# ULTRASONIC STIMULATION OF TOTAL SKIN GRAFTS UNION. AN EXPERIMENTAL STUDY IN RABBITS.

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## SUMMARY

*An experimental histological study was performed addressing the influence of therapeutic ultrasound on total-thickness skin grafts union in 20 adult female rabbits. Two 2-cm square-shaped grafts, were obtained at scapular regions with sides switched. The graft at right was irradiated on a daily basis (3 MHz, 0.5 W/cm2, for 5 minutes) from the third day on, during seven consecutive days; the graft at left received a sham treatment. The histological study in 5 μm-thick slides obtained from each graft and alternatively stained with Gomori trichromic, PCNA and picrosirius, included measurement of epidermal and dermal areas, proliferating cells on germinative layer counts, and neoformed vessels and collagen fibers direction. A significant increase of the number of proliferating cells (p=0.007) and of neoformed vessels (p=0.0001) was noticed on irradiated grafts, but not on epidermal and dermal areas. The authors concluded that the T-U/S induces morphological changes in biological processes involved on total-thickness skin graft union, such as germinative cells proliferation and new blood vessels formation, suggesting the potential for its clinical use in human beings.*


**Keywords:** *Skin transplantation; Ultrasonic Therapy; Rabbits.*

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## INTRODUCTION

Skin autologous grafts are certainly the most largely employed procedure for providing coverage to skin defects resulting from a wide variety of causes. They can be used in burn lesions, traumatic abrasions or avulsions, skin necrosis following surgery or trauma, congenital defects undergoing surgical fixing, and so on, in an almost infinite list of conditions, which roughly all plastic or orthopaedic surgeons routinely face.

In spite of that, the outcomes of skin grafting procedures are not always fully satisfactory, but, to date, there is no well-established means to stimulate union and to maximize aesthetic and functional outcomes of grafts. On the other hand, physical agents, such as electricity, magnetic or electric fields, and ultrasound may positively influence the results of the healing process or different tissues regeneration such as the skin, bones, muscles, tendons and peripheral nerves. Therapeutic ultrasound irradiation (UST) effectively reduces edema, increases local blood flow, relaxes muscles, relieves pain, and hasten tissue repair and modifies scar formation[1-8]. Concerning skin, there are evidences showing that UST promotes regeneration [5,9], a reason for its use in varicose ulcers treatment [2,10] or in pressure sores[11] have already been investigated regarding its ability to hasten and improve union and quality of skin grafts [12].

In what concerns to the latter, there is nothing established yet, and this is the purpose of the present trial: to study the role played by UST on total thickness skin-free grafts union in rabbits.

## MATERIAL AND METHODS

This study was approved by the Committee of Ethics on Experimental Animals Use, Medical School, Ribeirão Preto (SP). Twenty female New Zealand rabbits were used, and their average weight was 2.5 kg (range: 2.2 - 2.7 kg). Before and after surgery, the animals were kept in individual cages with free access to food and water. All rabbits received a subcutaneous injection of a weight-dependent dose of antibiotic agent (Penicillin procaine, 400,000 UI) immediately before surgery, for infection prophylaxis purposes.

Surgical procedure: the animals were anesthetized with an initial endovenous dose of sodium pentobarbital (Nembutal Abbott®, 30 mg/kg of body weight) applied on the ear marginal vein; occasionally, if an animal showed to be in pain, an additional dose was administered during the procedure. All scapular region was prepared for surgery as usual (trichotomy, antisepsis with 20% iodine alcohol solution) and the surgical field was covered by surgical drapes. Two squared areas of 4 cm2 (2 x 2 cm) were designed with the aid of a metal matrix, each one at each side of the scapular region and medially to the scapula, sectioned with a blade nr. 15 and fully removed as a skin-free graft, and then wiped for any subcutaneous tissue and fat debris. Both donator beds were reviewed for rigorous homeostasis and the grafts

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were sutured again, switching sides (the right one on left's bed and vice versa) with isolated stitches of 5/0 mononylon, being one at each angle and one at each side. Sutured grafts were covered with petrolate gauze, and a Brown's tie-over dressing was placed (Figure 1).

**Ultrasonic irradiation:** the dressing was removed on the third postoperative day and grafted area was carefully cleaned with saline solution in order to remove any blood or secretion debris, sprinkled with 20% iodine alcohol solution and allowed to dry. In order to avoid involuntary mobilization of the graft over the bed, all the region was abundantly covered by sterile liquid petrolatum and a plastic film (home polyvinyl chloride, 0.001-mm thick), which was covered by coupling gel of the ultrasonic irradiation head-stock. Irradiation was applied directly on the graft, using a portable clinical use equipment (SONACEL® Plus III, series 1050R)*, equipped with a small-gauge headstock specially made (1.35 cm diameter, 1.43 $cm^2$ area) and prepared for pulsed irradiation (1:2, or 50%) at 3 MHz frequency and 0.5 $W/cm^2$ power (SATA). The equipment was calibrated with a precision dosimeter (Ultrasonic Power Meter, model UPM-DT-1) prior to irradiation and periodically thereafter.

Daily 5-minute sessions were performed, starting immediately after dressing removal and repeated for 7 consecutive days, always at the same time, for following a 24-hour regimen between sessions. The ultrasonic headstock was gently moved over the graft in order to avoid its detachment from bed. An identical procedure was performed on the right grafted area (Group 1) and on the left one (Group 2), but with the equipment turned off in the latter, in order to cause the only common effect of massaging on both sides. A slightly compressive dressing was applied after irradiation sessions, keeping a petrolate gauze on grafted area in order to prevent adhesions.

**Histological preparations:** animals were killed on the 11th postoperative day with an endovenous injection of a massive dose of anesthetic agent, and each grafted area was resectioned, each one comprehending a squared area of skin measuring 4 cm x 4 cm with the graft in between. The resectioned skin was fixed on a carton frame, identified and sunk in a 10% formaldehyde water solution for 2 days for fixation, and after that, the graft was cut out from the remaining receptor skin and routinely processed for histological studies. Series of 5$\mu$m thick cross-sectioned areas were obtained, starting from one of the edges towards graft core, and alternatively stained with Gomori trichromic, with proliferation cell nuclear antigen (PCNA)[13] or with Picrosyrius. Each graft yielded about 500 serial cuts, 50 of which (1:10) were examined and 10 (1:5) were effectively analyzed. Each slide was mounted with five sections of the irradiated graft, side by side with five of the control graft of the same animal, in order to enable comparison. Sections were examined under light microscope (Carl Zeiss Axiophot) or under polarized light microscope (sections stained with Picrosyrius), equipped with a video camera attached to a microcomputer equipped with Snappy software (Video Snapshot) for image capture.

Sections stained with Gomori trichromic, which clearly show the boundaries between skin cells layers, were used for measuring epidermis and dermis area; using low magnification (25 x) and the Scion Image 402 software, three distinctive fields of the epidermis and dermis were selected on each section so that the length of each layer was the same for each field, with the only variable being thickness. With length and thickness measurements in hands, the epidermis and dermis area ($\mu m^2$) was calculated for each respective section. Still on these sections and with a high magnification (400X) neoformed vessels were sectioned on reticular dermis, next to the transition between the graft and receptor bed, on five different fields of each section, followed by calculation of an average value for each section, each graft, and lastly, for each group.

Proliferating cells were counted on the germinative layer of the skin, on sections stained with PCNA and examined under direct light microscope (Jenamed 2). First, three distinctive fields were selected, as described above, with all cells being counted, with high magnification (400 x). Then, only those cells strongly stained were counted, with frequency being expressed as a percentage of the total amount.

Collagen fibers orientation was assessed under moderate magnification (100 x) on sections stained with the Picrosyrius, using a polarized light microscope, with findings being merely descriptive.

Statistical analysis: non-paired data for epidermis and dermis area measurements were submitted to statistical analysis with the Wilcoxon's non-parametric test, at a significance level of 5% ($p \leq 0.05$). The Student's T-test was used for paired data analysis for proliferating cells and neoformed vessels counts, again at 5% significance level ($\leq 0.05$).

## RESULTS

**Gross findings:** no evident gross difference was observed between irradiated grafts (Group 1) and control grafts (Group 2) on the 11th postoperative day. Small scattered areas of necrosis were show, near to graft's edges and borders, in both groups. Bilateral partial epidermolysis (irradiated and control grafts) was found in four animals, and unilateral (control side) in one animal, which aesthetic appearance seemed to be better than those of necrosis-free grafts. There was no infection case.

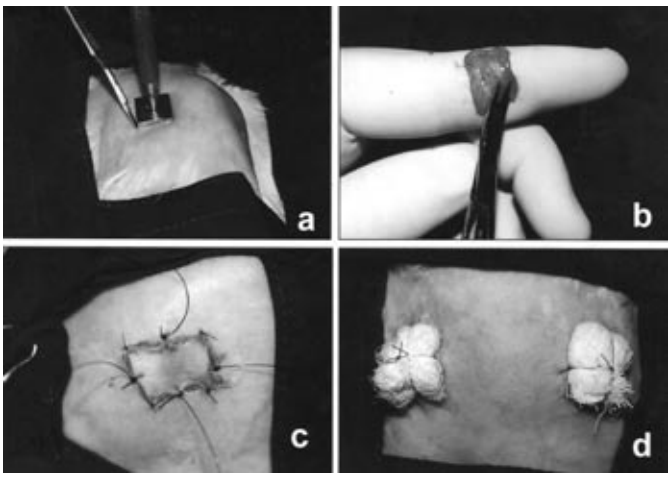


***Figure 1 -*** *Surgical procedure steps. Skin is marked with the 4 $cm^2$ (2 x 2 cm) metal matrix (a); graft being cleaned from any remaining subcutaneous tissue debris, and (b) already sutured and back to inverted bed; (c); Brown´s tie-over dressing(d).*

**Epidermis and dermis area:** the average epidermis area measured 246,392 $\mu$m2 (median: 220,641 $\mu$m2) in Group 1, and 200,626 $\mu$m2 in Group 2 (median: 173,664

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$\mu$m2), but the differences between groups were not significant (p=0.07). The average dermis area measured 2,157,730 $\mu$m2 (median: 2,164,660 $\mu$m2) in Group 1 and 2,109,150 $\mu$m2 (median: 2,102,330 $\mu$m2) in Group 2, with differences between both being not significant as well (p=0.21) (Figure 2, Table 1).

**Proliferating cells and neoformed vessels:** there was a significant increase (p=0.007) on the number of proliferating cells in Group 1, because they accounted for an average of 12.18% of the total amount, compared to 7.34% in Group 2 (Figure 3, Table 2). Similarly, there was a significant increase (p=0.0001) on the number of neoformed vessels on dermis reticular layer in Group 1, where these were counted on a range of 6.27 vessels by field, in average, compared to 3.07 vessels by field in Group 2 (Figure 4, Table 3).

**Collagen fibers orientation:** both in Group 1 and in Group 2, the usual pattern of main longitudinal collagen fibers parallel to the surface and secondary cross-sectional oriented towards skin surface, only with a slight disarrangement was maintained. No important difference was seen between both groups (Figure 5).

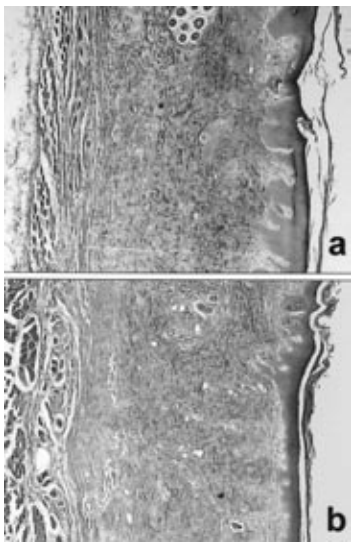


***Figure 2 -*** *Histological cross-sections showing an irradiated graft´s trend to epidermis thickness augmentation (a), compared to control (b) (Gomori´s trichromic, 25x).*

***Table 1 - Epidermis and dermis area*** *($\mu$m2) on irradiated and control grafts.*

| | Epidermis | | Dermis | |
|---|---|---|---|---|
| | **Irradiated** | **Control** | **Irradiated** | **Control** |
| **Median** | 220,641 | 173,664 | 2,164,660 | 2,102,330 |
| **Average ± SD** | 246,392 ± 92,394 | 200,626 ± 77,576 | 2,157,730 ± 411,701 | 2,109,150 ± 501,475 |

## DISCUSSION

Either they are temporary or permanent, skin autologous grafts are very important and appropriate for skin coverage of nearly any kind of loss, either acquired or traumatic. Most of cases, skin grafts union occurs with no intercurrences, but sometimes they may be lost due to a variety of local or generalized causes. Since overall health status of a patient is good, and the receptor bed is appropriate, a surgeon may decide on taking a partial or total thickness graft among fragmented grafts, as a mesh or slide. In any case, once the procedure is finished and the grafted region is protected with a proper dressing, there's little to do but wait for the outcome, in average, five to seven days later, when dressing is removed. In about 20% of the cases, the total skin graft undergoes some degree of necrosis, most of times affecting granular, lucid and corneal layers, in which cases the graft becomes united, although with a compromised aesthetic result. When basal or germinative layer, and the spindle cells layer are involved, the graft is fully compromised, requiring a new repair procedure.

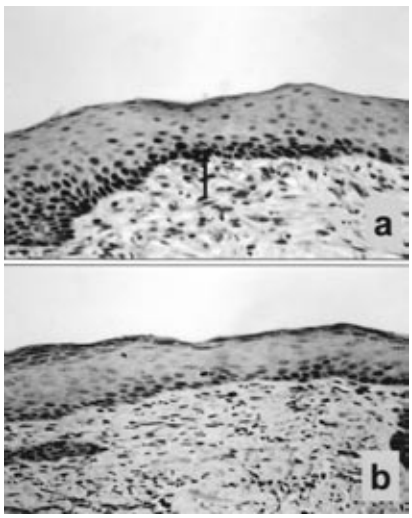


***Figure 3 -*** *Histological cross-sections showing increased concentration of proliferating cells on basal layer (arrow) of the irradiated graft (a), compared to control (b) (PCNA, 400x).*

***Table 2 -*** *Percentage (%) of proliferating cells by field compared to total number of cells.*

| | **Irradiated** | **Control** |
|---|---|---|
| **Average ± SD** | 12.18 ± 3.01 | 7.34 ± 1.9 |

The UST is used for treating a variety of pathological conditions of the musculoskeletal system [2], but little is known about its effects on skin. However, there are evidences showing that it stimulates skin injuries healing process, since Dyson et al.[9] showed the beneficial effects of pulsed UST (0.5 W/cm$^2$, applied for 5 minutes a day, in alternate days, during 21 days, starting on the 14th postoperative day) on injuries healing affecting skin total thickness, produced on rabbits' ears; according to those authors, the UST hastened healing process in its early phases, but no difference was seen after the 35th day, when compared to untreated injuries. Also, Dyson and Suckling[2] used pulsed UST (0.8 MHz, 0.2 W/cm$^2$, applied during 5 minutes on a daily basis) for treating varicose ulcers of lower limbs and reported a significant reduction of injury areas, a result also reported by Roche and West[10] with different UST parameters (3 MHz, 1 W/cm$^2$) for ulcers with areas smaller than 5 cm$^2$. Ivanov[12] gave a short statement saying that UST hasten union and enhances outcomes of skin grafts fixed on receptor bed with cyanoacrilate. However, to date, the biological phenomena involved in skin graft response to ultrasound irradiation are not totally clear, which fact motivated the present experimental study, giving particular attention to germinative cells proliferation and neoformed vessels stimulus.

In the experimental model used, the total thickness skin graft was preferred to the partial thickness one, because, in the previous, thickness is standardized since the beginning, furthermore, total thickness ones are shown to me more difficult to unite, facilitating UST effects detection and the comparison between irradiated and control grafts. The procedure was performed at the scapular region for two reasons: first, this region is out of the reach of the animal itself, thus more protected from any contamination; secondly, the skin on this region shows more appropriate tegumentary characteristics to the study, including its thickness. Receptor beds were carefully prepared, giving special attention to homeostasis and hematoma prevention, which could detach a graft from its bed, ultimately leading to necrosis. Grafts were sutured to beds' edges in order to keep its original dimensions and to enable a compressive Brown's tie-over dressing to be applied, which was removed on the third day for starting UST irradiation; in

a clinical situation in humans, this dressing is usually removed one week later, when the graft is already healed on bed.

Irradiation with UST started on the third postoperative day, because in this early phase tissue healing can be hastened [9]. Furthermore, irradiation is usually applied around skin defect in order to stimulate healing from wound periphery towards the center, but, in the present investigation, it was directly applied on the graft, aiming to stimulate vascular neoformation from the receptor bed, and cell proliferation both from it and from the graft itself. The graft was properly protected during irradiation in order to avoid its detachment from bed, which would certainly result in necrosis; on the other hand, the material used for protecting it (liquid petrolatum, PVC film) causes no influence whatsoever over its metabolism and does not interfere in its union [14]. Pulsed UST was regulated with 3MHz frequency and 0.5 W/cm$^2$ power, because the strongest physiological effects are achieved with these parameters. Indeed, with high frequencies, only the most superficial tissues are affected, and with low powers, UST's thermal effects are minimized, while in its other properties, they are emphasized. Furthermore, the ultrasonic headstock was continuously kept at upright position to graft surface and in total contact with it, being continuously moved for avoiding the formation of localized heating zones that could injury it [15].

The results of irradiation were purposely analyzed in an early phase of union, on the 10th postoperative day and after seven irradiation sessions. During this period, graft union, which is much faster in rats, is still an ongoing process and in which differences between irradiated and control grafts are more evident; in a more advanced phase, with grafts fully united, there are nearly no differences between each other, including cell proliferation and neoangiogenesis stimulation. Although not spectacular, the results achieved were conclusive in demonstrating that UST did stimulated cell proliferation on epidermis germinative layer, as seen on histological sections stained with PCNA[16], and the neoformation of blood vessels on dermis reticular layer, as previously described [17], which is a response that certainly favors graft union. However, the highly significant increase on cells proliferation (p=0.007) did not imply in a correspondent increase on epidermis area, which presented only a trend to augmentation (p=0.07), which probably means that other factors may affect skin grafts union. Dermis morphometry data (number of cells and area) were not significantly different between irradiated and control grafts (p=0.21). Also, no difference was seen regarding collagen fibers orientation in both experimental (Picrosyrius stain), which means that it doesn't importantly change on skin grafts in general.

The authors conclude that UST has stimulated morphological changes at cell level, resulting in an increased germinative layer proliferation, in new blood vessels formation, and in an early union of total thickness skin graft, an effect that may b e useful in clinical situations in human beings.

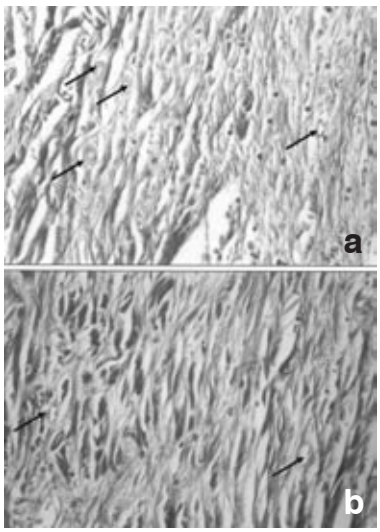


***Figure 4 -*** *Increase of neoformed vessels concentration (arrows) on irradiated graft dermis (a) compared to control (b) (Gomori´s trichromic, 400x).*

***Table 3 -*** *Number of blood vessels by field.*

| | Irradiated | Control |
|---|---|---|
| **Average ± SD** | 6.27 ± 2.59 | 3.07 ± 1.75 |

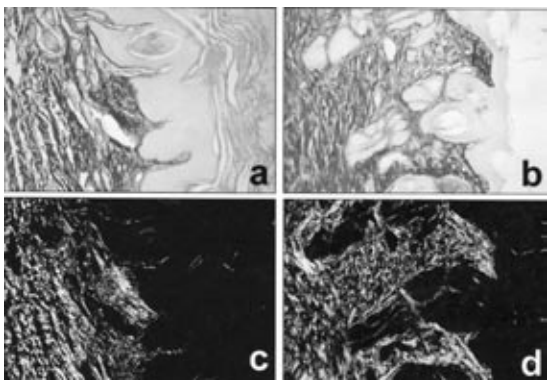


***Figure 5 -*** *Stained histological sections (epidermis on the right; dermis on the left) examined under direct light (a and b) and polarized light (c and d), showing absence of difference on collagen fibers orientation on irradiated graft (a and c) and on control graft (b and d) (Picrosyrius, 100x).*


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