EFFECTS OF LOW-POWER PULSED ULTRASOUND ON SECOND-INTENTION HEALING OF TOTAL SKIN INJURIES IN RATS

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SUMMARY

We evaluated the effects of low-power pulsed ultrasound on skin injury healing at dorsal region of rats. Sixty male rats were used (Wistar, mean weight: 300 g) divided into two groups, namely: 1) simulated irradiation; 2) effective irradiation (basic rate of 1.5MHz, pulse cycle rate of 1KHz, pulse width of 200 μs, power of 30mW/cm2 –SATA, 10 minutes of application in alternate days). These were further divided into subgroups, according to the time of injury assessment, as 3, 7, and 14 days, and healing was assessed by planimetrical and histomorphometrical analysis. A significant increase ($p<0.05$) of the healing area was seen for Group 2 (141.88±18.50 mm2) compared to Group 1 (117.38±15.14 mm2), at the 14th day. There was a significant reduction of the number of inflammatory cells ($p<0.05$), associated to an increment of angiogenesis for Group 2 (2196.56 cel/mm^2±234.93) in comparison to Group 1 (2611.68 cel/mm^2±423.82), at the 3rd day. No significant differences were seen in collagen formation, or on dermis and epidermis area between groups. It was concluded that low-power pulsed ultrasound does not cause any deleterious effects and can moderately stimulate skin second-intention healing in experimental environments, showing a potential to clinical use in human beings.

Keywords: Total skin lesion; Low intensity ultrasound; Secondary healing.

INTRODUCTION

Healing process occurs in order to restore anatomical and functional integrity of a tissue. Many biochemical and cellular events, on which the quality of formed scar depends, are involved in this process, which results from tissue response to injury. Tissue repair process is divided, in general, into three phases, with not so distinguishable limits, but superposed in time: 1) inflammation; 2) granulation tissue formation with extracellular matrix deposing, and; 3) remodeling[1].

There are evidences that tissue repair may be stimulated by physical agents, such as ultrasound. The beneficial effect of the ultrasound has been shown on several tissues, including skin, especially for angiogenesis, granulation tissue, number of fibroblasts and collagen synthesis increases, and reduction of leukocytes and macrophages, among others[2]. Occasional beneficial effects of the ultrasound would be of great importance, for example, for treating chronic skin ulcers, in which an increased healing speed, a reduced number of inflammatory cells and the improvement of neoformed tissue quality have already been shown, moreover in clinical trials[3].

Ultrasound's beneficial effects have been shown, especially with low power and pulsed mode, which minimizes also the risk of tissue injuries that may strongly occur[4] and with continuous mode[5]. Low power and pulsed mode are features of an equipment developed in our laboratory, which has been employed in a vast number of experimental and clinical trials on many biological tissues healing, particularly bone[6,7], but also muscle[8,9] and skin[10,11,12].

Considering the small number of controlled studies on

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the subject, this study had as objective to evaluate and quantify the effects of low-power ultrasound irradiation on skin healing, in an experimental total skin injury model in rats, by measuring injury area and by means of histological and histomorphometric studies.

MATERIALS AND METHODS

Sixty adult male Wistar rats with average body weight of 300 g (ranging from 250 to 350g) were used, and this study was approved by the Committee on Ethics in Animal Experiments of the Medical College of Ribeirão Preto, University of São Paulo, under protocol number 053/2004 during the 20th general meeting occurred in 03/07/2005. The animals were kept in appropriate cages in groups of five animals each, receiving meals and water ad libitum. The animals were randomly assigned to two groups, according to treatment protocol, as follows:
Group 1: simulated irradiation (n=30);
Group 2: effective low-power irradiation (n=30).
In each group, the animals were distributed into three subgroups (A, B and C, respectively) of ten animals each, according to the follow-up period after treatment, of 3, 7, and 14 days, respectively, when animals were sacrificed and the skin of the region of interest was dried in blocks for processing and histological and histomorphometric analysis. Normal skin fragments, removed when injuries were produced, and randomly selected, constituted Group 3, of normal control.

Surgical procedure

The animals were anesthetized with appropriate dosages of sodium pentobarbital (Nembutal®, Abbott). A broad trichotomy was performed at the right scapular region, then submitted to antisepsis with 2% iodine alcohol, and protected with sterile surgical drapes. Then, a total skin injury of 1 cm in diameter was produced, with a specifically made punch. The skin was pulled to form a fold, which was placed on a hard surface; once this was done, the punch was used in rotation movements, thus cutting a circular segment of skin, however, without trespassing it, and taking care not to injury skin deep surface on the other side of the fold. Occasionally, a scalp was used to finish skin section. The injury was protected by a moistened bandage, which was refreshed everyday, being removed only for ultrasound application.

Ultrasound irradiation

Immediately after injury, the first ultrasound irradiation session was performed, with the remaining other being performed on alternate days, so that in subgroups 1A and 2A (3 days) two applications were delivered; in subgroups 1B and 2B (7 days), four applications were delivered, and in subgroups 1C and 2C (14 days), seven applications were delivered. In order to optimize ultrasonic waves' contact with the tissue, injuries were soaked with saline solution and then covered with a plastic film (PVC, domestic use). The ultrasound attachment gel was spread over the film, allowing stationary ultrasonic irradiation 10 minutes with no contact between the headstock and the injury. Identical procedures were performed for Groups 1 and 2, with equipment turned off for Group 1 (sham irradiation) and turned on for Group 2 (effective irradiation).
The ultrasound equipment used was developed and built at the Laboratory of Bioengineering EESC-USP, with the following features: basic frequency of 1.5 MHz, pulsed mode with pulse repetition frequency of 1 kHz, pulse width of 200 μs, power of 30mW/cm2 (SATA) and effective irradiation area (EIA)of 22 mm.

Injury area record

Injury perimeter was recorded, immediately after its production and at the various postoperative checkpoints for both experimental groups. Records were obtained by means of an in loco transference on a thin transparent sheet, previously sterilized, using a thin ball tip pen. Those records were copied with a digital scanner and stored, for subsequent processing and computer analysis using a specific program for image processing and analysis (Matlab 6.0, release 13), able to measure the planimetric area. Records were reproduced in conjunction with the scale, in millimeters, for area calculation, from punch standard measurement of 1 cm diameter (78.54 mm^2).

Histological analysis

Skin fragments designed to histological analysis were fixed in 10% formol for 24 hours. After that period, they were included in paraffin blocks and submitted to 5 μm-thick cross-sectioned cuts, with two slides being prepared with four sequential cuts each. One slide was stained with Masson trichromic, which enables to view skin layers and inflammatory cells, and the other slide was stained with Sirus Red, for collagen identification, quantification and differentiation.

Morphometrical analysis

The histomorphometrical evaluation was performed on slides stained with Masson trichromic, employing a light-transmission microscope (Zeiss, model Axiophot II) mounted with a digital camera (JVC, model TK1270) attached to a microcomputer containing imaging analysis programs (KS400 V2.0, Carl Zeiss Vision, and Image Scion), for digital images storage and processing. Three sequential pictures of injury's inner portion were captured in each histological section (magnification lens 10x, optovar, 1x). Inflammatory cells density was quantified on subgroups 1A and 2A (3 days), and 1B and 2B (7 days); epidermis and dermis areas were measured on subgroups 1B and 2B (7 days), and 1C and 2C (14 days). Data achieved were transferred to a worksheet and, then, submitted to statistical analysis.

Slides stained with Sirius Red, for quantifying collagen, were analyzed with a polarized light microscope (Nikon, model E-800, magnification lens 20x) mounted with a digital camera (Nikon, model DXM-1200) and connected to a microcomputer equipped with a specific imaging processing and analysis program (Image Pro-Plus, Release 4.5.1.22, Media Cybernetics, Inc). The following were quantified: total collagen, and type-I and type-III collagen in all subgroups of both experimental groups, as well as in normal skin, for subsequent comparison purposes.

Statistical analysis

In order to analyze the results on inflammatory cells density, dermis and epidermis area, and collagen percentage, the two-way variance analysis (Anova) was used, that is, postoperative follow-up periods (3, 7 and 14 days) and experimental groups (1 and 2). The Tukey's post hoc test was employed to compare periods, being considered as significant when $p<0.05$.

For analyzing injury area measurement results, the non-parametric Mann-Whitney test was used for comparing two independent samples, with the same significance level ($p<0.05$).

RESULTS

Injury Area Record

The injury area variable was established by comparing the differences between values seen immediately after its production and on the last day of each subgroup A, B and C (3, 7 and 14 days) of groups 1 and 2 (simulated irradiation and effective irradiation).

The statistical analysis evidenced that there was no statistically significant difference between average or median areas of injuries in subgroups 1A and 2A ($p=0.064$; Figure 1), nor in subgroups 1B and 2B ($p=0.144$; Figure 1). However, for subgroups 1C and 2C, differences between baseline and final areas were significant, showing an increased cicatricial area in the group submitted to effective irradiation (141.88 ± 18.50 mm^2) compared to the group submitted to simulated irradiation (117.38 ± 15.14 mm^2) with $p<0.05$ ($p=0.002$); (Figure 1/ Table 1).

Histological analysis

Subgroups A (3 days): wound's initial matrix formation was observed, with the presence of a large amount of fibrin, being higher in Group 1 (Figure 2-A) than in Group 2, which, however, presents a larger contingent of young collagen fibers (Figure 2-B). It was also observed a large amount of neutrophils and monocytes in both groups, higher for Group 1 (Figure 2-A) than for Group 2 (Figure 2-B), which presented a higher amount of neoformed vessels (Figure 2-B).

Subgroups B (7 days): collagen fibers were more numerous and with a more mature appearance in both groups, particularly in Group 2, in which they more homogenously and regularly distributed (Figure 2-D) than in Group 1 (Figure 2-C). Neoformed vessels and fibroblasts were seen in both groups. Re-epithelization is occurring in both groups, but notably more advanced in Group 2, in which traces of keratinization may already be seen (Figure 2-D), when compared to Group 1 (Figure 2-C).

Subgroups C (14 days): collagen fibers were already more differentiated and packaged, acquiring the characteristic configuration related to mechanical forces usually acting on the skin (Figure 2-E), in a more pronounced aspect for Group 2, where they were much close to normal, with additional development of dermal papillae (Figure 2-F). Furthermore, partially repaired fibroblasts and skin annexes (sebaceous glands, pilary folicles) were seen, in a larger amount in Group 2 (Figure 2-F) than in Group 1(Figure 2-E). The newly-formed epidermis has already initiated its post-migratory differentiation process, being thicker in Group 1 (Figure 2-E) than in Group 2 (Figure 2-F), thus showing a faster healing process in Group 2. Keratin cover is seen in both groups (Figure 2-E and F).

Histomorphometrical analysis

Inflammatory cells density, epidermis and dermis area, and collagen type I/ III percentage and total collagen were evaluated (Table 1).

Inflammatory cells density: the inflammatory cells density was 2,611.68 cells/mm^2 in subgroup 1A and 2,196.56 cells/mm^2 in subgroup 2A, being reduced to 2,351.22 cells/mm^2 in subgroup 1B and to 2,466.42 cells/mm^2 in subgroup 2B. Differences were significant between subgroups 1A and 2A ($p<0.05$), but not for subgroups 1B and 2B (Table 1). In subgroups 1C and 2C, inflammatory cells were virtually absent, as well as in Group 3, with normal skin.

Epidermis and dermis area: Epidermis and dermis areas were assessed for subgroups B and C of both groups and for Group 3. In Group 1, epidermis area was reduced from 0.31 mm^2 to 0.25 mm^2 from subgroup B to subgroup C; in Group 2, it was reduced from 0.33 mm2 to 0.30 mm2, with differences between groups and subgroups being significant only when compared to values obtained from normal skin in Group 3 ($p<0.001$). The dermis area was reduced from 2.18 mm2 in subgroup 1B to 2.05 mm2 in subgroup 1C, but increased from 2.31 in subgroup 2B to 2.32 in subgroup 2C, with differences between subgroups and groups being significant only when compared to Group 3 ($p<0.05$)(Table 1).

Type-I collagen: it was more abundant than type-III collagen in

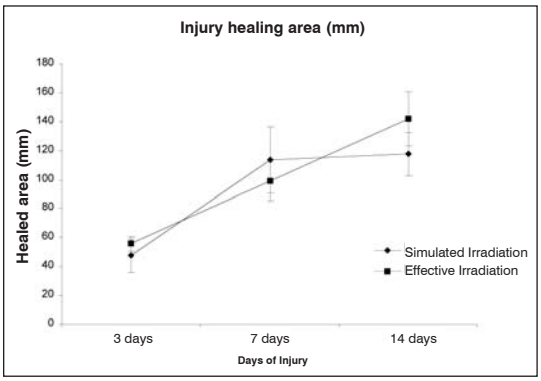

Figure 1 – *Graph of the evolution of healing area in groups 1 (sham irradiation) and 2 (effective irradiation), and in its subgroups A (3 days), B (7 days) and C (14 days).*

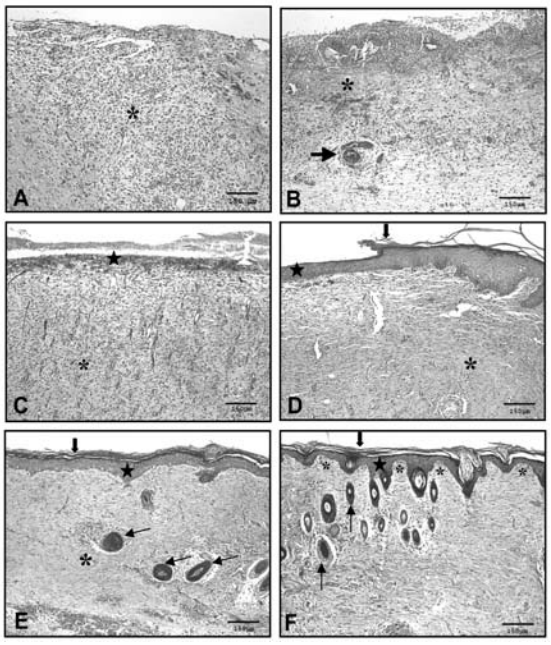

Figure 2 – *Photomicrography of group 1 animals' skin (sham irradiation) (left) (A, C, E) and of group 2 (effective irradiation) (right) (B,D,F) in subgroups: A (3 days) of injury (A,B), B (7 days) of injury (C,D) and C (14 days) of injury (E,F) Fibrin (*), Inflammatory cells (smaller in group 2, if compared to group 1), neoformed vessels (→), re-epithelization process (★), keratinization process (↓) and skin annexes (→).*

longer follow-up periods (7 and 14 days). In subgroups A of Groups 1 and 2, the percentage was 1.57% and 2.76%, respectively, raising to 10.30% and 10.67%, respectively, in subgroups B, and to 16.88% and 16.64%, respectively, in subgroups C. No group or subgroup reached to normal value observed for Group 3 (52.02%). Differences in percentage were significant when subgroups of a same group were compared ($p<0.001$) and between Groups 1 and 2 to Group 3 ($p<0.001$) (Table 1).

Type-III collagen: in all groups and subgroups, percent values for type-III collagen have always been superior to normal values observed for Group 3. In subgroups A of Groups 1 and 2, the percentage of type-III collagen was 1.06% and 1.65%, respectively, with differences between both being not significant. It rose to 1.41% in subgroup 1B, but dropped to 1.49% in subgroup 2B, then rising to 5.56% and 3.5% in subgroups 1C and 2C, respectively. Differences were significant when comparing the subgroups in each group ($p<0.001$) and between Group 3 and subgroups A ($p<0.004$), B ($p<0.003$) and C ($p<0.001$) from both Groups 1 and 2 (Table 1).

Total collagen: in all groups and subgroups, percent values for total collagen were always

	Density (cell/mm²)	Area Epidermis (mm²)	Area Dermis (mm²)	Type-I Collagen (%)	Type-III Collagen (%)	Total Collagen (%)	Healed Area (mm²)
1A	2611.68 ± 423.82	*	*	1.57 ± 1.35	1.06 ± 0.80	2.63 ± 1.30	47.73 ± 11.81
2A	2196.56 ± 234.93	*	*	2.76 ± 1.34	1.65 ± 0.60	4.41 ± 1.56	55.79 ± 5.29
1B	2351.22 ± 250.91	0.31 ± 0.24	2.18 ± 0.37	10.30 ± 3.13	1.41 ± 0.79	11.71 ± 3.54	113.56 ± 22.81
2B	2466.42 ± 488.16	0.33 ± 0.16	2.31 ± 0.71	10.67 ± 2.44	1.49 ± 1.15	12.16 ± 3.14	99.34 ± 14.03
1C	**	0.25 ± 0.14	2.05 ± 0.43	16.88 ± 5.90	5.56 ± 3.19	22.43 ± 4.24	117.38 ± 15.14
2C	**	0.30 ± 0.12	2.32 ± 0.50	16.64 ± 3.96	3.50 ± 1.39	20.14 ± 4.20	141.88 ± 18.50
N	**	0.16 ± 0.03	1.51 ± 015	52.02 ± 2.19	0.56 ± 0.21	52.58 ± 2.21	***

Labels: 1- Group with sham irradiation, 2- Group with effective irradiation, A- 3 days of injury, B- 7 days of injury, C- 14 days of injury, N- Normal non-injured skin. In this phase, there is no epidermis and dermis repair;** In this phase, there are no inflammatory cells,*** No healing area.*

Table 1 – *Histomorphometric parameters assessed.*

inferior to Group 3, in which an average value of 52.58% was recorded. In Group 1, it was 2.63% for subgroup A, reaching to 11.71% for subgroup B and to 22.43% for subgroup C. In Group 2, it was 4.41% for subgroup A, 12.16% for subgroup B, and 20.14% for subgroup C. Differences were significant when subgroups of Groups 1 and 2 were compared ($p<0.001$) and between these and Group 3 ($p<0.001$), (Table 1/Figure 3).

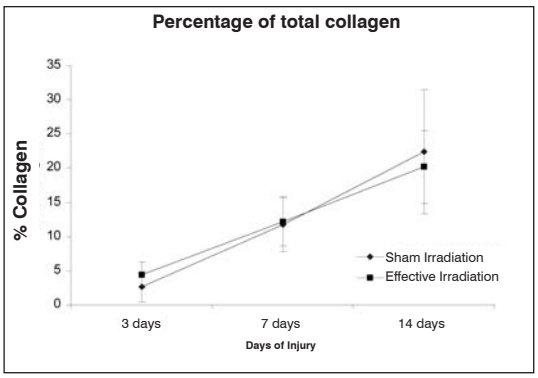

Figure 3 - *Percent values for total collagen in groups 1 (Sham Irradiation) and 2 (Effective Irradiation) and their subgroups A (3 days), B (7 days) and C (14 days).*

DISCUSSION

Ultrasound is probably the most used physical resource for treating soft tissues injuries, and it may hasten tissue repair in its different aspects[2,13]. With ultrasonic irradiation, it is possible to improve both healing speed and the quality of cicatricial tissue. Indeed, there are many reports about the effects of ultrasound on bone, tendons, muscles, ligaments, cartilage and skin healing process, showing benefits with low dosages and harms with high dosages[4].

In addition, many experiments have demonstrated the superiority of pulsed ultrasound when compared to continuous mode. Pulse ultrasound is characterized by pauses between duty cycles, minimizing thermal effects and maximizing the mechanical effect of irradiation, which causes an increased collagen synthesis, reaching 30% against 20% with continuous ultrasound, with the same power[5]. Many trials evidenced, also, that the biological effects of ultrasound related to cavitation and micromassage, such as support cells degranulation, changes on cell membrane's function, increased levels of intracellular calcium, fibroblastic activity stimulation, resulting in an increased protein synthesis, neoangiogenesis, and collagen's elastic tension, with those effects being more pronounced with pulsed ultrasound[2,13], regardless of the other parameters used in irradiation, such as basic frequency, power, pulse width, and pulses repetition frequency.

More recently, the beneficial effects of pulsed ultrasonic irradiation on healing and regeneration process of different kinds of tissues were related to low power, to the use of equipment built specifically with this feature[6]. Low-power ultrasound efficiency was proved in many trials on neo-osteogenesis stimulation[7] and on repair of other kinds of tissues, such as the skin[10,11,12], muscles[8,9], cartilage[14] and peripheral nerves[15].

Chronic skin injuries, such as ulcers of various causes, are difficult to heal, encouraging the search for means and devices that could benefit their healing process. Ultrasound is one of those devices, since its beneficial effects are experienced from acute inflammatory phase up to skin scar remodeling phase. Nevertheless, it use in skin tissue repair with very low powers has been little investigated, which constitutes the purpose of the present study.

The experimental model used here was that of extensive total skin injuries, that is, skin being fully removed from a relatively large area, exposing the fascial tissue for muscle coating. For providing a higher reproducibility of the method, a cutting circular punch was used, which performed more than 90% of skin cut, being the remaining 10% complemented by a scalp. In Group 1, irradiation was simulated, that is, the equipment was used as in Group 2, but turned off. The purpose of this strategy was to submit animals in Group 1 to the same protocol as Group 2, thus assuring that the effects found would be related only to ultrasonic stimulus.

The ultrasonic irradiation equipment was the same as the one previously used and validated in other studies, for stationary use. For use in human beings, with large injuries, recommended irradiation time is 20 minutes a day, but, in the present investigation, due to relatively small injuries, we determined 10 minutes of application in alternate days. In contrast with irradiation on injury margins, as mentioned in other studies[11,12], here we preferred to apply ultrasound directly on the injury, which was isolated from ultrasound headstock by means of a PVC film, under which saline solution was introduced and over which the headstock attachment gel was spread, which enabled infection prophylaxis while assuring transmissibility of ultrasonic waves[16]. With injury's bloody bed being constituted of muscular fascia and muscle, rich in multiple-potential mesenchymal cells, it is worthy to think that direct irradiation to that site would be more efficient than on injury margins.

The skin injury area evolution follow-up is the parameter mostly employed in daily clinical practice of physical

therapists and doctors, reason of its use in this study. The injury area measurement by the employed method (computed planimetry) was selected because of its low cost, user friendship, and relevant clinical applicability, with several reports available on its use for following up the evolution of open injuries[17,18]. By this parameter, the effects of ultrasonic irradiation appeared only on the last follow-up period, of 14 days (subgroups C), when the difference between groups was significant, which had not happened on periods of 3 days (subgroups A) and 7 days (subgroups B).

At the qualitative histological analysis, the effects of ultrasonic irradiation on Group 2 manifested as a smaller amount of fibrin, a lower number of neutrophils and monocytes, a larger contingent of neoformed vessels, a higher number of collagen fibers in a more mature arrangement and earlier re-epithelization, including the emergence of annexes and keratin granules. The morphometrical analysis corroborated those data, beginning by the number of inflammatory cells, which gradually reduced in both groups, with a more pronounced drop in Group 2, although the difference between both groups was not significant, the same occurring with epidermis and dermis area. Regarding total collagen and type-I collagen, a significant increase was seen between subgroups of both groups, characterizing healing process advancement, while type-III collagen, which was already above normal at the first checkpoint (subgroup A) for both groups, presented with a significant increase between this and the last checkpoint (subgroups A and C), becoming about ten times higher than normal in Group 1, and seven times higher in Group 2. Nevertheless, the differences between both groups were not significant for any of those parameters. Furthermore, neither in group 1 nor in group 2, collagen percentage reached the normal values of Group 3, clearly showing that the healing process was still at its early stages for both groups on the 14th follow-up day. Those results somehow show an apparent harmless of ultrasonic irradiation on skin healing, which is not true if more attention is given to qualitative differences, which indicate a faster healing process on Group 2. Thus, ultrasonic irradiation would not quantitatively change the process, but only qualitatively.

The results achieved in this study are consistent to those reported in literature, which report that ultrasonic irradiation indeed stimulates skin healing[2,3,16], with a reduction of the injury area occurring, probably, as a result of the action on myofibroblasts at injury site, increasing its contractibility[19]. Nevertheless, variations in parameters such as time, frequency and application dosage, among different authors, contribute for questions to remain unanswered, as well as to the existence of meta-analysis studies, which suggest that there aren't enough biophysical evidences to support the effects of ultrasonic irradiation on healing[20].

The authors conclude that the ultrasonic irradiation as employed in this study positively influenced second-intention healing process on rats' skin, by hastening it, and that the method may potentially be used for clinical application in human beings, with adjusted application parameters, especially regarding time and frequency.

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