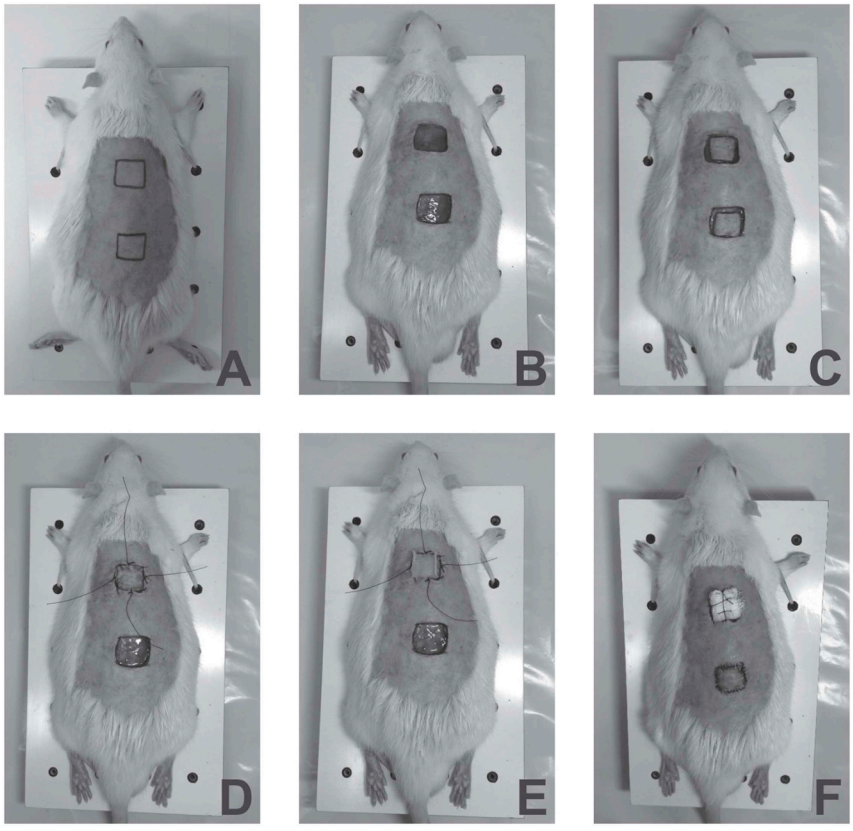

Fig. 1. (A) Demarcation of the grafts. (B) Removal of cutaneous segments. (C) Inversion of cutaneous segments. (D) Fixation of the graft with simple points at the vertices and between their points, with leftover nylon rope for lashing. (E) Rayon® embedded in physiological solution. (F) Brown dressing or tie-over type in the cranial graft, while the caudal graft was sutured with continuous stitches.

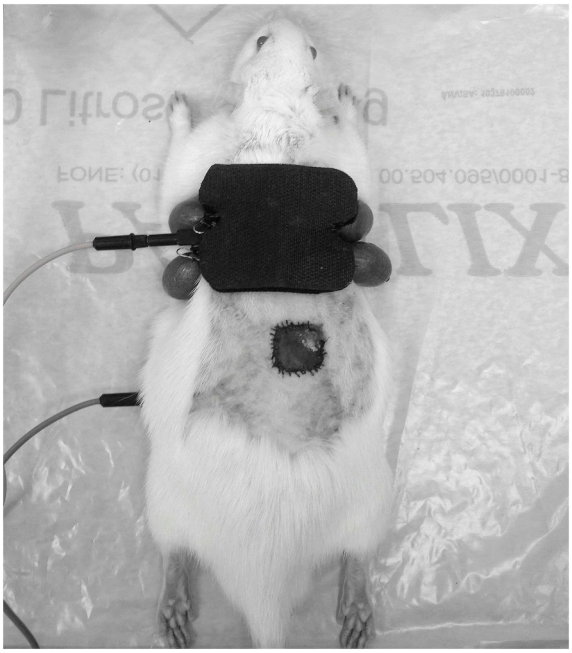

Fig. 2. Application of HVPC performed with anodic and cathodic stimulation, motor level, 10 Hz, minimum voltage to produce contraction was 20 μs and 100 μs pulse interval.

consecutive days, always at the same time to observe the 24-h interval between sessions [14,16,17].

The therapeutic intention was applied with an active aluminium electrode (2 × 2 cm) positioned on a graft (cranial graft), and the dispersive (4 × 4 cm) positioned in the ventral region of the animal. The dispersive electrode was duly coupled with a sponge soaked in water, and the active electrode, coupled with gauze, soaked in sterile saline solution (Fig. 2).

2.4. Histological and immunohistochemistry

The percentage of graft contraction was analysed by ImageJ® software (US National Institutes of Health, Bethesda, Maryland, USA) on day 3 and on day 10 after surgery. The values of the animals were evaluated with the initial perimeter corresponding to the values after the surgery (3rd day) and final perimeter on the 10th day after surgery.

$$\text{Percentage of tissue contraction (\%)} = \frac{\text{Initial area} - \text{Final perimeter} \times 100}{\text{Initial area}}$$

Graft sample was collected for histological analysis, submitted in 10% formol fixative medium. The procedures performed to prepare the samples involved fixation, dehydration, diaphanisation, imbibition and inclusion or embedding. At fixation, the skin segments were fixed in 10% formalin buffer solution for 48 h. Dehydration consisted the passage of the piece in increasing concentrations of ethyl alcohol (50, 70, 90 and 100%) in order to eliminate all contained water from the material. The diaphanisation process consisted three xylene exchanges to remove the alcohol from the previous stage and preparation of the part for the immersion in liquid paraffin. In the soaking, the piece was placed in a container with liquid paraffin at a temperature of 56 °C. Three exchanges were made so that the paraffin would infiltrate throughout the interior of the part and in the inclusion or embedding, which was carried out after imbibition with the material included in liquid paraffin. Then, the piece was sent to microtomy and non-serial cuts with 5–6 μm thickness were stained with haematoxylin and eosin to quantify inflammatory infiltrates and gap area.

Immunohistochemistry reactions were performed on histological sections of the paraffin wax material by means of the antigen-antibody reaction, followed by the marker reaction visible under the microscope. The dewaxed and hydrated slides underwent an antigen retrieval process by incubation in a steam pan in buffered medium for 40 min. After cooling the material, the endogenous tissue peroxidases were removed by the addition of hydrogen peroxide, and non-specific binding of the primary antibody was avoided by the addition of horse serum. The slides were then incubated with primary antibody to VEGF and factor VIII for 12 h (overnight) in a humid chamber. Next day, the slides were incubated with secondary antibody for 30 min, followed by the conjugated polymer also for 30 min, and then stained with DAB for 1 min. All reagents were from the PicTure™ MAX Polymer Kit (ZYMED® Laboratories). The slides were counterstained with Harris haematoxylin for 1 min and mounted with Entellan for further light microscopy analysis.

2.5. Statistical analysis

For the statistical analysis of graft contraction, epidermal thickness, thickness of inflammatory infiltrates, gap area, VEGF and factor VIII were treated with analysis of variance (ANOVA) and Duncan's post-test. Data processing was performed using the SAS software (Cary, NC), version 9.3, and the critical level was set at 5% ($p < 0.05$).

3. Results

The percentage of graft contraction with values of $F_{5.54} = 4.284$ and $p < 0.005$ was significantly different ($p < 0.05$) in group 4 when comparing with groups 1, 2, 3, 5 and 6, which all presented a higher percentage of contraction of the graft area (Table 1).

Data on the thickness of the inflammatory infiltrates of the graft between the groups had values of $F_{5.54} = 4.284$ and $p < 0.005$. A significant difference ($p < 0.05$) was observed among groups 1, 5 and 6 when compared to groups 2, 3 and 4, and groups 2 and 3 when compared to groups 1, 4, 5 and 6, as well as in group 4 when compared to all other groups (Table 2).

Data on the thickness of the GAP (space between the transplanted tissue and the recipient bed) had values of $F_{5.54} = 4.284$ and $p < 0.005$. A significant difference ($p < 0.05$) was observed among groups 1, 2 and 3 when compared to groups 4, 5 and 6, group 4 when compared to the other groups, and groups 5 and 6 when compared to groups 1, 2, 3 and 4 (Table 3).

The vascular endothelial growth factor (VEGF), with a value of $F_{5.54} = 4.284$ and $p < 0.005$ was significantly different ($p < 0.05$) between group 4 and all other groups (Table 4).

The graft factor VIII had values of $F_{5.54} = 4.284$ and $p < 0.005$. A significant difference ($p < 0.05$) was observed among groups 1 and 2 when compared to groups 3, 4, 5 and 6; group 3 when compared to groups 2, 4, 5 and 6; group 4 when compared to groups 3, 4 and 5; group 5 when compared to groups 1, 2 and 4; groups 5 and 6 when compared to groups 1, 2 and 3; and finally group 6 when compared to groups 1, 2 and 3 (Table 5).

Table 1
Mean percentages and standard deviations of graft contraction in the groups studied (%). SD, standard deviation. *p ≤ 0.05 versus groups 1, 2, 3, 5 and 6.

	Groups	Mean ± SD
1	Sham	11.1 ± 5.52
2	HVPC-Anodic	8.90 ± 3.67
3	HVPC-Cathodic	9.20 ± 4.27
4	Nicotine	16.6 ± 5.09*
5	Nicotine/HVPC-Anodic	10.6 ± 3.10
6	Nicotine/HVPC-Cathodic	12.6 ± 3.73

Table 2
Mean values of the thickness of the inflammatory infiltrates and standard deviations of the graft in the studied groups (μm). SD – Standard Deviation. #p ≤ 0.05 vs 2, 3 e 4. βp ≤ 0.05 vs 1, 4, 5 e 6. £p ≤ 0.05 vs 1, 2, 3, 5 e 6.

	Groups	Mean ± SD
1	Sham	115,5 ± 8,9$^{\#}$
2	HVPC-Anodic	89,1 ± 9,03$^{\beta}$
3	HVPC-Cathodic	94,7 ± 8,7$^{\beta}$
4	Nicotine	140,7 ± 13,65$^{£}$
5	Nicotine/HVPC-Anodic	121,5 ± 14,44$^{\#}$
6	Nicotine/HVPC-Cathodic	127,5 ± 13,11$^{\#}$

Table 3
Mean values of the GAP area and graft standard deviations in the studied groups (μm). SD – Standard Deviation. #p ≤ 0.05 vs 4, 5 e 6. βp ≤ 0.05 vs 1, 2, 3, 5 e 6. £p ≤ 0.05 vs 1, 2, 3 e 4.

	Groups	Mean ± SD
1	Sham	5,3 ± 0,7$^{\#}$
2	HVPC-Anodic	3,0 ± 0,93$^{\#}$
3	HVPC-Cathodic	4,4 ± 2,17$^{\#}$
4	Nicotine	20,9 ± 7,88$^{£}$
5	Nicotine/HVPC-Anodic	13,9 ± 8,06$^{\beta}$
6	Nicotine/HVPC-Cathodic	12,6 ± 5,23$^{\beta}$

Table 4
Mean values and standard deviations of the amount of vascular endothelial growth factor (VEGF) of the grafts, in the groups studied. SD – Standard Deviation. #p ≤ 0.05 vs 1,2, 3, 5 e 6.

	Groups	Mean ± SD
1	Sham	1,5 ± 0,40
2	HVPC-Anodic	2,7 ± 1,08
3	HVPC-Cathodic	2,3 ± 1,69
4	Nicotine	0,6 ± 0,25$^{\#}$
5	Nicotine/HVPC-Anodic	2,0 ± 1,29
6	Nicotine/HVPC-Cathodic	1,4 ± 0,45

Table 5
Mean values for factor VIII and graft standard deviations in the studied groups (μm). SD – Standard Deviation. #p ≤ 0.05 vs 3, 4, 5 e 6. βp ≤ 0.05 vs 2, 4, 5 e 6. £p ≤ 0.05 vs 1, 2, 3 e 5. *p ≤ 0.05 vs 1, 2 e 4. Ωp ≤ 0.05 vs 1, 2 e 3.

	Groups	Mean ± SD
1	Sham	4,1 ± 1,25$^{\#}$
2	HVPC-Anodic	5,2 ± 1,70$^{\#}$
3	HVPC-Cathodic	3,7 ± 0,78$^{\beta}$
4	Nicotine	0,6 ± 0,51$^{£}$
5	Nicotine/HVPC-Anodic	2,4 ± 0,74*
6	Nicotine/HVPC-Cathodic	1,7 ± 1,32$^{\Omega}$

4. Discussion

Smoking is described as damaging the repair processes and promoting inhibition of endothelial cell function as well as fibroblasts and vascular endothelial growth. Additionally, it compromises peripheral circulation and reduces wound contraction [18–21]. Thus, it is relevant to search for therapeutic interventions that may minimise the deleterious effects of nicotine in tissues, which in the present study occurred through the evaluation of the effects of HVPC on the integration of total skin grafts in rats submitted to the action of nicotine.

Electrical stimulation is a widely used resource in the management of difficult-to-repair wounds and is considered an efficient therapeutic intervention, which is reflected in the reduction of treatment time and promoting important socioeconomic impact [9,16,22,23]. The intervention by means of HVPC deserves to be highlighted [24,25]. The viability of experimental flaps affected by exposure to nicotine has been studied, demonstrating the deleterious influence of the substance [25–27].

The study evaluates the effectiveness of a high voltage current to reduce the level of pain, improve the quality and the process of wound healing, processes that involve the circulatory increment necessary for the viability of the graft. Clinical and experimental studies present positive results from the application of HVPC with variation from 30 to 60 min [8–12,28,29].

Among the expected complications related to cutaneous grafts, lack of integration to the recipient bed is highlighted, followed by the dreaded excessive contraction and in the presence of infection, the contraction is almost half the initial area. Additionally, in its absence, graft/receptor bed contracts in one-third of the initial area. Thus, partial thickness grafting may lead to skin necrosis and fibrosis [30,31].

In the present study, the percentage of tissue contraction of the grafts was higher in the group of animals that were exposed to nicotine and did not receive stimulation, with a mean percentage of contraction of 16.6%. This is similar to the result found previously [32] where the authors observed 12.04% contraction of the total thickness graft. It is likely that the findings were found by the fact that autologous skin grafts are fragile and can contract significantly during the healing process, especially when they are altered due to the use of substance that can cause insufficient blood supply, resulting in degeneration and necrosis [33].

The group exclusively submitted to nicotine action presented a higher rate of inflammatory infiltrate, possibly due to the deleterious influence of the substance. The presence of inflammatory infiltrate in the graft, regardless of its location, is related to the reduction of the survival of the graft [34]. The response to this variable was greater in the stimulated and nicotine groups, in relation to the groups that were only stimulated, and when compared to the group that received only the nicotine. It is likely that this is due to nicotine, because it promotes peripheral vasoconstriction and decreases the blood flow that nourishes the skin, resulting in ischemia and consequently making tissue repair difficult [35]. In addition, electrical stimulation can accelerate the resolution of the inflammatory process, advancing to the stages of remodelling [36].

The animals stimulated with HVPC with positive polarity presented a lower rate of inflammatory infiltrates when compared to sham groups and other groups under the action of nicotine. One hypothesis would be the cascade of events during and after the inflammatory and proliferative healing process [37]. Responses inherent to polarity are not yet fully substantiated, since both polarities may interfere with the wound healing [38].

The graft integration rate in the present study was observed through the evaluation of the gap thickness (space between the transplanted tissue and the recipient bed). The isolated nicotine group achieved a larger area, which corresponds to lower integration rate of the graft, a probable consequence of the action of the substance on the tissues involved [39]. The groups stimulated with positive or negative polarity and previously submitted to nicotine presented lower values when compared to nicotine alone. These results may suggest that despite the presence of nicotine, the use of the therapeutic resource may have minimised the formation of gaps in tissue integration. The groups that did not receive nicotine interference and were only stimulated with high voltage with positive or negative polarity, presented lower values of the gap area when compared to other groups. This may infer how harmful the effects of the drug in tissue integration are and suggests that this may have occurred because the resource influences the viability and repair of the skin grafts.

The VEGF is related to angiogenesis and plays an important role in the tissue repair process and survival of flaps [40]. In addition, the effect of VEGF on the expression of VEGF in the presence or absence of VEGF is not known. It is likely that the influence of the therapeutic resource applied is related to the response found. Angiogenesis may be

associated with the effects of electrical current on endothelial tissue, which has been shown to induce further release of chemical mediators that stimulate cell proliferation [41].

The inflammatory phase begins immediately after injury, with vasodilation and increased vascular permeability that promotes chemotaxis. In the present study, the quantification of blood vessels was performed using the immunohistochemical technique and anti-factor VIII antibody, also called the von Willebrand factor. Factor VIII (FVIII) is an essential blood coagulation protein and a key component of the fluid phase blood coagulation system [42], being highly specific for vascular tissue and used as a marker of vascularisation in some studies. This study opens a wide range of possibilities for the use of HVPC in clinical practice, however, it still needs to be further explored for current modulation in order to increase the integrity of skin grafts in tissues that have the presence of nicotine.

Additionally, Rosinczuk et al. [43], suggest that the development of therapies based on the use of mechanical forces, or of bandages with appropriate mechanical properties, prevents improper scarring. In addition, knowledge of the mechanisms of mechanical signal transduction and its involvement in the activation of certain genes opens up new ways for combination therapies that use mechanical and drug therapy.

The limitation of the study is the lack of analysis of interleukins and other cytokines. The evaluation of growth factors and cytokines could provide additional responses regarding electrical stimulation in wound healing, since changes in the pattern of growth factor expression are associated with poor healing.

Additional studies are needed to improve the use of the electoral stimulation with other parameters. Thus, new pre-clinical and clinical trials are needed to translate these effects to humans.

5. Conclusion

Considering the findings of this study, it can be concluded that HVPC can positively influence the integration of the skin graft in rats submitted to nicotine. Specifically, anodic HVPC is shown to promote greater integration in relation to a lower percentage of tissue contraction, a lower number of inflammatory infiltrates and a greater amount of vascular endothelial growth factor and newformed blood vessels. Whereas, the cathodic polarity has presented smaller amount of tissue gap.

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