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# Effect of ultrasound and dexpanthenol on collagen organization in tegumentary lesions

## Efeito do ultrassom e do dexapantenol na organização das fibras colágenas em lesão tegumentar

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## Abstract

**Objective:** To analyze the effect of ultrasound (US), dexpanthenol (d-P) and a combination of these treatments (US+d-P) on collagen fiber organization in tegumentary lesions in rats by birefringence analysis. **Methods:** Wistar rats (50) were anesthetized (Thionembutal - Sodic = 50mg/Kg), 1cm$^2$ of dorsal region skin was removed, and the animals were divided into five groups: control (C), gel (G), US (3 MHz, 0.1 W/cm2, 1 minute, continuous), d-P (10%) and US+d-P. After daily treatment for 7 and 14 days, 6µm thick sections of lesioned areas were stained in picrosirius and measurements of the collagen birefringent area (µm$^2$) were obtained using polarized light microscopy (Zeiss Axiolab-ZEISS- Germany) with histological image analysis software (KS 400 2.0 - Kontrol Eletronics, Munique, Germany). The means were compared by ANOVA followed by the Tukey test ($p<0.05$). **Results:** The US+d-P group showed a significantly greater ($p\leq0.001$) birefringent area (1586.43±162.14) than the other experimental groups: C (139.36±35.35), US (317.55±129.9) and d-P (192.41±3657) by the 7$^{th}$ day of treatment, indicating acceleration of the wound healing process. By the 14$^{th}$ day of treatment, the US+d-P, US and d-P groups presented greater birefringence than the control group, but did not differ from each other. **Conclusion:** The combination of treatments (US+d-P) accelerated collagen fiber synthesis and organization in the early stages of cutaneous repair.

**Keywords:** ultrasound; dexpanthenol; collagen; physical therapy.

## Resumo

**Objetivo:** Analisar o efeito do ultrassom (US), do dexapantenol (d-P) e da associação dos tratamentos (US+d-P) na organização de fibras colágenas na lesão tegumentar em ratos por meio da análise da birrefringência. **Métodos:** Foram utilizados 50 ratos Wistar, anestesiados com Thionembutal Sódico (50mg/Kg), dos quais foi retirado 1cm$^2$ de pele da região dorsal, divididos em cinco grupos: controle (C), gel (G), US (3 MHz, 0,1 W/cm$^2$, 1 minuto, modo contínuo), d-P (10%) e US+d-P. Após sete e 14 dias de tratamento diário, foram removidos segmentos dessas áreas e obtidos cortes de 6µm de espessura que, posteriormente, foram corados em Picrosirius. Os cortes foram observados em microscopia de polarização utilizando um software responsável pela medida de birrefringência das fibras colágenas (KS400 2.0 - Kontrol Eletronics). As médias das áreas birrefringentes (µm$^2$) de cada grupo foram submetidas à análise de variância pela ANOVA, seguida do teste de Tukey ($p\leq0,05$). **Resultados:** A média de área birrefringente do grupo US+d-P (1586,43±162,14) foi maior ($p\leq0,001$) que a dos grupos experimentais (C: 139,36±35,35, US: 317,55±129,9 e d-P: 192,41±36,57) no 7º dia de tratamento, indicando uma aceleração na síntese e organização das fibras colágenas na região lesionada. No 14º dia de tratamento, os grupos US+d-P (2858,47±510,17), US (1779,94±482,78) e d-P (2546,88±304,45) apresentaram área birrefringente maior que a do grupo C, porém não diferiram entre si. **Conclusão:** A associação dos tratamentos (US+d-P) acelerou a síntese e a organização das fibras colágenas apenas no estágio inicial de reparo tegumentar.

**Palavras-chave:** ultrassom; dexapantenol; colágeno; fisioterapia.

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# Introduction

Collagen is the most abundant protein in animal organisms and it is also the main protein structure of the connective tissue. There are several types of collagens, although types I, II, III and IV are the most common[1]. Collagen type I the is most prevalent in the dermis, although types III, IV and V can also be observed on a smaller scale.

Collagen molecules aggregate through crosslinking, and this highly organized net of chemical interactions turns the collagen into an organic crystal, which explains why it is resistant to tensions and pressures[2].

Variations in the organization and molecular order of collagen bundles may be detected and measured during the repair process by analysis of their anisotropic properties (birefringence)[3].

Total birefringence is dependent on the geometry of the molecules, the partial volume (concentration), the aggregation level, the orientation of the bundle components and the oscillatory strength of all electronic transitions in the molecule, as well as the collagen crosslinkings[3-5].

The repair process of a wound has three phases that continually overlap: inflammatory, proliferative and remodeling, and it involves several biochemical and cellular alterations[6]. Regardless of the tissue, collagen is the most important component in the repair process.

Skin lesions are treated with vitamins such as dexapanthenol or d-panthenol (d-P), which is a pro-vitamin of the B complex that, when topically applied, is converted into pantothenic acid, a natural skin constituent[7]. Ebner et al.[8] observed *in vitro* and in vivo that fibroblast activation with d-P leads to an acceleration of the epithelization of wounds, creating an epithelium with a high organizational level of epidermal structures.

As a therapeutic modality, ultrasound (US) has been used both in the tissue repair process and in the treatment of wounds[9-12]. Studies have demonstrated an increase in collagen synthesis, fibroblast proliferation and local microcirculation with ultrasound treatment[10,13,14], although according to ter Haar14, in many of these effects, the mechanism of action is unknown. Some studies have demonstrated a risk of alterations in healthy skin[15] from the continuous use of 3 MHz US at intensities from 1 to 2 W/cm2 as well as *in vitro*[16] skin samples irradiated above 2.5 W/cm2.

Due to the difficulty of transdermal drug permeation, the use of physical accelerators such as US in a therapy called phonophoresis[17,18] has been studied. The research group responsible for the above-mentioned study also verified that not only did US accelerate the tegumentary repair process but its combination with d-P[19] further accelerated that process. Their study also showed increased re-epithelization of the wound after seven-days of treatment, although it did not quantitatively study collagen fibers, which justifies the present study. The present study tried to verify if these treatments alone and/or in combination could alter the organization of collagen, which is of great importance in the healing process.

Since US requires a coupling agent, the addition of d-P to that agent may accelerate collagen synthesis in lesions thus treated. Therefore, the aim of this study was to analyze the effect of US, d-P and a combination of these treatments in tegumentary lesions in rats by histomorphometric analysis of collagen fiber birefringence.

# Methods

## Animals

This study was approved by the Committee for Ethics in Animal Experimentation of the Universidade Federal de São Carlos (UFSCar), São Carlos, SP, Brazil (protocol nº 020/2006). Fifty male Wistar rats, weighing approximately 250g each, were maintained in the Biotery of the Universidade Metodista de Piracicaba (UNIMEP), Piracicaba, SP, Brazil, with a controlled light/dark cycle (12h/12h) and water and chow ad libitum in individual cages in which the litter was changed daily.

## Experimental procedures

After anesthesia with sodium phenbarbital (50mg/Kg), the animals were trichotomized in the dorsal area, from which 1cm$^2$ of skin was removed, including the hypodermis, using a scalpel and a hollow template. All animals received only one application, in the lesioned area, of polyvinyl-pyrrolidone-iodine (PVPI) 10.0g with 10% stabilizer and softener after the surgery.

## Experimental groups

The animals were divided into five experimental groups (n=10): control (C), gel (G), gel + ultrasound (US), gel + dexapanthenol (d-P), gel + US+ dexapanthenol (US+d-P). Five animals from each group received treatment for seven days and the other five animals for 14 days; euthanasia was carried out on the 8th and 15th day, respectively. The daily treatment of all animals occurred only in the morning period and was carried out by the same researchers.

### *C group*

The animals of group C received no treatment after the surgical procedure.

*Gel group*

Carbopol 940® was the gel used; it consists of carbomer, propylene glycol, triethanolamine, methylparaben and distilled water. Two grams of gel were applied daily over the entire lesion with a spatula.

*d-P group*

This group received gel plus 10% d-P, with 2g applied daily over the lesion, as in the previous group.

*US and US+d-P groups*

US (Sonomaster Dual, KW Eletrônica Ltd., Amparo, Brazil) was applied daily to the lesion of the US and US+d-P groups. The parameters were: a 3 MHz frequency[11,20], a 0.1 W/cm$^2$ intensity[11], continuous emission mode21 and an application time of 1 min/cm$^2$[22] with the transducer being moved slowly and continually until the end of the application[23], and an ERA of 4 cm$^2$ ± 30%.

The US intensity was calibrated at 3 MHz before and during the experiment using distilled and degassed water in an US scale (UPM-DT-10 Ohmic Instruments, Easton, USA). Ultrasonic wave transmissivity in Carbopol 940® gel and d-P gel was verified according to the method of Guirro, Guirro and Ferreira[24], considering a variation of 10% in US and 0.07% in scale. The procedure was repeated five times for each gel and none of the preparations showed a statistically significant attenuation (data not shown).

## Histological processing

Twenty-four hours after the final day of treatment, the animals were euthanized by cervical displacement and the lesioned area was removed including 2 mm of the surrounding intact skin. Cross-sectional segments of the central area of these lesions were fixed in 10% buffered formalin solution and histologically processed for embedding in paraplast. From each segment three 6μm histological slices stained with Picrosirius 1% (Sirius red in saturated solution of picric acid) were prepared on microscope slides for blinded analysis. Using polarization microscopy (Zeiss Axiolab, ZEISS, Germany) in conjunction with histological image analysis software (KS 400 2.0 - Kontrol Eletronics, Munique, Germany), the birefringence of the collagen fibers was quantified by a sole observer in five areas (10,000 μm$^2$ each) per slice that were distributed throughout the entire lesion.

## Statistical analysis

GraphPad Prism 4.0 (GraphPad Software Ind, San Diego CA, USA) was used for statistical analysis. The Kolmogorov-Smirnov test was used to verify the normality of data distribution. Afterwards, the analysis of variance (ANOVA) test was used, which was followed by the Tukey test. A value of $p<0.05$ was considered statistically significant. The obtained data were submitted to polynomial regression in order to evaluate the birefringent areas in light of the different treatments and treatment lengths.

# Results

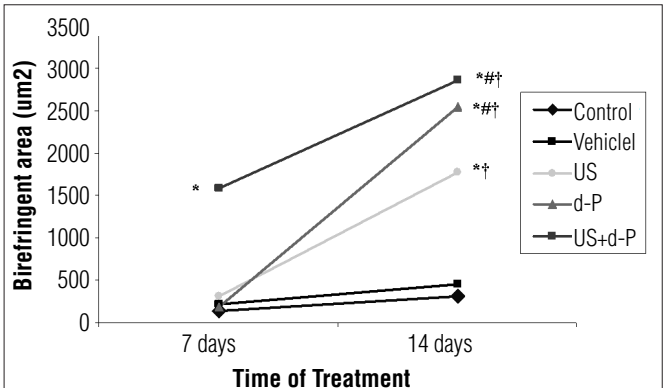
The US+d-P group showed a greater birefringent area ($p<0.001$) on the 7$^{th}$ day of treatment than the other groups (Table 1 and Figure 1). On the 14$^{th}$ day, the d-P group as well as the US+d-P group showed greater birefringent areas than the groups receiving isolated treatments (US). The US group was significantly different ($p<0.001$) from both the C and G group, in which there was no intergroup difference.

The data were also analyzed by means of polynomial regression. All groups showed an increase of the mean birefringent area during the treatment time, although the US, d-P and US+d-P groups showed a significant improvement

**Table 1.** Values (mean±SD) of birefringent areas (μm$^2$) on the lesioned regions of experimental groups treated per 7 e 14 days.

| Groups | 7 days | 14 days |
|---|---|---|
| Control | 139.36±35.35 | 316.96±55.15 |
| Vehicle | 218.13±93 | 448.42±148.87 |
| Ultrasound | 317.55±129.9 | 1779.94±482.78*† |
| Dexpanthenol | 192.41±36.57 | 2546.88±304.45*#† |
| Ultrasoud + Dexpanthenol | 1586.43±162.14*# | 2858.47±510.17*#† |

* Different of control group in the same period of treatment ($p<0.001$); # Different of ultrasound group in the same period of treatment ($p<0.001$); † Different of the same group in relation at 7 days of treatment ($p<0.001$).




* Different of control group in the same period of treatment ($p<0.001$); # Different of ultrasound group in the same period of treatment ($p<0.001$); † Different of the same group in relation at 7 days of treatment ($p<0.001$).

**Figure 1.** Polynominal regression of birefringent areas in relation of treatment time for all experimental groups. ultrasound (US), dexpanthenol (d-P), ultrasound + dexpanthenol (US+d-P).

($p<0.001$) that did not occur in the C and G groups ($p>0.05$), as demonstrated in Figure 1.

Figure 2 shows bright areas corresponding to the birefringence of collagen fibers in the injured area of animals in the experimental groups treated for seven and 14 days. On the 14th day, the tissue was more mature and, consequently, the collagen fibers were brighter.

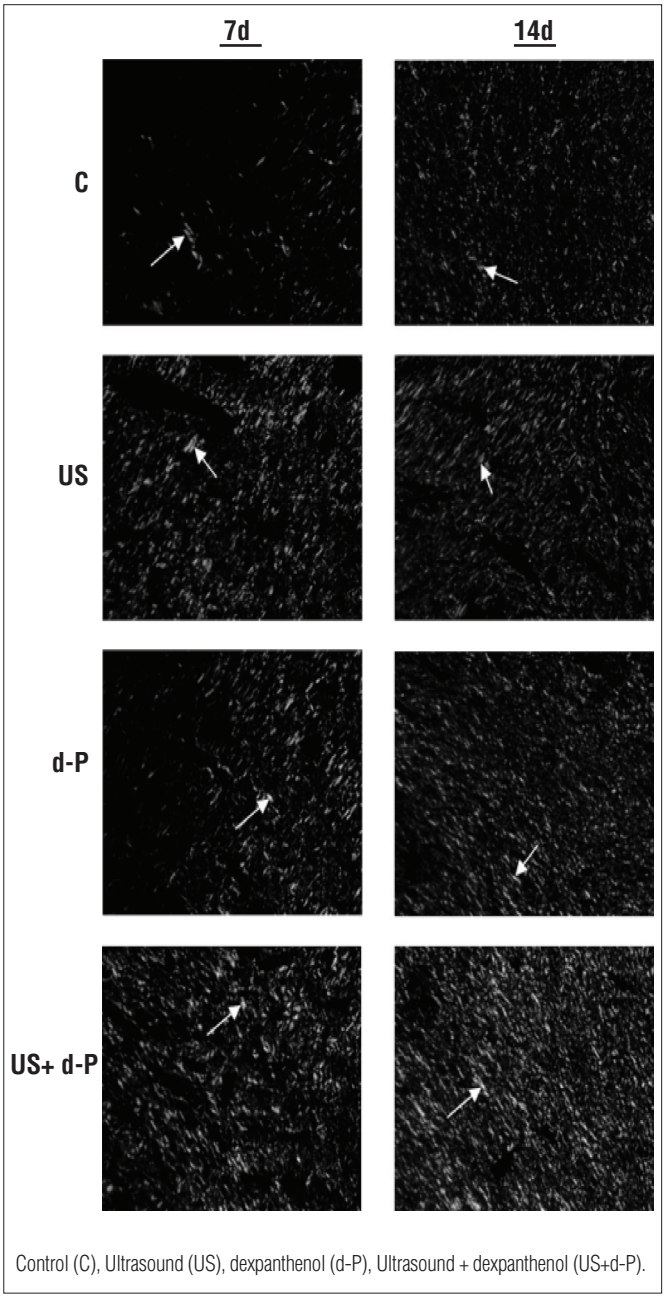



**Figure 2.** Microscopy of polarized light of the injured areas showing the arrangement and characteristic glow of collagen fibers (white arrows). It is observed that the group US+d-P presents a larger amount of collagen fibers on day 7 (7d) compared to groups US and d-P. On the 14th day (14d), d-P is the group that most similar to the group (US+d-P) (200x, Picrosirius 1%). Control (C), Ultrasound (US), dexpanthenol (d-P), Ultrasound + dexpanthenol (US+d-P).

## Discussion

The regeneration of wounds is a highly organized process involving cellular interactions, the formation of granulation tissue and the deposition of proteoglycans and collagen, which is the main component of a mature scar[6].

To provide therapeutic effects in the tissue repair process, such as in the interaction between cells and matrix, physical and pharmacological resources have been used, including the separate application of US[9-12] and d-P[7,8] in isolated treatments.

On the 7th day of treatment in the present study, the d-P group's collagen fiber birefringent area did not differ from the C group's according to the Tukey test (Table 1). However, on the 14th day of treatment, the mean birefringent area of the d-P group was significantly greater ($p<0.001$) than the C group's (Table 1, Figure 2). It is probable that d-P promoted an increase in collagen synthesis by means of fibroblast activation, which confirms the results of Ebner et al.[8]. Since the synthesis and remodeling rate of the collagen is slow, the effect only appeared on the 14th day.

According to Young and Dyson[11], US treatment accelerates inflammatory reaction and cellular proliferation in the initial stages of epidermal regeneration in rats, as well as increases collagen deposition and the tensile strength of the wound. Demir et al.[13] used US at intensities of 0.1 W/cm$^2$ and 0.5 W/cm$^2$ and observed inflammatory phase acceleration in the wound repair. Using the same parameters as the present study but with pulsed US, these authors observed a decrease in the length of the inflammatory phase time. In the proliferative phase, there was an increase in hydroxyproline and fibroblast numbers, as well as stimulated collagen synthesis.

Similar results were observed in the present study in that there was no significant difference in the birefringent areas (Table 1 and Figure 1) of the US group treated for seven days compared to the other groups. However, on the 14th day, which is in the proliferative phase, there was an increase in comparison to the C group.

The combination of treatments (US+d-P) has been studied by our research group *in vitro*[25] as well as in tegumentary regeneration[19]. In a qualitative analysis, Polacow et al.[19] observed stimulated re-epithelialization of wounds and increased fibroblast numbers, as well as better organized healing tissue after a seven-day-treatment. However, they did not study the effects of this combined treatment on the synthesis and organization of collagen.

In the present study, under the same experimental conditions, the results showed that on the 7th day of treatment the US+d-P group had a significantly greater birefringence area ($p<0.001$) than either of the isolated treatments, which demonstrates accelerated maturation of the collagen fibers

present in the injured tissue (Table 1). Our results reinforce the beneficial effect of this association, because if there are more fibroblasts[19], there is also increased synthesis of extracellular matrix, which confirms that fibroblasts direct this process since they liberate growth factors that contribute to wound healing[10,12,26]. The cavitation effect of ultrasound increases the influx of calcium in the monocytes, acting as a second messenger and promoting the synthesis and liberation of factors that contribute to wound healing[11,27].

According to KoeKe et al.[28], who studied the effect of US associated with 10% hydrocortisone, phonophoretic transdermal delivery positively influences the repair process of tendons when compared to isolated treatments with US and hydrocortisone.

On the 14th day of treatment, the US+d-P group showed greater birefringence, although it did not differ from the group treated with d-P alone, since, during that treatment period (from seven to 14 days) the wounds were in the remodeling phase and the synthesis rate and deposition of collagen were undergoing reductions in the injured tissue.

Data were submitted to polynomial regression (Figure 1) in order to evaluate the birefringent areas with respect to the different treatment lengths, and the results of this analysis showed that all the experimental groups except C and G showed a significant increase ($p<0.001$) in birefringence on the 14th day compared to the first seven days of treatment. This reveals the natural maturity of collagen fibers in this phase of the repair process, which corresponds to the beginning of the remodeling phase. However, the combined treatment was very effective until the 7th day. Future studies involving this combination of treatments and including histological and biochemical analyses of the lesions during the inflammatory phase (around the 3rd day) should be developed in an attempt to understand its mechanism of action.

Lasers, another physical therapeutical resource, have also been used for treating wounds[29,30]. However, as Vogt et al.[31] observed, the healing process in a humid environment is faster than in a dry environment, which has also been confirmed by several other studies reporting that fibroblasts and endothelial cells increase with the isolated use of linkage gel[9,19]. This study demonstrates that if d-P is added to this gel, added benefit may be obtained, especially when used in conjunction with US.

Although the present study approached a very restricted aspect of tegumentary regeneration, the results complement other studies that have analyzed this association of treatments, which seems promising for the treatment of lesions, but needs further basic study.

The results confirmed our hypothesis that the combination of treatments (US+d-P) accelerates collagen synthesis in tegumentary lesions, suggesting that it may accelerate the healing process in an early stage of treatment.

## Conclusion

Isolated treatment with d-P and US in rat skin lesions, as well as a combination of the two treatments, stimulated collagen maturation. The combined treatment (US+d-P) was more effective in the first phase of the healing process (seven days) in that it accelerated the maturation of collagen fibers, which propitiates faster healing.

## References


1. Veit G, Kobbe B, Keene DR, Paulsson M, Koch M, Wagener R. Collagen XXVIII, a novel von Willebrand factor A domain-containing protein with many imperfections in the collagenous domain. J Biol Chem. 2006;281(6):3494-504.
2. de Campos Vidal B, de Carvalho HF. Aggregational state in the molecular order of tendons as a function of age. Matrix. 1990;10(1):48-57.
3. Vidal BC. Form birefringence as applied to biopolymer and inorganic material supraorganization. Biotech Histochem. 2010;85(6):365-78.
4. da Cunha A, Parizotto NA, Vidal Bde C. The effect of therapeutic ultrasound on repair of the achiles tendon *(tendo calcaneus)* of the rat. Ultrasound Med Biol. 2001;27(12):1691-6.
5. de Campos Vidal B. Image analysis of tendon helical superstructure using interference and polarized light microscopy. Micron. 2003;34(8):423-32.
6. Oberyszyn TM. Inflammation and wound healing. Front Biosci. 2007;12(8):2993-9.
7. Idson B. Vitamins and the skin. Cosm Toil. 1993;108:79-94.
8. Ebner F, Heller A, Rippke F, Taush I. Topical use of dexpanthenol in skin disorders. Am J Clin Dermatol. 2002;3(6):427-33.
9. Bayat M, Razavi N, Hosseini A, Hekmat H. The effects of ultrasound therapy on skin scars of rabbit. Arch Irn Med. 2001;4(2):72-5.
10. Lowe AS, Walker MD, Cowan R, Baxter GD. Therapeutic ultrasound and wound closure: lack of healing effect on x-ray irradiated wounds in murine skin. Arch Phys Med Rehabil. 2001;82(11):1507-11.
11. Young SR, Dyson M. Effect of therapeutic ultrasound on the healing of full-thickness excised skin lesions. Ultrasonics. 1990;28(3):175-80.
12. Altomare M, Nascimento AP, Romana-Souza B, Amadeu TP, Monte-Alto-Costa A. Ultrasound accelerates healing of normal wounds but not of ischemic ones. Wound Repair Regen. 2009;17(6):825-31.
13. Demir H, Yaray S, Kirnap M, Yaray K. Comparison of the effects of laser and ultrasound treatments on experimental wound healing in rats. J Rehabil Res Dev. 2004;41(5):721-8.
14. ter Haar G. Therapeutic applications of ultrasound. Prog Biophys Mol Biol. 2007;93(1-3):111-29.
15. Bem DMD, Maciel CD, Zuanon JA, Neto CB, Parizotto NA. Análise histológica em tecido epitelial sadio de ratos Wistar (*in vivo*) irradiados com diferentes intensidades do ultrassom. Rer Bras Fisioter. 2010;14(2):114-20.
16. Boucaud A, Montharu J, Machet L, Arbeille B, Machet MC, Patat F, et al. Clinical histologic and electron microscopy study of skin exposed to low-frequency ultrasound. Anat Rec. 2001;264(1):114-9.
17. Tang H, Mitragotri S, Blankschtein D, Langer R. Theoretical description of transdermal

transport of hydrophilic permeants: application to low-frequency sonophoresis. J Pharm Sci. 2001;90(5):545-68.

18. Escobar-Chávez JJ, Bonilla-Martínez D, Villegas-González MA, Rodríguez-Cruz IM, Domínguez-Delgado CL. The use of sonophoresis in the administration of drugs throughout the skin. J Pharm Pharm Sci. 2009;12(1):88-115.
19. Polacow MLO, Dib-Giusti HHK, Leonardi GR, Vieira CEC, Guirado GN, Zague V, et al. Efeito do ultra-som e do d-pantenol na regeneração tegumentar. Rev Bras Fisioter. 2005;9(3):365-71.
20. Fyfe MC. A study of the effects of different ultrasonic frequencies on experimental o edema. Aust J Physiother. 1979;25(5):205-7.
21. Cameron MH, Monroe LG. Relative transmission of ultrasound by media customarily used of phonophoresis. Phys Ther. 1992;72(2):142-8.
22. Oakley EM. Application of continuous beam ultrasound at therapeutic levels. Physiotherapy. 1978;64(6):169-72.
23. Byl NN, McKenzie A, Wong T, West J, Hunt TK. Incisional wound healing: a controlled study of low and high dose ultrasound. J Orthop Sports Phys Ther. 1993;18(5):619-28.
24. Guirro ECO, Guirro R, Ferreira AL. Efeitos da estimulação ultra-sônica pulsada de baixa intensidade no processo cicatricial: estudo experimental em ratos. Rev Ciênc Tecnol. 1995;4(8):37-47.
25. Zague V, Polacow ML, Pires-de-Campos MS, Leonardi GR. Evaluation of the ultrasound influence in the cutaneous penetration of d-panthenol: test in vitro. J Cosmet Dermatol. 2005;4(1):29-33.
26. Moulin V, Auger FA, Garrel D, Germain L. Role of wound healing myofibroblasts on re-epithelialization of human skin. Burns. 2000;26(1):3-12.
27. ter Haar G. Therapeutic ultrasound. Eur J Ultrasound. 1999;9(1):3-9.
28. KoeKe PU, Parizotto NA, Carrinho PM, Salate AC. Comparative study of the efficacy of the topical application of hidrocortisone, therapeutic ultrasound and phonophoresis on the tissue repair process in rat tendons. Ultrasound Med Biol. 2005;31(3):345-50.
29. Bayat M, Azari A, Golmohammadi MG. Effects of 780-nm low-level laser therapy with a pulsed gallium aluminum arsenide laser on the healing of a surgically induced open skin wound of rat. Photomed Laser Surg. 2010;28(4):465-70.
30. de Oliveira Guirro EC, de Lima Montebelo MI, de Almeida Bortot B, da Costa Betito Torres MA, Polacow ML. Effect of laser (670 nm) on healing of wounds covered with occlusive dressing: a histologic and biomechanical analysis. Photomed Laser Surg. 2010;28(5):629-34.
31. Vogt PM, Reimer K, Hauser J, Rossbach O, Steinau HU, Bosse B, et al. PVP-iodine in hydrosomes and hydrogel - a novel concept in wound therapy leads to enhanced epithelialization and reduced loss of skin grafts. Burns. 2006;32(6):698-705.