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ORIGINAL RESEARCH

Effect of high-voltage electrical stimulation and topical insulin on experimental cutaneous lesions

Efeito da estimulação elétrica de alta voltagem e insulina tópica em lesão cutânea experimental

Resultados de la estimulación eléctrica por alta voltaje e insulina tópica en lesión cutánea experimental

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ABSTRACT | This study aims to evaluate the effect of cathodic high voltage electrical stimulation (HVES), associated with topical insulin, on rat integumentary lesions. For this purpose, 42 Wistar rats (240±30 g) were submitted to surgical removal of 1 cm^2 of dorsal skin and divided into six groups (n=7), treated for seven consecutive days: Control (C), placebo electrical stimulation (PES), cathodic electrical stimulation (ES), topical insulin (TI), placebo insulin (PI) and HVES associated with topical insulin (ES+I). HVES was administered 24 hours after surgery, 30 minutes per day, with a frequency of 100 Hz and a mean voltage of 60 V, maintained at the motor threshold. Lesion areas were recorded macroscopically on the first, fourth and eighth day, submitted to histological treatment for inclusion in paraplast® and staining in Hematoxylin and Eosin. Epithelialization and the numerical profile of the cells were obtained by histometric analysis. The Shapiro-Wilk and Anova one-way test was followed by the Bonferroni ($p<0.05$). There was a significant reduction in the area of the lesion by the eighth day of treatment, both in the ES and ES+I groups, when compared with other groups. Reepithelialization did not differ between groups, but the distance between the edges of the lesion was lower in the ES and ES+I groups. These same groups showed a significant increase ($p<0.05$) in the number of fibroblasts and a decrease in leukocytes. Thus, we can conclude that cathodic HVES accelerated the lesion repair process, with the topical application of insulin showing no additional effect.

Keywords | Electric Stimulation; Insulin; Rats Wistar/injuries.

RESUMO | O objetivo deste estudo foi avaliar o efeito da estimulação elétrica de alta voltagem (EEAV) catódica, associada à insulina tópica, em lesão tegumentar de ratos. Para tanto, foram utilizados 42 ratos *Wistar* (240±30 g), submetidos a retirada cirúrgica de 1 cm^2 de pele do dorso em 6 grupos (n=7), tratados por 7 dias consecutivos: Controle (C), estimulação elétrica placebo (EP), estimulação elétrica catódica (EE), insulina tópica (IT), insulina placebo (IP) e EEAV associada a insulina tópica (EE+I). A EEAV foi administrada 24 horas após a cirurgia, 30 minutos por dia, com frequência de 100 Hz e voltagem média de 60 V, mantida no limiar motor. Áreas das lesões foram registradas macroscopicamente no primeiro, quarto e oitavo dia, sendo submetidas a tratamento histológico para inclusão em paraplast® e coloração em Hematoxilina e Eosina. A epitelização e o perfil numérico das células foram obtidos por análises histométricas. Utilizou-se o teste de Shapiro-Wilk e Anova one way seguida de Bonferroni ($p<0,05$). Observou-se redução significativa na área da lesão no 8º dia de tratamento, nos grupos EE e EE+I em relação aos demais grupos. A reepitelização não diferiu entre os grupos, mas a distância entre as bordas da lesão foi menor nos grupos EE e EE+I. Nestes mesmos grupos houve aumento significativo ($p<0,05$) no número de fibroblastos e diminuição de leucócitos. Pode-se concluir que a EEAV catódica acelerou o processo de reparação da lesão, não demonstrando efeito adicional com a aplicação da insulina tópica.

Descritores | Estimulação Elétrica; Insulina; Ratos Wistar/lesões.

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RESUMEN | El propósito de este estudio fue evaluar el resultado de la estimulación eléctrica por alta voltaje (EEAV) catódica, asociada a la insulina tópica, en lesión cutánea de ratas. Para ello, se utilizaron 42 ratas Wistar (240±30 g), les sometieron a cirugía de retirada de 1 cm^2 de piel del dorso en 6 grupos (n=7), y les trataron por siete días consecutivos: control (C), estimulación eléctrica placebo (EP), estimulación eléctrica catódica (EE), insulina tópica (IT), insulina placebo (IP) y EEAV asociada a la insulina tópica (EE+I). Se aplicó la EEAV 24 horas después de la cirugía, 30 minutos por día, con frecuencia de 100 Hz y voltaje de media tensión de 60 V, y la mantuvo en el umbral motor. Se registraron las áreas de las lesiones macroscópicamente en el primer, cuarto y octavo día, y las sometieron al tratamiento histológico para inclusión en paraplast® y tinción hematoxilina-eosina. Se obtuvo la epitelización y el perfil numérico de las células por análisis histométricos. Se empleó la prueba Shapiro-Wilk y ANOVA one way de Bonferroni ($p<0,05$). Se redujo significativamente el área de la lesión en el octavo día del tratamiento en los grupos EE y EE+I comparados a los demás grupos. La reepitalización no fue distinta entre los grupos, sin embargo, la distancia entre los bordes de la lesión fue menor en los grupos EE y EE+I. También en estos grupos aumentó significativamente ($p<0,05$) el número de fibroblastos y disminuyeron los leucocitos. Se concluye que la EEAV catódica aceleró el proceso de reparación de la lesión, pero no ocurrió resultado con la aplicación de la insulina tópica.

Palabras clave | Estimulación Eléctrica; Insulina; Ratas Wistar/ lesiones.

INTRODUCTION

Lesion repair is a combination of complex biological and molecular events, with strong interference between cells and their surrounding microenvironment. It has several phases: coagulation, inflammation, migration, proliferation and remodeling, which overlap in time and space[1]. These events change the composition and organization of the extracellular matrix and the local expression of various growth factors[2].

Scientific evidence has shown that high-voltage electrical stimulation (HVES) has grown in both research and clinical area as a prominent way to regenerate lesions[3–5]. Several studies have linked HVES with the healing of chronic ulcers, though studies linking this type voltage use with acute lesion regeneration are still scarce[3,6–9].

Several studies have shown success of the use of insulin in the treatment of chronic lesions both in diabetic humans[1,10,11] and in rabbits[12]. Liu et al.[1] showed that topically applied insulin accelerated the reepithelialization and maturation involved in lesion repair, proving that it stimulated the migration and differentiation of keratinocytes.

Ideally, to treat a cutaneous lesion, one must institute prophylactic measures. However, once a leasion appears, early intervention is necessary to avoid or minimize recurrent risks, as well as to facilitate the cicatrization process.

Clinical use of resources that accelerate lesion regeneration, such as HVES and insulin, has shown positive cellular results with cutaneous lesions, even when not linked to diabetes[13]. This study's hypothesis is that association between electrical stimulation and topical insulin has the potential to increase the inherent effects of both resources.

The objective of this study was to evaluate the effects of cathodic high-voltage electrical stimulation, topical insulin, and the combination of both in the integumentary regeneration of rat lesions.

METHODOLOGY

The study used 42 Wistar rats, aged from 3 to 4 months (240±30 g), randomly divided into six groups (n=7): 1) Control (C) – untreated lesion; 2) Placebo electrical stimulation (PES) – lesion treated with HVES; 3) Eletrical stimulation (ES) – lesion treated with cathodic HVES; 4) Insulin (I) – lesion treated with topical insulin; 5) Placebo insulin (PI) – lesion treated with dermatological cream without any active ingredient; and 6) ES + insulin (ES+I) – lesion treated with cathodic HVES + topical insulin.

Treatment occurred during a period of seven consecutive days, and the animals were kept in individual cages, equally supplied with ration and water *ad libitum*, and exposed to a light/dark cycle of 12 hours each.

The animals were weighed and anesthetized prior to the surgery with an intramuscular injection of Dopalen (Ketamine Hydrochloride) and Rompum (Xylazine Hydrochloride) in a ratio of 2:1, at a dose of 0.09 ml/100 g and 0.06 ml/100 g. Dorsal hair was removed manually

prior to the extraction of 1 cm^2 of skin, including the hypodermis, achieved with the use of a graphite-cast hollowed-out template and a scalpel 11.

Treatment occurred one day after surgery. The application of HVES lasted 30 minutes, on seven consecutive days, under anesthetic induction similar to the one used during surgery. Frequency was of 100 Hz and voltage ranged from 20 to 100 V, using the motor threshold of each rat as a parameter. Tension was increased throughout the application time to avoid current accomodation.

An active silicon-carbon composite electrode measuring 2 cm^2 was placed on the lesion, over sterile gauze moistened with saline solution, while the dispersive electrode measuring 4 cm^2 was placed in the abdominal region, over sterile gel. We used the Neurodyn High Volt – ANVISA 10360310008 – IBRAMED® equipment. PES group was exposed to the same procedures, though the equipment was turned off.

Treatments using topical insulin and placebo insulin used enough cream to cover the lesion area (0.7 grams cream). The topical insulin (0.5 U/g) was supplied by the Pharmacy of the University Hospital of UNICAMP, under the patent: PI 0705370-3 UNICAMP. For the ES+I group, insulin was applied only after electrical stimulation.

Standardized photographic record of the cutaneous lesion was performed with a digital camera (SONY-CYBERSHOT 8.1), positioned 40 cm from the perpendicular surface of the lesion. A millimeter ruler shared the same plane with the lesion. Records were obtained on the first day, after the surgery, on the fourth and on the eighth day. Lesion was measured using the Image Pro-plus 6.2 software (MediaCybernects). Each image calibration used the ruler, with the margins selected automatically. Lesion area was also calculated automatically and expressed in cm^2.

Linear measures of reepithelialization were obtained adding the edge of the lesion and the end of the regenerating epithelium. Center lesion measurements occurred between the extremities of the regenerated epithelium, both in 15 non-serial cuts per animal, using a Carl Zeiss millimeter eyepiece with a 4x objective. These measurements were adjusted according to the micrometric coefficient of Mandarin-de-Lacerda et al.[14].

The sample was stained using Hematoxylin-Eosin and to quantify fibroblasts and leukocytes, we used a light microscope with a 100x objective, adapted with a cross-linked eyepiece (Carl Zeiss, KF 10x/18) in 15 areas of 1000 μm^2 each.

Lesion area was analyzed with the repeated measures ANOVA test, followed by the Bonferroni *post-hoc* or the Tamhane test ($p<0.05$). The Shapiro-Wilk test was used to normalize the epithelization data and the cell numerical profile, followed by the Anova one way test and the Bonferroni *post-hoc* ($p<0.05$).

RESULTS

Table 1 shows that all experimental groups experienced a reduction of the lesion area between the first and eighth day. On the other hand, the intergroup analysis showed that the ES group saw a significantly higher reduction percentage of the lesion area, when compared with the other groups ($p <0.05$), except the ES+I group.

Table 1. Mean ± Standard Deviation (in cm^2) of the lesion area during different days (first, fourth and eighth) and reduction percentage of the lesion area in the following groups: control – C, placebo electrical stimulation – PES, electrical stimulation – ES, insulin – I, placebo insulin – PI, electrical stimulation + insulin – ES+I

Groups	1st day	4th day	8th day	% reduction
Control	1.16 ± 0.15	0.83 ± 0.12	0.46 ± 0.16*	60.3%
Placebo electrical stimulation	1.34 ± 0.18	0.90 ± 0.17	0.58 ± 0.17*	56.7%
Electrical stimulation	1.28 ± 0.17	0.71 ± 0.09	0.27 ± 0.10	78.9%
Insulin	1.11 ± 0.08	0.91 ± 0.17	0.41 ± 0.12*	63%
Placebo insulina	1.31 ± 0.17	1.19 ± 0.30	0.48± 0.08*	63.3%
Electrical stimulation + insulin	1.11± 0.08	0.79 ± 0.05	0.33 ± 0.08	70.3%

*$p<0.05$ versus ES

Table 2 shows the linear measure values of the reepithelialization process, that is, the extension between the edges of the epidermis and the free edge at the center of the lesion, and also the central distance of the lesion, that is, the distance between the growing epithelia of both edges. This table shows similar values for all experimental groups. The best lesion closure rate occurred in the groups treated with HVES (ES and ES+I), different from group I ($p<0.05$).

Table 3 shows the number of fibroblasts and leukocytes present in the lesion. Higher means of fibroblasts can be observed in the HVES-treated groups, especially in the isolated group (ES) and in the group associated with topical insulin (ES+I), both with equal values. These same groups showed a reduced number of leukocytes. The ES group showed the most significant lesion reduction, after ES+I group ($p <0.05$).

Table 2. Mean ± Standard Deviation (in μm) of reepithelialization (epithelial growth) and distance between lesion edges (lesion closure) of the following groups: control – C, placebo electrical stimulation – PES, electrical stimulation – ES, insulin – I, placebo insulin – PI, electrical stimulation + insulin – ES+I

Groups	Reepithelialization	Distance between edges
Control	1615.93 ± 1345.62	2159.51 ± 809.51
Placebo electrical stimulation	1504.04 ± 1436.76	2156.60 ± 7470.80
Electrical stimulation	1437.70 ± 506.81	1120.40 ± 840.43*
Insulin	1443.75 ± 228.82	3202.46 ± 1417.73
Placebo insulin	1845.30 ± 692.77	2683.02 ± 1162.61
Electrical stimulation + insulin	1936.76 ± 437.80	1108.38 ± 780.69*

*p<0.05 *versus* I

Table 3. Mean ± Standard Deviation of the number of fibroblasts and leukocytes per 1000 μm² on the regenerating dermis of the following groups: control – C, placebo electrical stimulation – PES, electrical stimulation – ES, insulin – I, placebo insulin – PI, electrical stimulation + insulin – ES+I

Groups	Fibroblasts	Leukocytes
Control	40.56 ± 7.4*$	11.80 ± 5.47*
Placebo electrical stimulation	39.8 ± 3.5*$	10.37 ± 2.3*#
Electrical stimulation	53.3 ± 5.21	5.00 ± 1.69#
Insulin	46.9 ± 3.9*$	10.46 ± 1.9*#
Placebo insulin	43.18 ± 4.9*$	17.18 ± 3.33*
Electrical stimulation + insulin	55.71 ± 9.5	6.86 ± 2.01#

*p<0.05 versus ES; $ p<0.05 versus ES+I; #p<0.05 versus PI

DISCUSSION

Some factors interfere in the healing process, and are classified as local (infection, local tissue perfusion, type of damaged tissue) and systemic influences (age, nutrition, tissue oxygen tension, diabetes, immunological condition, associated diseases)[15]. For more reliable results, this study chose not to take these influences into consideration. Therefore, all rats used were the same age and race, had the same mechanism of injury, same diet and were kept in a controlled environment. We also considered the stress level, by using a group of placebo animals, who underwent surgery, handling and anesthesia daily, and provided the results presented by the control group.

Electrical stimulation is believed to accelerate the lesion reparation process, by imitating the electric current that naturally occurs in the skin when damaged[16]. Our results show that all groups had their lesion reduced during the acute phase, including the control and placebo groups. The combined use of tratments (ES+I) produced a high healing rate (70%). However, it was the ES group that had the best reduction percentage (78.9%), statistically standing out from the other groups ($p<0.05$).

The application of different polarities can interfere with tissue response, since different cells present in the cicatrization process may migrate due to the charge used, a fact identified by Kloth and McCulloch[17], who observed negative poles attracting cells like neutrophils and macrophages, which would then suffer autolysis to counteract the necrosis.

This study used a negative pole, without increase in reepithelialization, in other words, epithelium growth. However, the distance between the epithelial edges, symbolizing lesion healing and its contraction, was significantly better in HVES-treated groups, both by itself (ES) and associated with topical insulin (ES+I). The percentage of reduction in lesion areas was also significantly higher in the ES group. These results confirm the effects of cathodic HVES in the healing of cutaneous lesions.

The dermis cells showed a significant decrease in the number of leukocytes in the ES-treated groups and a significant increase in fibroblasts in the ES and ES+I groups, proving the benefits of the cathodic HVES. No other groups showed similar results. The group treated with placebo insulin had a significant increase in the number of leukocytes, reflecting the exacerbation of the inflammatory process, caused by the lack of any active therapeutic measure, such as electrical stimulation or topical insulin.

The difference between the reepithelialization measurements and the distance between the edges of the lesion, both evaluated in this study, are justified by the action of myofibroblasts, cells responsible for the contraction of the lesion. These cells align and attach themselves to the thicker collagen fibers, pulling them together and thus greatly contributing to the healing process[18,19].

The number of fibroblasts confirms study data, indicating that electrical stimulation improves lesion regeneration, since it promotes cell migration, stimulates fibroblasts and increases protein synthesis[17,20].

Isolated application of topical insulin showed no significant difference in all analyzed variables, though insulin is known to stimulate growth and the development of different types of cells[21]. It is also important in the reepithelialization process, which involves proliferation, migration and differentiation of keratinocytes in the edges of the lesion[22].

Lima et al.[23] used topical insulin with the same formulation and provenance as our study and observed accelerated healing of the cutaneous lesions of diabetic rats. On the other hand, our study used non-diabetic rats and the lesions were acute, which might explain the difference in results. The distance between the edges of the lesion in the group treated with topical insulin did not differ from the control and placebo groups, highlighting the electrical stimulation groups as the ones with the best lesion closure.

Apikoglu-Rabus et al.[21] studied the effect of topical application of insulin on acute lesions in diabetic and non-diabetic rats. Their study showed an accelerated repair process with the use of insulin. The non-diabetic rats used in this study failed to produce similar results. We believe the difference lies with the experimental protocol, since the authors of such study applied insulin twice a day.

In accordance with Sekhejane and Hourold[24], this study proves that topical insulin is beneficial when treating lesions, even if non-diabetes-related lesions, by evidencing its role in the reduction of the inflammatory reaction.

This study allows for future research, regarding the combined use of high-voltage electrical stimulation and topical insulin in an experimental model with diabetic animals, with reduced growth factors, proliferation and cell migration[25].

CONCLUSION

The cathodic HVES accelerated the lesion repair process, with the addition of topical insulin showing no additional effect.

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