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Comparative macroscopic study between Oil extracted *Annona* seed AlGaInP Laser and Therapeutic Ultrasound for Skin wounds

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Abstract: *Recent studies suggest the effectiveness of AlGaInP Laser, Therapeutic Ultrasound (UST) and the oil extracted Annona seed as therapeutic resources in the treatment of cutaneous wound healing. The objective was to comparatively evaluate the macroscopic process of healing of skin wounds in mice. The sample consisted of 32 male mice (Mus musculus) divided into four groups: group 1 (control), group 2 (pulsed UST 3 MHz), group 3 (topical application of oil extracted Annona seed) and group 4 (660nm laser AlGaInP). The animals were anesthetized and then shaved. The surgical incision was made with a punch of 8 mm diameter perpendicular to the back of the animal. The treatments were started 24 hours after injury. Each group was divided into subgroup A (n = 4, five applications) and B (n = 4 eleven applications) as observation period, with euthanasia at 7 and 14 days, respectively. Statistical analysis was obtained through non-parametric Mann Whitney and significance at p <0.05, by GraphPad Prism v program. 5.0. It was concluded that all treatment groups were effective in regression of cutaneous wound 14 days of the healing process when compared to the control group.*
Keywords: *Healing. Ultrasound. Laser. Annona.*

I. Introduction

A cutaneous wound consists in the disruption of the continuity of body tissue, generally due to physical trauma, chemical, mechanical, among others [1]. It is known that skin is one of the constituents of the integumentary system, which reverses the entire surface of the body and has the function [2]: protection, temperature regulation of the body, excretion and tactile production of vitamin D. Thus, given tissue injury, tissue loss is what triggers the formation of scar given the stimulus to the defense of the body and start the healing process, which is complex and dynamic and involves biochemical and physiological events, which should work in harmony for the benefit restoration of tissue [3-5].

However, in view of such a complex and dynamic process, it is known that it can be influenced and scarring is not always successful. Variables in general (age, nutritional status, associated with underlying diseases such as diabetes, cardiovascular changes and coagulation, among others) or local (surgical technique, hematoma formation, infections, foreign body reaction, dryness during healing, among others), can interfere with the same, and has long been an object of study and as the factors that affect it [3,4].

In the last years, researchers within the Physiotherapy have used several equipment to facilitate the scarring process of cutaneous wound with proven scientific results [1,6-9]. In this context, it refers to the low-power laser (LPL), such as AlGaInP, the AsGa and HeNe, Therapeutic Ultrasound, LED (light emitting diode) and High Frequency Generator (HF) as current resources, according to the literature, are shown with beneficial and effective action.

Regarding the LPL, phototherapy has been shown to be highly effective in the treatment of tissue repair due to stimulate the proliferation of fibroblasts, the synthesis of collagen, the synthesis of ATP and lymphocyte activity, whose evidence is mainly resulting from experimental studies in animals [10]. In recent years, several studies have shown benefit in the use of laser AlGaInP 660 nm wide situations, such as in the treatment of soft tissue injury (ligaments, tendons), joint diseases, open wounds, pressure ulcers, among others [1, 11,12]. Since it is in the red range of the electromagnetic spectrum and is healing, whose influence in injured tissues triggers photobiomodulation process, besides causing therapeutic effects such as local analgesia, reducing edema and anti-inflammatory action [11, 13].

Therapeutic Ultrasound, which in turn is another resource widely used in physiotherapy. When the 3 MHz frequency, is frequently used in physiotherapy for dermatologic disorders, ulcerative lesions on the skin, tendons and ligaments surface [13]. In pulse mode, provides athermal and mechanical physical effects to the tissues, thus contributing to increased cell permeability and the passage of metabolites across the cell membrane, increased protein synthesis, the flow of calcium ions, among numerous other factors contributing positively to tissue healing [11, 14, 15].

On the other hand, the use of herbal folk medicine for resources also makes growing in tissue regeneration. As an example of plants used in natural medicine for this purpose, the Annona muricata, popularly known as soursop, is grown in tropical regions and well known for its various medicinal properties in all its constituents, from root to leaf, as hypotensive effect, insecticide, antibacterial, antitumor, soothing, sedative, antispasmodic, among others [16,17]. However, scientific research as to its veracity is scarce and its use by the population often based only on common sense.

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Several active ingredients extraction protocols of the various constituents of Annona muricata are described in literature [18-20], which showed that its main chemical constituents are acetogenins, alkaloids, phenolics and essential oils, whose anti-inflammatory and antioxidant properties can be promising in tissue healing.

Before this, aimed to evaluate the macroscopic process of healing of cutaneous wounds in mice through the wound regression percentage, using comparatively therapy Ultrasound Therapy, Laser 660nm AlGaInP and oil extracted from the seeds of Annona.

II. Materials And Methods

The experiment was conducted at the Laboratório de Fisiologia of the Faculdade Integral Diferencial (FACID / Devry), Teresina, PI, Brazil. The study was approved by the Comitê de Ética de Uso em Animais - CEUA of FACID / Devry, under Protocol No. 029/13.

The sample was composed of 32 male mice (Mus musculus), weighing between 20 and 30 grams were randomly divided into four groups according to treatment and subdivided into subgroups A and B, according to the observation period after the treatment of 7 and 14 days. The animals were housed in polypropylene cages and maintained throughout the experiment in hygienic conditions, temperature and lighting and fed a standard diet of the vivarium, feed (Labina TM) and water "ad libitum". The experimental groups according to the type of treatment were:

- Group 1: control, n=8;
- Group 2: UST pulsatile 3 MHz, indirect application with paper film on the skin wound, pulse frequency of 16Hz to 10% ERA 3.5 cm² intensity of 0.3 W/cm 2,2 min, n=8;
- Group 3: Topical application of 0.2 ml of oil extracted from the seed Anonna, n=8;
- Group 4: AlGaInP laser 660 nm, continuous, 30mW, 5J / cm², scan mode within the skin wound and 0.06310 contact area/cm², 2 min, n=8.

The animals were weighed and then anesthetized with the administration by intramuscular injection of ketamine hydrochloride 10%, in 0.1 ml dose per 100 g of body weight associated with the same dose of xylazine hydrochloride 2%. The lesion was induced in the back of the animals, placed in the supine position and began with the trichotomy of the region. To perform the experimental injury was employed a surgical instrument (punch) of 8 mm in diameter, positioned perpendicularly to the back.

The first therapies with UST pulsatile 3 MHz, topical applications of the oil extracted from the seed and Annona irradiation with laser AlGaInP of 5 J / cm2, were 24 h after the injury in groups 2, 3 and 4, respectively, and extended daily, without breaks on weekends, until the eve of the sacrifice of animals. The animals of subgroup A (sacrifice on day 7) received their treatment corresponding to five times, whereas the animals of group B (sacrifice day 14) received 11 times (Figure 1).

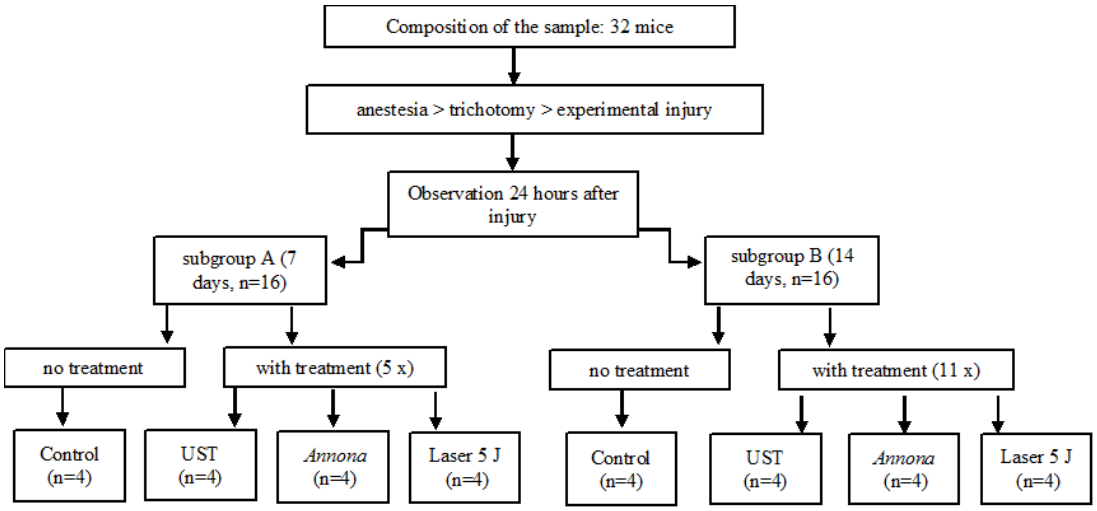


Figure 01. Sample distribution scheme in the experiment

The study was performed using a low power laser AlGaInP (Laserpulse Ibramed) and Therapeutic Ultrasound Sonopulse Ibramed (1 and 3 MHz frequency), both with the application parameters for healing purposes already described.

As for the oil extracted from Annona, it was obtained from the same seeds, which were removed from the ripe fruit (Anonna muricata), washed with water, blotted dry with a towel, and exposed to a shaded environment. The seeds were crushed in a blender and extracted successively with Hexano9 in a conical container. The vial was shaken every 5 minutes for 30 minutes and then the solvent was filtered and concentrated in an evaporator, in which the extract was concentrated under vacuum. The solvent was added to the material again, repeating the process two more times. The final product obtained was the crystalline Anonna seed oil, pH between 6 and 7, which was applied topically daily and the concentration originally obtained. The sacrifice of the animals took place on the 7th and 14th days, when there was the removal of tissue for macroscopic analysis. The mice were euthanized by an overdose of sodium thiopental 50 mg/kl. For macroscopic analysis, the areas of injuries were recorded immediately after production, on 7 and 14 days of treatment by digital camera brand Nikon COOLPIX L820, resolution of 16 Megapixels, fixed tripod, kept at a constant distance of 30 cm from surgical area. For the determination of the wound area and image analysis, we used the ImageJ software to calculate the area of the same.

The statistical treatment was obtained with the aid of non-parametric Mann Whitney test, using GraphPad Prism Program, v. 5.0. Values were considered significant when $p < 0.05$.

III. Results

According to Fig. 02, macroscopic analysis of the skin wound regression percentage in mice at 7 and 14 days was observed in the intergroup analysis that significant results in regression of skin wounds were found in all treatment groups when compared to the group control only 14 days. Compared to 7 days, although we have not achieved significant results, which may be related to the small sample available in this experiment, the control group showed the, on average, less regression of cutaneous wound when compared to the other groups.

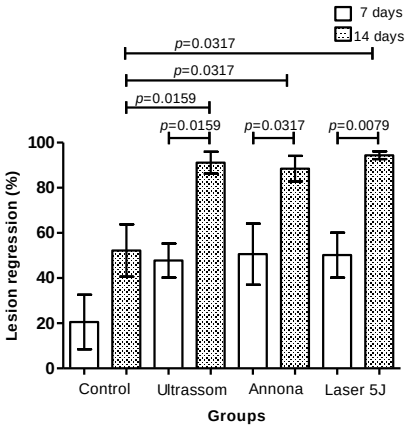


Figure 02. Intragroup and intergroup analysis wound regression percentage of the different groups at 7 and 14 days.

Concerning the intergroup analysis, it can be seen that in all groups there was a significant increase in regression of the wound at 14 days compared to 7 days of treatment - except for the control group, which was not found significant, and especially for LPL group 5 J/cm2, which showed even more significant results than the other groups. Fig. 03 summarizes, an illustrate manner, all found, in accordance with the trends presented in statistical analysis.

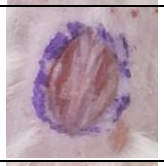
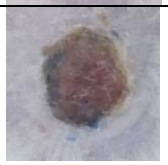
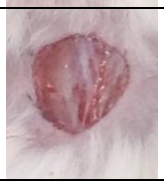
Groups	Days after the skin wound		
	0	7	14
Control			
Therapeutic Ultrasound Pulsed 3MHz			
Seed oil from *Annona*			
Laser Al GaInP 5J/cm^2			

Figure 03. Lesion skin wounds induced by 8 mm punch during the experimental periods (0, 7 and 14 days) mice.

IV. Discussion

In the current study it was observed that both physiotherapeutic resources as herbal study were effective in the acceleration of tissue repair, especially in the later stage of the healing process. Studies suggest that LPL as well as UST, facilitates tissue repair of skin wounds [1,6,9,12-15]. However, in the literature there is a shortage of developed scientific research on the healing action of the constituents of Annona.

In a study that sought to show the effects of laser therapy (AsGa, 904 nm, 5 Hz, 50 millijoules and depth of 5 mm) and ultrasound (continuous, 3 MHz, 0.5W/cm2), in tissue regeneration from the analysis of four medical records of patients with healing change symptom (necrosis in the surgical wound). After abdominoplasty treatments ranging between 6 and 30 sessions in total, proliferation and acceleration was observed in tissue repair, reducing the tissue tension, leading the total closure of the injury [21].

It may be observed that in the aforementioned study revealed LPL efficiency and UST associated with the treatment of healing changes, although with different parameters than were used in this study, since the physical therapy approach was introduced on the 11th day after surgery average. It is known AsGa laser that is in the invisible range of the electromagnetic spectrum and is generally not indicated for deep and superficial lesions, and may have been employed because of the extent and depth of postoperative injury. Regarding the UST, it was probably used continuous mode due to necrosis appearance in the inflammatory phase of the healing process, making it necessary to stimulate the blood supply and improves collagen fiber realignment with reduced tissue tension.

In a study [1] which compared the treatment of LPL (AlGaInP, 670 nm, 30 mW, 6 J/cm², 1 point, 120s) with the high frequency generator (HF) (amplitude range 80% small Standard electrode 120 s) and the combination therapy of both healing of skin wounds in rats at 3 and 7 days of treatment. HF, as well as LBP, showed significant results in regression of wounds ($p < 0.05$) and the combination therapy was more significant ($p < 0.01$). As compared to this study (Image 2), the same number of days evaluated Laser group of 5 J/cm² was not effective in comparison to the control after 7 days of incisional injury, which can be associated to the dose and mode of application many different.

Were also found positive effects of LPL (630 nm) in the early and middle of the healing process of cutaneous wound in diabetic mice [22] through dose of 3.6 J/cm², which was compared in different power: 5 mW/cm² (12 min), 10 mW/cm² (6 min) and 20 mW/cm² (3 min), applied 5 times / week for 2 weeks. It was observed that all lasers groups promoted significant results in wound contraction in the control group at 3, 6 and 9 day of treatment, but none on the 12th day.

Low LPL dosage used in the aforementioned study was effective only in the initial phase of wound healing compared to the control group. This present study (Image 2), which was used higher doses of powers of LBP showed significant laser results only in the remodeling phase, suggesting that higher doses of LPL are more effective in remodeling phase than in the acute phase whereas at this stage smaller doses could better meet the principle of the inflammatory cascade. As in the other aforementioned earlier study [22].

In a controlled and randomized study that investigated the effects of treatment of UST (pulsatile, 2 ms on and off 8ms, 0.5W/cm², 5 min/day) and LPL (AsGa, 904 nm, 6 mW, 1 J/cm², 16Hz, 10 min/day) in healing wounds in rats for 10 days, we observed a reduction of the inflammatory phase in both treatments. As well as beneficial effects on maturation and proliferation of fibroblasts, however the laser was more effective, more significant results in the 4th and 10th day of the initial stages of healing. [23] Similarly, in the present study, as UST as LPL were both effective in accelerating tissue healing, especially the group that received treatment as laser therapy, in which the results were even more significant.

In another research, it was observed that UST (pulsed 20% duty cycle, 0.5W/cm 2, 3 min) was able to promote regression of the most advanced form of normal wound on ischemic wounds than in the early stages wounds healing. In ischemic wounds, in addition to delayed healing we have also found an increased number of inflammatory cells [24].

Regarding the use of herbal resources, medicinal plants are often seen by the population as alternative treatments for curing diseases and symptoms. The basis for the use of Annona constituents in various diseases and disorders, is still bound to the common sense of the population, requiring more scientific research for more evidence and conclusions about the effectiveness of this herbal medicine in the healing process of skin wounds. For which to know the factors that affect the process of tissue repair from its various constituents.

Studies reveal their indiscriminate use in the treatment of various diseases such as cancer, based only on information from family and friends. In a study that sought to interview 59 patients undergoing treatment for cancer and the use of medicinal plants, were described 14 species of plants with antineoplastic purpose and, among them, the Annona, popularly known as soursop, was one of the most cited. The method of preparation and method of use for this was reported infusion of leaves and fruit, grinding dried leaves, fruit juice and / or pulp, among others [25].

An assessment on medicinal plants as herbal alternative and its intended use by the population of certain municipalities of the Espítiro Santo, the soursop (Annona) was cited as a reference against cancer and in favor of healing through leaves tea. Other recent studies have also suggested as Annona plant species used in wound healing [27] as well as their inhibitory effect of metalloproteins, which are associated with tumor progression of many human malignancies, which may partly explain its antitumour effects [28].

Other authors highlight the widespread use of leaves of Annona muricata against skin diseases and abscesses in folk medicine, but no scientific evidence for the use of them²º. Because of this, a study was done that aimed to evaluate the healing of excisional wounds in rats with ethyl acetate extract from leaves in question from ointment applied in two different doses (5 and 10% m / m, 0.2 ml). Both doses exhibit a promising potential for cure, with acceleration of wound contraction, epithelialization and collagen synthesis, and anti-inflammatory effects, increase antioxidant activity and decrease in the concentration of malondialdehyde [20].

Corroborating the above findings, methanolic and aqueous extracts of A. muricata leaves also showed significant reduction of free radical activity and increased elimination of them with better protection against damage induced by H^2O^2 DNA (pBR322) by methanol extract compared the aqueous. Furthermore, the phytochemical analysis of such detected the presence of polyphenols and tannins, steroid, flavonoid, terpenoid, among others, which confirms the protective Annona elements muricata against free radicals, as the phenolic compounds are one of the most efficient antioxidants present in plants [18].

Other researchers have also reported applications of Annona muricata constituents in the treatment of painful conditions and inflammatory disorders in African traditional medicine. In this context, the observed toxic symptoms, nociceptive tests and behavioral changes in rats have shown analgesic effects via interaction with peripheral mechanisms and opioid system, as well as anti-inflammatory property by inhibiting the chemical mediators of inflammation (cyclo -oxigenases COX-1 and COX-2), by means of the lyophilized extract (immature fruit pericarp and seeds in question) [19].

V. Conclusion

All groups were effective in wound regression when compared to the more advanced group control in the stages of tissue healing and, in this study, the LPL performed even more significant than the other treatment groups. However, in general, it appears that in the literature there is no consensus on the ideal parameters for the

application of LPL, and the pulsatile UST in skin wounds, but it is known for its beneficial effects in accelerating tissue repair.

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