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ORIGINAL ARTICLE

Measurement of healing area using planimetry after applying low intensity ultrasound to the skin of rats

Mensuração de área de cicatrização por planimetria após aplicação do ultra-som de baixa intensidade em pele de rato

Ferreira AS[1], Barbieri CH[1], Mazzer N[1], Campos AD[2], Mendonça AC[1]

Abstract

Background: Planimetry is a method used to evaluate the progression of skin wound healing. Computerized planimetry is still an experimental method, but its advantages have been demonstrated in several investigations. **Objective:** To evaluate the effects of low-intensity pulsed ultrasound on the healing of a skin lesion produced on the dorsal region of rats, by means of computerized planimetry. **Methods:** Sixty male Wistar rats of mean weight 300g were used. They were divided into two groups according to the treatment applied: 1) simulated irradiation (control); 2) effective irradiation (fundamental frequency 1.5MHz, pulse repetition frequency 1KHz, pulse width 200µs, SATA intensity 30mW/cm^2 and application for ten minutes on alternate days). Each group was divided into three subgroups according to the length of time for which ultrasound irradiation was applied of three, seven and 14 days, respectively, and healing was evaluated by means of planimetry; a tracing of the wound was obtained on special paper and this was digitized and measured by means of a graphing software. Statistical analysis was performed using the Mann-Whitney non-parametric method. **Results:** The healed area was significantly greater ($p<0.05$) in group 2 (141.88±18.50mm^2) than in group 1 (117.38±15.14mm^2) on the 14th day. There were no significant differences between the subgroups for the other experimental periods. **Conclusions:** Low-intensity pulsed ultrasound irradiation stimulated secondary skin healing under these experimental conditions. Computerized planimetry was shown to be a low cost method that was easy to use and present clinical applicability.

Key words: computerized planimetry; skin injuries; low-intensity ultrasound; healing.

Resumo

Contextualização: A planimetria é um método utilizado para avaliar a evolução da cicatrização de feridas. A planimetria computacional é um método ainda em experimentação, mas cujas vantagens têm sido demonstradas em várias investigações. **Objetivos:** Avaliar os efeitos do ultra-som pulsado de baixa intensidade sobre a cicatrização de lesão cutânea produzida na região dorsal de ratos, por meio da planimetria computacional. **Materiais e métodos:** Utilizou-se 60 ratos machos Wistar (peso médio de 300g) divididos em dois grupos com 30 animais cada, de acordo com o tratamento: 1) irradiação simulada (controle); 2) irradiação efetiva (Freqüência fundamental de 1,5MHz, freqüência de repetição de pulsos de 1KHz, largura de pulso de 200µs, intensidade de 30mW/cm^2 SATA, dez minutos de aplicação em dias alternados). Cada grupo foi subdividido em três grupos, de acordo com o período de irradiação ultra-sônica, de três, sete e 14 dias, respectivamente, e a cicatrização foi avaliada por meio da planimetria, um decalque da lesão sendo obtido em papel especial, digitalizado e medido ao computador por meio de um programa gráfico. Análise estatística pelo método não-paramétrico de Mann-Whitney. **Resultados:** Houve aumento significante ($p<0,05$) da área cicatrizada no grupo 2 (141,88±18,50mm^2) em relação ao grupo 1 (117,38±15,14mm^2), no 14º dia. Não houve diferenças significantes entre os grupos nos demais períodos. **Conclusões:** O ultra-som pulsado de baixa intensidade estimula a cicatrização cutânea por segunda intenção em condições experimentais. A planimetria computacional mostrou-se um recurso de baixo custo, fácil manuseio e de aplicabilidade clínica.

Palavras-chave: planimetria computacional; lesões cutâneas; ultra-som de baixa intensidade; cicatrização.

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[1] Graduate Program in Orthopedics, Traumatology and Rehabilitation, the School of Medicine of Ribeirão Preto, Universidade de São Paulo (USP) – Ribeirão Preto (SP), Brazil.
[2] Department of Social Medicine, USP.
Correspondence to: Cláudio Henrique Barbieri, Departamento de Biomecânica, Medicina e Reabilitação do Aparelho Locomotor, Faculdade de Medicina de Ribeirão Preto (USP), Campus Universitário, CEP 14049-900, Ribeirão Preto (SP), Brazil, e-mail: chbarbie@fmrp.usp.br

Introduction

The skin healing process occurs to restore the anatomical and functional integrity of tissues. To achieve that, the body makes use of a complex mechanism which involves chemotaxis, cellular division, neovascularization, extracellular matrix protein synthesis and scar remodeling. Wound contraction is an important step in the closing of skin lesions. In animals such as rabbits and rats, whose tegument is loosely linked to the underlying tissues, it rarely causes these tissues to lose their function. Nevertheless, in humans, insufficient contraction delays closing and leads to bleeding and infections, whereas an intense contraction may lead to tissue contractures and consequently deformity and dysfunction. However, taken in isolation, the contraction of a wound seldom leads to its permanent closing. This is caused mainly by the formation of the granulation tissue and to the reepithelization[1-3]. The possibility of speeding up the healing process and the closing of skin lesions with therapeutic chemical agents or physical resources has been the object of investigation of countless researchers.

Ultrasound therapy is a form of treatment that is often used in physical therapy practice. Ultrasound is a type of high-frequency mechanical energy in which the energy is transmitted through the vibrations of the molecules of the environment through which the wave is being irradiated. It is broadly used in the treatment of soft-tissue lesions and can speed up tissue recovery in its different phases. It accelerates the healing and enhances the quality of the scar tissue[4-9]. It affects the phases of the physiological mechanism (acute inflammation, proliferation, remodeling) from the beginning, at which stage it promotes the liberation of histamine and of growth factors by degranulation of macrophages, mastocytes, and platelets. Ultrasound can also affect the fibroblasts and the endothelial cells, increasing collagen synthesis, and thus increasing tissue resistance to traction[5,9].

Ultrasound can be produced in the form of continuous or pulsed waves. In the continuous mode, it is characterized by the production of biophysical and thermal effects, whereas in the pulsed mode it reduces the thermal effect due to the cyclical interruption of energy emission, while maintaining the biological effect. It has been suggested that the non-thermal effects of ultrasound, including cavitation and acoustic micromassage, are more important for the treatment of soft tissue lesions than the thermal effects. Inadequate doses of ultrasound in either mode may be damaging, which calls for a perfect understanding of its biological effects, action mechanisms and adequate dosage, according to the characteristics of the tissue, in order to maximize the safety and efficiency of the treatment. For this reason, the equipment must be calibrated and periodically tested to meet safety standards.

The beneficial effects of ultrasound have been demonstrated on the healing of several biological tissues, such as bone[10], muscle[11,12] and skin[1,13,14]. Particularly at low intensities, the use of specifically designed equipment with these features[10] and in the pulsed mode minimizes the risk of tissue lesions that might occur at more elevated intensitites[4,9]. These effects would be of great relevance, for instance, in the treatment of chronic skin ulcers. There are reports of increase in the healing speed, reduction of the number of inflammatory cells and improvement in the quality of the newly formed tissue, especially in clinical investigations[1,14,15].

The evolution of a lesion or a skin wound and the efficacy of a particular treatment may be followed and measured at regular intervals by several methods, until skin continuity has been reestablished. Planimetry is a non-invasive evaluation method used for superficial wounds, with no complicating factors which, in general, exist in deep lesions. It only describes the external surface of the wound, without offering any indication of the quality of the underlying repair tissue[3,16].

The study of skin wound contraction through sequential planimetry in rats is apparently a simple and precise methodology of this phase of the healing process and is widely used[15,17,18]. In addition to its use in investigations with animals, this method has also been applied to humans, regardless of wound morphology[1,14,19]. The present study sought to evaluate the effects of low frequency ultrasonic irradiation on skin healing using planimetry to measure the damaged area of an experimental model of skin lesion with total thickness in rats.

Methods

The present study was approved by the Ethics in Animal Testing Committee of the School of Medicine of Ribeirão Preto of Universidade de São Paulo (USP) (approval report number 052/2004). All proceedings were carried out at the Bioengineering Laboratory of the aforementioned institution. Sixty adult male Wistar rats weighting from 250 to 350g were used, derived from the central vivarium of the Ribeirão Preto campus of USP. The animals were kept in collective cages, each containing five rats, under suitable environmental conditions (temperature control and ventilation), and received an unrestricted supply of food and water. The animals were randomly divided into two groups (1 and 2) of 30 animals each, according to treatment. Group

1 was submitted to simulated irradiation (control, with the equipment turned off), and group 2 received effective ultrasound irradiation. In each group, the animals were distributed in subgroups of ten animals each (A, B and C), according to the period of treatment, of three, seven, and 14 days respectively.

Surgical procedures

Initially, the animals were anesthetized with an intraperitoneal injection containing a single dose of pentobarbital sodium (Nembutal®, Abbott, 60mg/kg). Next, the right scapular area was shaved; the animal was positioned on the operation table, and an antiseptic (2% iodine alcohol solution) was applied to the skin. With the animals prepared in this way, a 1cm-diameter skin lesion was resected with a punch instrument manufactured especially for that purpose. Because the skin of rats is very elastic, it was necessary to fold it and press it against the hard surface of the surgery table to produce the lesions. After the procedure, the animals were kept in their collective cages.

Ultrasound application

The equipment used was developed for medical and biological purposes at the Bioengineering Laboratory of the Department of Materials, Aeronautical and Automotive Engineering of the School of Engineering of São Carlos (EESC) of USP. It has the following features: 1.5MHz fundamental frequency; pulsed mode with a 1KHz frequency of pulse repetitions; 200μs pulse width; $30mW/cm^2$ SATA intensity; and 22mm effective radiation area (ERA). The time of irradiation at each session lasted ten minutes. For the ultrasound application, the animals were placed in a PVC trap-like device consisting in a 4.5cm-diameter closed tube with perforations for ventilation and a side window, where the lesion was exposed. In this way, the animal was immobilized without the use of force. The treatment was started immediately after the lesion was produced, and every other day thereafter. The irradiation was applied twice to subgroup A, four times to subgroup B, and seven times to subgroup C. The ultrasonic probe was placed directly on the lesion, which was prepared as follows: first, the wound was washed liberally with a saline solution; next, it was covered with plastic wrap that was previously sterilized and stretched out over the surface to avoid air bubbles; then, the plastic wrap was covered with the hydrogel coupling for the ultrasound probe. Irradiation was administered in the stationary mode, with the probe resting directly on the lesion for ten minutes. The same procedure was carried out for group 1 (control) with the equipment switched off, and group 2 (irradiated) with the equipment switched on.

Record of lesion area

The perimeter of the lesion area was recorded immediately after it was produced, and on day three for subgroup A, day seven for subgroup B, and day 14 for subgroup C, for both experimental groups (1 and 2). The perimeter of the lesion was traced *in loco* with the use of a previously sterilized sheet of tracing paper and a fine ballpoint pen (Figure 1).

These records were scanned and stored for later processing and computer analysis, using the software Matlab 6.0 release 13 especially designed for image processing and analysis and developed at the Computer Vision Laboratory of the Department of Electrical Engineering of EESC-USP. The records were reproduced with a millimeter ruler to measure the areas, using as a reference point the standard area of the 1cm-diameter punch employed to produce the lesions ($78.54mm^2$). Based on the internal area of the punch, a $100mm^2$ template had to be made as the software does not read fractions.

Image processing through computer vision

the eight-bit grayscale scanned images (Figure 1A) were converted into a binary code to obtain the outline of the lesion area (Figure 1B). Binarization can be described as the separation of the area of interest from the rest of the figure, called background, leaving the image with only two hues of gray, namely zero (black) or one (white) corresponding to the area of interest and the background, respectively. By means of morphological operations, the internal area of the outline (center of the lesion), initially characterized as background, was modified and filled in so that it belonged to the area of interest (Figure 1C). The lesion area was then calculated by counting the number of pixels (unit of space) of the area of interest, consisting of the amount of zero or black pixels present in the image. The conversion of pixels to mm^2 is based on the image of a $100mm^2$ template. The progress of the wounds over time was evaluated. We compared the results among the subgroups for each group (group 1 and 2). We also compared the results between groups (control and irradiated), by comparing the results from subgroup 1A and 2A, subgroup 1B and 2B, and subgroup 1C and 2C. The non-parametric Mann-Whitney test for two independent samples was used for all comparisons with a level of significance smaller than 5% ($p<0.05$).

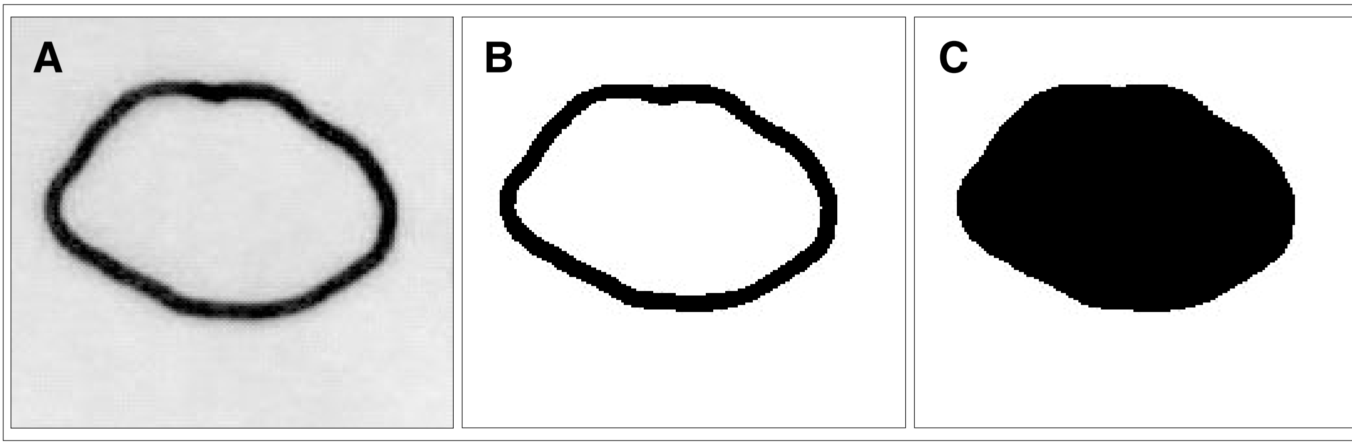

Figure 1. Sequence of the digital image processing to calculate the lesion area. The tracing (A), binarization of the outline (B), and filling out of the internal area (C) are shown.

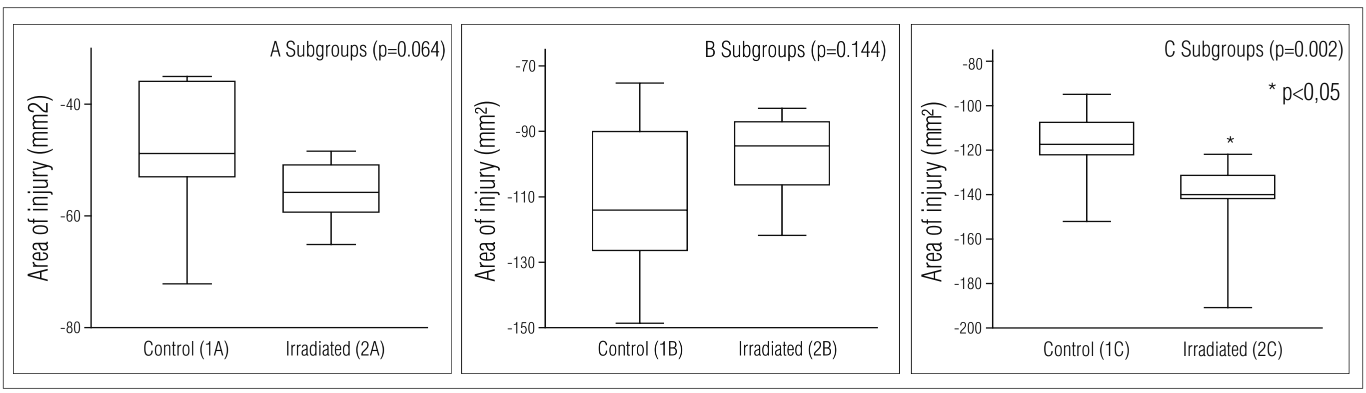

Figure 2. Comparison of the results of the control and irradiated groups, on the first and third days after the lesion for the A subgroups (three days); on the first and seventh days after the lesion for the B subgroups (seven days); and on the first and 14th days for the C subgroups (14 days).

Results

The control group and the irradiated group had an increase in the healed area over the course of the 14 days that followed the skin lesion. The initial mean area of the lesion, immediately after it was produced in subgroup 1A (control - three days), was 115.73mm^2 and decreased to 68.00mm^2 by day three, with a mean difference of 47.73mm^2. In subgroup 2A (irradiated three days), the mean lesion area was 119.85mm^2 at the outset and 64.06mm^2 on day three, with a mean difference of 55.79mm^2. In subgroup 1B (control – seven days), the mean lesion area was 133.24mm^2 at the outset and 19.69mm^2 on day seven, with a mean difference of 113.55mm^2. In subgroup 2B (irradiated - seven days), the mean lesion area was 118.03mm^2 at the outset and 18.69mm^2 on day seven, with a mean difference of 99.34mm^2. In subgroup 1C (control - 14 days), the mean lesion area was 127.55mm^2 at the outset and 10.17mm^2 on day 14, with a mean difference of 117.38mm^2. In subgroup 2C (irradiated - 14 days), the mean lesion area was 148.69mm^2 at the outset and 6.81mm^2 on day 14, with a mean difference of 141.8mm^2.

Figure 2 shows the box plot representation of the differences in area found in subgroups A (three days), B (seven days), and C (14 days). The lower and upper edges of the box represent the first and third quartiles, the line inside the box represents the median, and the external horizontal lines represent the minimum and maximum value in the sample

The statistical analysis showed that there was no significant difference between the mean or median lesion areas of subgroups 1A (control - three days) and 2A (irradiated - three days; p=0.064) or between subgroups 1B (control - seven days) and 2B (irradiated - seven days; p=0.14). In contrast, there was a significant difference between subgroups 1C (control - 14 days) and 2C (irradiated) (p=0.002; as in Figure 2).

Discussion

The efficacy of the low-intensity ultrasound (LIU) was confirmed in several investigations about its influence on the stimulation of the neo-osteogenesis[10,20] and on the recovery of other kinds of tissues, such as skin[1,13,14,21] and muscle[11,12].

Although several studies have already shown the beneficial effects of LIU on the process of healing and regeneration of various types of tissues, little is known about its effects on skin healing. Therefore, the primary goal of the present investigation was to determine whether LIU effectively accelerates the closing of skin lesions, by using computerized planimetry to measure the lesions at each evaluated phase.

The lesions were produced on the dorsal region of rats, a region that is less susceptible to infection as it has less contact with feces and saliva. A total skin lesion was produced, i.e. that included the entire thickness of the skin down to the fascial plane. The aim was to remove any central proliferating layers which could foster quick spontaneous healing, so that the wound would have a maximum stimulus from the physiological responses, including scar contraction and central epithelization. This would be a way of reproducing a severe skin lesion, which always has a slow spontaneous healing process.

Macroscopic observations of the integrity of the skin of rats were made throughout the experiment, and no adverse reactions were found during this period for either group. Nevertheless, keeping the animals in collective cages could cause new tissue damage due to the contact between them, thus compromising the analysis of the healing process. Although that was a possibility, the process of keeping the animals in the same enclosure was the same for all groups and it did not affect the results.

The specialized literature offers a vast array of time and frequency patterns of pulsed LIU for skin tegument repair. Alves[13] treated for five minutes each of the four areas adjacent to burn lesions inflicted on the skin of rats, comprising a total of 20 minutes per day. Hilário[14] treated patients with trophic leg ulcers by irradiating the peripheral area of the lesion three times a week, with an irradiation time of 20 to 40 minutes, depending on the ulcer area. Peschen et al.[15] treated patients with chronic venous ulcers with three weekly ten-minute sessions of the sub-aquatic technique. Anastácio[1] also treated patients with vascular ulcers with three weekly ten-minute irradiation sessions in each area adjacent to the lesions. Campanelli[21] treated patients with plantar ulcers with three weekly ten-minute sessions for each adjacent spot, the number of which depended on the extent of the lesion.

The initial protocol for pulsed LIUS recommended a 20-minute daily application[10], but depending on the size of the lesion, several surrounding areas had to be irradiated, making the treatment to be long and costly. For this reason, some researchers have reduced the application time, and following this rationale, we shortened the duration to ten minutes per session, and tested whether such a length of time would be sufficient to accelerate the recovery process. For the same reasons, the frequency of the applications was reduced to alternate days, totaling three weekly sessions, which made the process more viable both clinically and economically.

The treatment with pulsed LIU began on the first day, immediately after the lesion was produced, to interfere with the healing process from its initial stage of acute inflammation as recommended by several authors[9,22,23]. The times for evaluation were set at three, seven, and 14 days according to Carvalho[22] because they concentrate most of the events which accompany the stages of skin healing[3,9,16,22,24].

The employment of the stationary application technique, which consists of applying the transducer directly to the treated area, had already been validated in previous studies[1,10-14]. However, because the wound left deep tissues exposed, this had to be done in the most sterile manner to avoid infections that might compromise the healing process. Thus, the deep tissues were doused in saline solution and isolated with plastic wrap, over which a layer of gel was applied. This technique isolated the wound but did not interfere with the transmission of the ultrasonic waves[25].

According to Simões[16], wound healing is a complex event that involves the interaction of several cellular and biochemical components and occurs spontaneously, without external interventions, but when treated by other means, tends to take place faster and with better functional and aesthetic results. In the case of chronic skin lesions, such as ulcers from various causes which rarely heal spontaneously, the use of external resources that might speed up the process are plainly justifiable, for they could mean the difference between healing or not. Ultrasound is one of these resources given that its beneficial effects are noticeable from the acute inflammatory phase up to the scar remodeling phase, effectively resulting in a faster healing process as observed on day 14, in which there was a significant difference in the exposed area compared to the first day.

Knowledge of the physiological events by which wound healing takes place is of great importance, and its contraction is an important phase in the closing of lesions that heal by secondary intention. It is considered by some investigators to be controlled by the myofibroblasts as they are found in great number near the edges of the contracted wounds, associated with the centripetal movement of the preexisting tissue[16,18,26]. The actual mechanism that triggers wound contraction is still controversial. It may start along the edges of the wound[2,24] or in its deepest parts, or in both places[3,24]. In the short term, the closing of a wound by means of the contraction of its edges is considered insufficient as there is usually a central area that closes by epithelization. Therefore healing is completed as the result of a combination of contraction and epithelization processes[18].

Epithelization is an early stage of the healing process. The proliferation of epithelial cells along the edges of the wound occurs in the first 24 hours, leading to a thickening of the marginal epithelium; meanwhile, the proliferated cells migrate on the dermis, led by fibrin matrix. The organs associated to the skin lose their normal structure and contribute to the progress of epithelization, which begin in the epithelial cells of hair follicles, fat glands and their ducts**.** Several epidermal growth factors released from the macrophage, such as TGF-β (transforming growth factor-β), EGF (epidermal growth factor), PDGF (platelet-derived growth factor) and IGF-I (insulin-like growth factor type I), speed up the epithelization[2,16,24].

In the relevant literature, there are references to the application of ultrasonic irradiation to the edges of the lesions[1,13,14,21], to their central area devoid of skin[25] or to both[9,15]. In the present study, the irradiation was applied to the edges and to the center of the lesion simultaneously due to the dimensions of the probe (22mm in diameter) and the affected area (10mm in diameter). This was done deliberately to stimulate the myofibroblasts, which are sensitive to ultrasound, thus increasing scar contraction, as reported by other authors.

The follow-up of the healing progress by measuring the circumference or area of the skin lesion is the most common parameter in physical therapy and medicine, which justifies its use in the present study. The measurement of the lesion area through computerized planimetry was chosen due to its low cost, easy use, and clinical applicability, as has been stated in several reports of its employment for follow-up of the progress of skin lesions in humans[17,27]. The computerized reproduction of the tracing, obtained directly from the wound with tracing paper, using image processing software and techniques allowed us to calculate its dimensions[17,27,28]. According to this parameter, the effects of the ultrasonic irradiation only appeared in the last period of observation, i.e. day 14 (C subgroups), when the differences between the groups were significant. That was not the case on day three (A subgroups) or on day seven (B subgroups).

The ultrasonic irradiation certainly had an effect early in the proliferating phase of healing, which starts around the third day after the lesion and continues for two to three weeks, resulting in the cellular infiltration of the wounded area, neoangiogenesis, collagen matrix deposition, reepithelization, and wound contraction. Cells such as fibroblasts and endothelial cells cluster in the wound area by means of a combination of migration and proliferation, and the stimulus that controls these events originates from several sources, such as the macrophages. They phagocytize the devitalized tissue and release growth factors, which are one of the most important sources. Thus, it is very likely that the proliferation of fibroblasts has been due, in part, to an indirect effect of the ultrasound, by means of the macrophages[9].

Young and Dyson[9] observed that, around the fifth day after they produced a skin lesion in rats, there was a considerably smaller contingent of inflammatory cells, but a greater amount of granulation tissue, in the animals submitted to ultrasonic irradiation. Also, the alignment of the fibroblasts in the wounded area parallel to their surface in the treated animals, compared to a random alignment in the untreated ones, was regarded as an indication of more advanced healing. According to the authors, these findings suggest that the stimulation to heal caused by ultrasonic irradiation must have occurred during the inflammatory phase of healing.

In animals like the rat, the scar contraction generated by cellular forces and contractile elements of the fibroblasts and myofibroblasts, which appear in the wound at the end of the first and beginning of the second week[16], is the main mechanism for the occlusion of the wound. Given that healing was faster in the irradiated animals, it is valid to propose that the ultrasonic irradiation also stimulated this mechanism. The post-operative observations were not extended to more than two weeks precisely because during this phase the events and differences between treated and untreated animals are more evident and the closing of the lesion is almost complete, as was observed in the present study. Its results are similar to those reported in the literature, according to which low-intensity pulsed ultrasonic irradiation, in fact, stimulates skin healing[1,13-15,21].

In view of the results obtained for the period between day seven and day 14, and by comparing the lesion area in the control and irradiated groups (p=0.002), we suggest that new research be carried out to determine on which day the low-intensity pulsed ultrasound for skin healing had the greatest efficacy.

Conclusions

The results achieved in the present study allow the conclusion that the low-intensity pulsed ultrasonic irradiation stimulates the healing of skin lesions by secondary intention, according to the applied parameters, particularly time and periodicity. Furthermore, computerized planimetry proved to be a low-cost resource that is easy to handle and applicable in clinical practice.

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