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High Intensity of Continuous Ultrasound in the Skin Repair Process in Rats: Risks to Tissue Integrity

Viviani de Marque Carrer[1], Auristela Duarte Moser[2]
[1](*Department of Postgraduate Program in Health Technology/ Pontifical Catholic University of Paraná, Brazil)*
[2](*Department of Postgraduate Program in Health Technology/ Pontifical Catholic University of Paraná, Brazil)*

Abstract: *Healing is a complex process involving different steps. Any change in one of them interferes with the normal repair process causing functional, morphological and aesthetic problems in the scar. Therapeutic ultrasound is a widely used and studied resource for cutaneous repair. However, divergences and parameters cause further scientific investigations. The experimental research sought to verify the effects of the high intensity of the therapeutic ultrasound in the cutaneous healing process of rats in the different stages 3, 7 and 21 days. Methodology: 60 animals, male rats, young adults, were irradiated in one SHAM group and another group with 3MHz continuous and intensity 2.0W / cm² for 5 minutes, 24 h after surgery. Results: The high intensity of the TUS in the continuous mode promoted burns, necrosis and poor healing in the irradiated areas. Conclusion: The high intensity of continuous TUS irradiated for 5 minutes was not therapeutic in skin repair of rats.*
Keywords: *Burn, Necrosis, Repair, ultrasound, wound healing*

I. Introduction

After injury, the inflammatory process begins. At this early stage the epidermis of the margins of the lesion by first intention become thicker and migrate from the deep margins to the upper part. The epidermis then joins the upper dermis, separating the necrotic tissue from the epithelium and the necrotic dermis where the crusty scar contains the dry blood clot, fragments of dead epidermis, collagen fibers, and elastin. Mitotic activity is restricted to the adjacent basal cells of the wound margins (1). Cells involved in inflammation can be clustered into cells present in vascular endothelial tissues, mast cells and macrophages, and cells that arrive from the bloodstream to platelets and leukocytes. Leukocytes are active and divided into polymorphonuclear cells and mononuclear cells. Mast cells are cells present in the damaged tissue and secrete various chemical mediators such as histamine, heparin, leukotrienes (2). The released histamine increases capillary permeability, makes vasodilation and facilitates the passage of fibrinogen. The proliferative phase lasts from 12 to 14 days (3) or up to 4 weeks (4). This phase is responsible for the closure of the lesion, subdivided into three phases: Reepithelialization, Fibroplasia and Angiogenesis (5, 6, 7). In the reepithelial phase there is migration of uninjured keratinocytes from the wound margins which proliferate, considering the growth factors PPARα and PPARβ, those responsible for the increase of the mitoses and hyperplasia of the epithelium. Keratinocytes have their cytoskeleton altered for keratin production. Its motion plane is also related to the water content present in the wound bed where resected wounds reepitheze more slowly (8; 7). When the reepithelialization is established, a new basement membrane is formed, starting from the edges of the wound, closing the new epidermis on the matrix (4). Due to the increase of fibrinogen, fibronectin and plasma proteins, a temporary stromal is created for the growth of fibroblasts. Migration and proliferation of fibroblasts are regulated by TGFα, PDGF, VEGF, FGF, mediators released by the macrophages and cytokines 1L1 and TNF formed at the site of the wound.

In the wound, growth factors produce and deposit large amounts of hyaluronic acid, fibronectin, collagens type I, III and VI. Then the fibroblasts bind to each other and to the extracellular matrix by tensioning around the contracting wound. The new tissue continues to grow inward from the margins of normal tissue. At the end of this stage the wound bed is completely filled by the granulation tissue that is slowly enriched with collagen fiber deposition promoting a fibrous mass, the scar (9). In the Remodeling or maturation phase there is a reorganization of collagen and an increase in the resistance of the scar. The remodeling involves successive stages of production, digestion and orientation of the collagen fibers (3). The order of deposition is related to fibronectin and is digested by the enzyme collagenase. At the end of this stage, scar staining is pale due to decreased melanocyte regeneration and absence of neocapillaris (10; 11). The scar is slowly considered to be avascular and may last for months or years. At this stage there is also a decrease in the number of fibroblasts and macrophages and an increase in the collagen content, whose fibers progressively align in the direction of increased wound tension. Several authors report the acceleration of wound healing and induction of collagen by the TUS (12; 13; 14). Therapeutic ultrasound promotes tissue heating, influences cellular metabolism, increases the extensibility of collagen constructs and can raise the local temperature by up to 45 ° C without causing injury (15; 16). The prevalence of the thermal effect on the mechanic occurs in intensities higher than 1.0 W /

cm², continuous mode in the frequencies 1 MHz or 3 MHz (17). There are discrepancies in the penetration depth of the ultrasonic emission and degrees of absorption (18; 19). Authors report that studies on TUS show methodological flaws and problems with equipment calibration (20). In this context, several authors suggest the application of low intensity ultrasound therapy in the healing process, the efficacy of the therapeutic ultrasound requires further clarification (21; 22; 23; 24; 25). There is unanimity among the authors: Need for ultrasonic therapy protocols regarding frequency, time of application, mode of emission and intensity.

II. Materials And Method

Sixty male Wistar rats, young adults, average weight 230g were used. The animals were kept in cages with the same environmental conditions, light / dark cycle of 12 hours, feeding, receiving water and feed *ad libidum*, for 22 days. The project followed the guidance of Law 6638 and the recommendations of the Brazilian College of Animal Experimentation. The animals were anesthetized with dissociation of the drugs Ketamine (60mg / kg) and Xilazine (10mg / kg) by administering 0.2ml for each 100g animal weight, intramuscularly, in the semitendinous muscle. The animal was then placed in the ventral decubitus position, fixing the lower and upper limbs to wooden supports to perform dorsal tricotomy. The 4 cm incision was measured with a pachymeter, obtaining exposure of the skin and subcutaneous tissue, sutured with simple nylon 2.0 thread. PVPI was used as antiseptic (26). After the end of the surgical procedure, the animals received Cephalexin (Cephalexin) at the concentration of 15mg / kg, subcutaneously, once a day for 5 days, with antibiotic effect (27). The ultrasound equipment has an analgesic effect (28; 29) and anti-inflammatory drugs (30). Two groups of 30 rats were randomly divided. Each group according to the intensities Control Group = Sham; Group 1 = 2.0W/cm². These were subdivided into 10 rats according to the healing stages: 1. Inflammatory phase (3 days); 2. Proliferative phase (7 days) and Remodeling phase (21 days). Therefore, 30 rats were exposed to ultrasonic therapy with 3MHz frequency, in continuous mode, intensity of 2.0W/cm² at 3, 7 and 21 consecutive days with onset of ultrasound therapy after 24 hours of injury for 5 minutes. The other 30 rats were exposed to ultrasonic therapy off.

Therapeutic ultrasound equipment was measured using a precision dosimeter (Ultra Sonic Power Meter®, model UPM-DT-1) and obtained a maximum deviation of 20%, the value being tolerated by NBR 60601-2 -5. Stimulation was performed at the same time with animals sedated with xylazine and ketamine in the above-mentioned proportions, with a 5-minutes treatment time remaining under anesthetic effect. After 3, 7 and 21 days of treatment the animals suffered euthanasia per lethal dose of intraperitoneal Sodium Thiopental (120mg / kg) according to Resolution 714 of the Federal Council of Veterinary Medicine of June 20, 2002. After euthanasia, a segment of the dorsum on which the scar was contained was removed. The segments destined for histology were fixed in 10% formaldehyde solution for 72h and 70% alcohol until the beginning of dehydration. The scar was sectioned into 5 mm and 1 cm portions for each side of the incision. Four cuts of each sample were selected from the extremities and center of the scar. After inclusion in paraffin blocks, they were submitted to cuts of 4 micrometers, being prepared the slides with 4 cuts in each blade. Histological sections were stained with Hematoxylin and Eosin to evaluate the basic structures of the cell as inclusions and alterations in the cytoplasm, and by the Sirius Red used to promote an increase in specific birefringence for the collagen types I and III structure for analysis of their fibers in the microscope with polarization (31). The microscope used was the Olympus BX50 brand with 3CCD pro-series capture cameras and the image capture program will be ImageProPlus version 4.5 of Cybermetics®. The images were captured by an Olympus® DP71 camera, sent to a Sony Trinitron® color monitor, frozen and scanned by an Oculus TCX® (coreco) scribing board, to be analyzed by the ImageProPlus 4.5 application for Windows on a computer. For each slide, a reading was performed in three fields following the path of the scar, with magnification of 400x, for the quantification of type I and III collagens stained in Sirius Red with polarized light. In the group treated with ultrasound 3MHz and 2.0W/cm², the collagen from the scar edges was quantified, as there was necrosis at the center of the lesion, making impossible any qualitative or quantitative analysis at the site itself. In addition, objective readings of 40x and 400x were used for the qualification of the dermis, epidermis, granulation tissue and repair. Statistical Analysis: For Hematoxylin and Eosin stained slides, we used a qualitative analysis by histological description of the epidermis, dermis and subcutaneous tissue. The results obtained by reading the types I and III collagens with Image ProPlus 4.5 Program were expressed as means, medians, minimum values, maximum values and standard deviations. For the comparison of groups and moments of sacrifice we used the analysis of variance model and the LSD test for multiple comparisons. The collagen III variable underwent a logarithmic transformation. Values of $p < 0.05$ indicated statistical significance.

III. Results And Discussion

The slides stained with H.E. were qualitatively evaluated with light microscopy using increases of 40 and 400x. The description of the blade verifies the thickening of the epidermis, preserved attachments, absence or presence of cellular proliferation involved in the repair and healing itself.

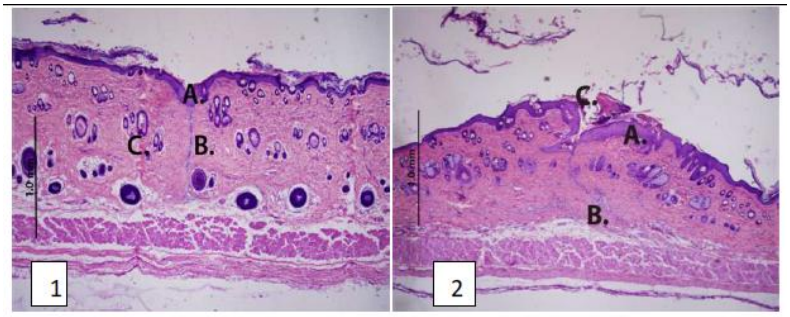

Fig.1. Inflammatory Phase. 1.1-Photomicrography of the animal of the control group- sham, 3 days of TUS. Hematoxylin-Eosin staining. Panoramic view, optical microscopy, 40x magnification. Note: A1.1. Stratum corneum thick; B1.1 Granulation fabric in formation and aligned; C1.1. Dermal annexes organized. 1.2- Photomicrography of the animal of group 1. Coloration Hematoxylin-eosin. Note: A1.2. Thickening of the stratum corneum; B1.2. Presence of granulation tissue; C1.2. Necrotic tissue.

As Fig. 1 shows a thickening of the epidermis can be observed. We identified an alignment of the granulation tissue at the incision area. Also, reorganization and presence of the appendages of the dermis, proximity between the edges of the lesion during the tissue regeneration phase for the control group. As Fig. 1.2 there is a disorganization and thickening of the epidermis. There is formation of granulation tissue at the edges of the incision in all animals of group 1 in addition to morphological alterations of the dermis attachments. An intense disorganization of the dermis was observed in the proximal region of the incision with disarrangement and misalignment of the deeper layers. Mortimer and Dyson (32); Leung et al. (33) observed degranulation and lesion in mast cells. Bem et al. (34) evaluated the TUS in mouse skin, continuous mode, 3 MHz, in intensities 0.5; 1.0; 1.5 and 2.0 W/cm² in 4 days, 2min .: Increased epidermal thickness, inflammatory infiltrate, thinning of collagen fibers at intensities 1.0; 1.5 and 2.0 W / cm². They suggest precautions in the use of TUS mainly in high intensity esthetic treatments, with the risk of causing internal lesions that may appear late.

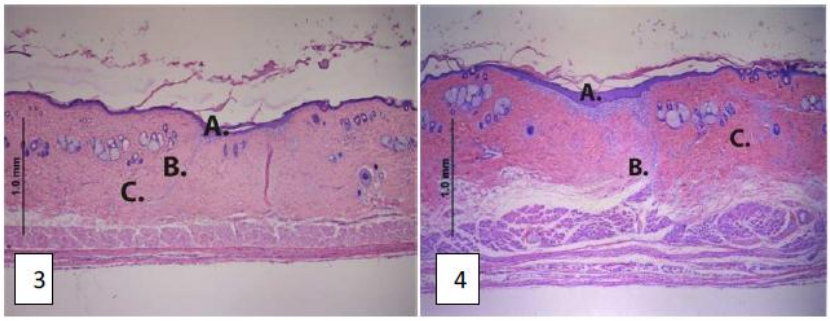

Fig. 2. Proliferative Phase. 2.3- Photomicrography of the animal of the group sham, treated TUS for 7 consecutive days. Panoramic view, optical microscopy, 40x magnification. Hematoxylin-eosin staining. Note: A2.3. Thick epidermis; B2.3. Concentration of granulation tissue; C2.3. Alignment of granulation tissue. 2.4- Photomicrography of the animal of group 1. Coloration Hematoxylin-eosin. Note: A2.4. Wide area of thick stratum corneum; B2.4. Distal granulation tissue concentration; C2.4. Dispersion of the granulation tissue in the dermis.

In Fig. 2 it is possible to observe in all the animals of group sham, decreased thickness of the stratum corneum, great concentration of granulation tissue in the region of the dermal papillae, presence of alignment of the granulation tissue along the region of incision in the dermis. In this phase we found that there were no appendages of the dermis near the edges of the incision. There is an organization in the deeper layers of the dermis. On the other hand, in the group 1 animals, a large area of epidermal thickening was observed, bilaterally at the site of the surgical incision, absence of dermal papillae along the epidermal layer at the lesion site. There is presence of granulation tissue dispersed between the layers of the dermis and epidermis, a large area with absence of appendages of the dermis and derangement of the dermis with involvement of the hypodermis and muscular layer.

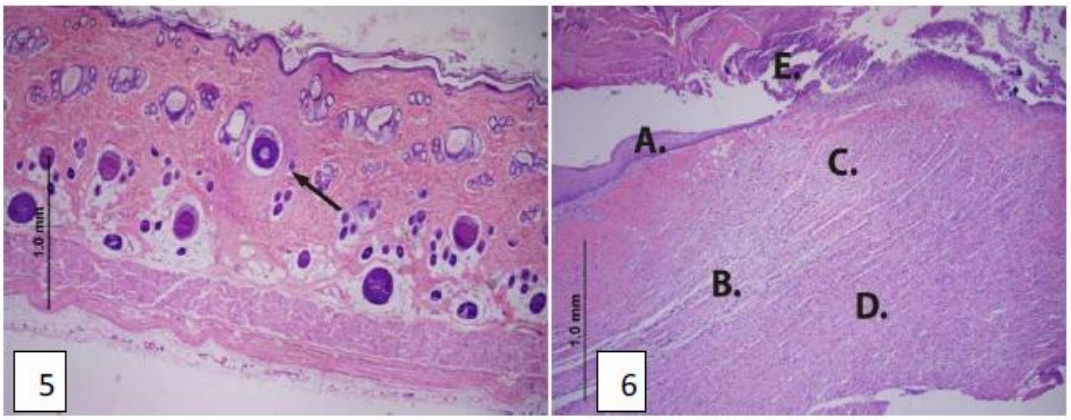

Fig. 3. Remodeling Phase-3.5. Photomicrography of animal from the group sham, treated by TUS in a period of 21 consecutive days. Panoramic view, optical microscopy, 40x magnification. Hematoxylin-eosin staining. Note: The arrow shows tissue repair area. Photomicrography of the animal of group 1. Note: A3.6. Thickening of the stratum corneum; B3.6. Absence of dermal papillae; C3.6 Absence of dermis attachments; D3.6 Intense granulation tissue; E3.6 Necrotic tissue.

It was possible to observe according to figure 3 that there was a similar pattern of tissue repair in all animals of group sham at 21 days. It is observed a reduction of the granulation tissue, presence of appendages of the dermis, which demonstrates the process of reorganization of the dermis and also of the epidermis in the regeneration of the area of injury. All animals in group 1 presented burns, crust, necrosis and lesion at the incision site, making quantification impossible. Therefore, material from the edges of the lesions was used. Large thickening of the stratum corneum, formation of crust and necrotic tissue was observed. Also, intense disorganization of the dermis with large area of distribution of granulation tissue, intense loss of the dermis attachments, absence of the normal pattern of tissue repair and presence of giant cells. There were losses of the hypodermic and muscular layers. In a study on ultrasonic therapy in rabbit ears, researchers observed an induction of venous thrombosis, increased lymphocytes, necrosis in addition to edema, erythema and heat, at the intensity of 3.0W/cm², in the continuous mode (35). Variable Collagen Type I. A significant difference between groups was considered for $p < 0.05$. When comparing the intensities of the groups in 3 days, with the quantification of the area of the type I collagen, its means and standard deviations, no significant results were obtained ($p = 0.089$). The same happened for the type I collagen variable for the time of 7 days $p = 0.175$. There was no significant difference between the quantified area related to high intensity applied. When comparing the group SHAM and the group 1 in 21 days of therapy with ulstrasound there was significance between ($p < 0.001$) for type I collagen and quantified area. The control group presented a higher mean number of collagen type I in the scar area (34.48% and ± 8.83) than the group treated with the intensity 2.0W / cm² (7.10% and ± 3.50).
When analyzing the moments of sacrifice of group 1, a significant difference ($p = 0.018$) was observed between the means of the amount of collagen type I in 3 and 7 days. In 3 days we have 13.64% of type I collagen in the scar area to ± 5.58 and 21.18% and ± 9.58 in 7 days. When comparing sacrifice times 3 days with 21 days in this group we have a significant decrease of the type I collagen variable ($p = 0.038$). There was a decrease in the amount of collagen type I of 13.64% and ± 5.58 in 3 days to 7.10% and ± 3.50 for 21 days. In this same group, the average amount of type I collagen was compared in the area of the incision edges between 7 and 21 days. There was a significant ($p < 0.001$) decrease in the amount of this collagen from 21.18% to 7.10% and a standard deviation of ± 9.58 to ± 3.50. Variable Collagen Type III. For the comparison in which there was a significant difference between the groups, the comparisons of groups two and two for the type III collagen were made. Considering each group, we tested the null hypothesis that the mean of collagen III is equal for the moments of sacrifice versus the alternative hypothesis that at least one moment has a mean different from the others ($p < 0.05$). In group 1 it was possible to observe statistical significance for type III collagen between 3 and 21 days ($p < 0.001$) and between 7 and 21 days ($p < 0.001$). It did not present statistical significance for type III collagen between 3 and 7 days ($p = 0.051$). The performance of ultrasonic therapy in the intensity 2.0W / cm² was notorious, where at all times it was worse when compared to the SHAM group. The non-significant increase of the type III collagen variable in 21 days in the intensity 2.0W / cm 2 indicated the delay of the healing process. Authors reporting high intensities may be less effective than low dosages in cutaneous repair (36; 37).

IV. Conclusion

The high intensity irradiated in the time of application of 5 minutes of the Therapeutic Ultrasound in the frequency of 3MHz, in the mode of continuous emission, was not effective in the process of healing by first intention in the cutaneous repair of rats. The therapy caused skin burns, tissue necrosis to the muscular layer of the incisional dorsal region, as well as presence of giant cells and reverberation of the repair process. Further studies should be performed at different intensities, frequencies and time of application.

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