Application of *Jatropha curcas* L. seed oil (*Euphorbiaceae*) and microcurrent on the healing of experimental wounds in Wistar rats[1]

Aplicação do óleo das sementes de *Jatropha curcas* L. (*Euphorbiaceae*) e microcorrente no reparo de lesões experimentais em ratos Wistar

José Roberto Passarini Junior[I], Fernanda Oliveira de Gaspari de Gaspi[II], Lia Mara Grosso Neves[III], Marcelo Augusto Marreto Esquisatto[IV], Gláucia Maria Tech dos Santos[V], Fernanda Aparecida Sampaio Mendonça[VI]

[I]Graduate student, School of Pharmacy, Herminio Ometto University Center, UNIARARAS, Araras-SP, Brazil. Acquisition and interpretation of data, involved in technical procedures.
[II]PhD, Full Professor, NUCISA, Herminio Ometto University Center, UNIARARAS, Araras-SP, Brazil. Interpretation of data and critical revision.
[III]Fellow Master degree, Postgraduate Program of Biomedical Sciences, Herminio Ometto University Center, UNIARARAS, Araras-SP, Brazil. Acquisition and interpretation of data, involved in technical procedures.
[IV]PhD, Full Professor, Postgraduate Program of Biomedical Sciences, Herminio Ometto University Center, UNIARARAS, Araras-SP, Brazil. Acquisition and interpretation of data, histomorphometric analysis.
[V]PhD, Full Professor, Postgraduate Program of Biomedical Sciences, Herminio Ometto University Center, UNIARARAS, Araras-SP, Brazil. Supervised all phases of the study, manuscript writing and critical revision.
[VI]PhD, Full Professor, Postgraduate Program of Biomedical Sciences, Herminio Ometto University Center, UNIARARAS, Araras-SP, Brazil. Main author. Tutor, responsible for conception, design, intellectual and scientific content of the study; critical analysis; final approval of manuscript.

ABSTRACT

PURPOSE: To investigate the effects of *Jatropha curcas* L. seed oil and microcurrent stimulation on the healing of wounds experimentally induced in Wistar rats.

METHODS: Forty-eight animals were divided into four groups: (A) control; (B) treated with microcurrent (10 μA/2 min); (C) treated with *J. curcas* seed oil, and (D) treated with *J. curcas* seed oil plus microcurrent. Tissues samples were obtained two, six, ten and 14 days after injury and submitted to structural and morphometric analyses.

RESULTS: The animals of groups A and C showed similar responses in terms of repair area, total number of cells, number of newly formed blood vessels, epithelial thickness, and percentage of area occupied by mature collagen fibers. Significant differences in all parameters analyzed were observed between animals of groups B and D and the control 10 and 14 days after experimentally induced injury. The morphometric data confirmed the structural findings

CONCLUSIONS: The application of *J. curcas* seed oil alone was not effective on experimental wound healing when compared to control, but microcurrent application alone or combined with the oil exerted significant differences in the parameters studied. These findings suggest that the positive results were due to microcurrent stimulation.

Key words: Jatropha curcas. Wound Healing. Histology, Comparative. Rats.

RESUMO

OBJETIVO: Investigar os efeitos do óleo das sementes de *Jatropha curcas* L.e microcorrente em lesões experimentais em de ratos Wistar.

MÉTODOS: Quarenta e oito animais foram divididos em quatro grupos: (A) controle, (B) tratado com aplicação de microcorrente, (C) tratado com óleo de sementes de *J. curcas* e (D) tratado com de óleo de sementes de *J. curcas* associado à microcorrente. Amostras de tecido foram obtidas no 2º, 6º, 10º e 14º dia após a lesão e submetidas às análises estrutural e morfométrica.

RESULTADOS: Os animais dos grupos A e C apresentaram respostas semelhantes quanto a seus efeitos sobre as medidas da área de reparo, número total de células e de vasos sanguíneos neoformados, espessura do epitélio e porcentagem da área ocupada por fibras colágenas maduras. Os grupos de animais B e D apresentaram resultados diferenciados e significativos em todos os parâmetros analisados nos dez e 14 dias após a lesão experimental. Os dados morfométricos confirmaram os achados estruturais.

CONCLUSÕES: A aplicação do óleo das sementes de *J. curcas* não promoveu respostas significativas no reparo das lesões experimentais quando comparadas ao controle, mas a microcorrente aplicada isolada ou combinada a este óleo apresentou diferenças significativos nos parâmetros estudados Este fato sugere que os resultados positivos se devem provavelmente a ação da aplicação da microcorrente

Descritores: Jatropha curcas. Cicatrização. Histologia Comparada. Ratos.

Introduction

Popular observation of the use and efficacy of medicinal plants in wound healing has contributed significantly to the study of their properties and therapeutic effects. In this respect, folk medicine raises the interest of researchers in studies that involve multidisciplinary areas[1]. Medicinal plants are important for pharmacological research and for the development of novel drugs. In the latter case, the plant or its constituents can be used directly as the therapeutic agent, but also as a starting material for the synthesis of drugs or as a model of pharmacologically active compounds. Phytopharmaceutical laboratories concentrate their efforts on identifying the active substances and mechanisms of different plants. The medicinal value of these plants lies in their phytochemical constituents that belong to different chemical families, such as alkaloids, essential oils, flavonoids, tannins, terpenoids, saponins, and phenolic compounds[2-4].

Jatropha curcas L. (family Euphorbiaceae), known as "pinhão manso" (physic nut), is native to tropical countries and can be cultivated in areas with poorly fertile soils and unfavorable climate. These characteristics make the plant a promising biofuel[5,6]. Although the oil has a high energy content, other components of this plant may also be used as an energy source[7,8]. However, this plant is not only economically and environmentally important as a source of biodiesel, but is also used as a folk remedy in many countries for the treatment of various diseases. All parts of the plant can be used for a variety of purposes. The latex derived from *J. curcas* has medicinal, pesticidal and antimicrobial properties and is widely used as a healing agent[9]. Villegas *et al.*[10], studying the wound healing activity of the latex in rats, found that this property is due to the presence of a proteolytic enzyme. The roots, stems, bark, leaves, seeds and fruits, fresh or cooked, are widely used in traditional folk medicine in many parts of Western Africa[11,12]. Esimone *et al*.[13] obtained promising results with an ointment containing fresh *J. curcas* leaf extract applied to excision wounds created in the back skin of albino rats. In a study using albino mice, Shetty *et al*.[14] showed that crude *J. curcas* bark extract was effective in accelerating the healing process of experimentally induced skin wounds. Direct application of crushed leaves of the plant to cuts and wounds has been shown to promote coagulation. The seeds of *J. curcas* are used as anthelminthic agent and for the treatment of gout, paralysis, ascites, and skin disease. The seed oil of the plant has been to shown to be effective in treating rheumatic diseases, parasitic skin diseases, itching, jaundice and fever, as well as a diuretic agent[14-17].

The wound healing process is a well-organized chain of biochemical and cellular events that promote platelet aggregation, activation of the coagulation cascade, inflammatory infiltration, cell differentiation, and tissue remodeling. This process involves the activity of an intricate network of blood cells, cytokines, and growth factors that restore the normal condition of skin or tissue[18]. Clinical evidence indicates that the repair of dermal and subdermal connective tissue can be accelerated by the external application of a low-intensity electrical current. Electrical stimulation using low-intensity (microamperage) currents is compatible with endogenous currents that act in the organism at the cellular level, accelerating wound healing and improving the quality of scar tissue[19]. Studies investigating the effects of electrical current stimulation using different amplitudes and frequencies have shown that these procedures promote cellular modifications and tissue responses in experimentally induced wounds[20-22]. Mendonça *et al*.[23] investigated the effect of microcurrent stimulation (10μA) on wound healing in rats and observed that this treatment is effective in promoting tissue repair, exerting positive effects on the newly formed tissue area, number of fibroblasts, number of newly formed vessels, and epithelial thickness.

The objective of the present study was to investigate the effects of *Jatropha curcas* L. seed oil and microcurrent stimulation on the healing of wounds surgically induced in the back skin of Wistar rats.

Methods

Forty-eight male Wistar rats (*Rattus norvegicus*), 120 days old and weighing on average 250g, obtained from the Center of Animal Experimentation, Herminio Ometto University Center, UNIARARAS, were used. The animals were maintained in individual cages at a constant temperature of 23 ± 2ºC under a 12-h light/dark cycle, and received commercial chow and water *ad libitum*. No differences in behavior were observed between animals during the study. The experimental procedures were approved by the Ethics Committee of Herminio Ometto University Center (protocol 058/2011) and were conducted in accordance with international regulations on animal testing and biodiversity[24,25].

Preparation of the extract

The seeds of *Jatropha curcas* L. (Euphorbiaceae) were selected, broken down into smaller pieces on dry ice, and ground with a knife mill. The material was then extracted with hexane in an extractor tank under pneumatic agitation for 1h. The procedure was repeated three times and the solvent was changed between extraction runs. The extracted material was concentrated by rotary

evaporation until only the oil was left.

Experimental model

A trichotomy was performed 48h before surgical intervention. After local asepsis, the animals were anesthetized with xylazine hydrochloride (20 mg/kg body weight) and ketamine hydrochloride (50 mg/kg). The site of incision was measured with a caliper in the craniocaudal direction (2 cm long and 2 mm deep) and was marked with a ballpoint pen. The incision was then made in the back skin of the animal and was left unsutured.

The animals were divided into four groups of 12 animals each: group A, control; group B, treated with microcurrent (10 μA/2 min/day); group C, treated with *J. curcas* seed oil; group D, treated with *J. curcas* seed oil and microcurrent (10 μA/2 min/day). Treatments were performed according to the protocol of Mendonça *et al*.[23].

Collection and preparation of samples for structural analysis

Three animals from each group were sacrificed with an overdose of the anesthetic at two, six, ten and 14 days after experimentally induced injury. The total wound area (120 to 160 mm^2) was removed for structural and morphometric analyses. Each sample was removed and fixed in 10% formalin in Millonig buffer, pH 7.4, for 24h at room temperature. Next, the specimens were washed in the buffer solution and processed for embedding in Paraplast®. Longitudinal sections (7 μm) were stained with hematoxylin-eosin for routine histology and with picrosirius-hematoxylin for the observation of collagen fibers. The specimens were examined and documented under a Leica DM 2000 photomicroscope at the Laboratory of Micromorphology, Herminio Ometto University Center, UNIARARAS.

Morphometric analysis

Images from cross-sections of the mid-region of the experimental wound were captured and digitized using a Leica DM 2000 photomicroscope. Digitized images were used for the determination of the morphometric parameters: tissue repair area (μm^2), total number of cells (fibroblasts and inflammatory cells ($n/10^4$ μm^2), number of newly formed blood vessels ($n/10^4$ μm^2), and thickness of regenerated epithelium (μm). For this analysis, three specimens were randomly selected among the sections obtained. For this purpose, three samples of 10^4 μm^2, using virtual Leica Image Measure™ grid, were collected from central region of experimental lesion in each animal. To measure the area of granulation tissue deposition in repair area was used tool contouring program from Sigma Scan Pro 6.0™ in the same sections cited above. The results were entered into spreadsheets (Biostat for Windows XP program) and compared by ANOVA and the Tukey post-hoc test. A level of significance of 5% was adopted[26].

Results

Tissue repair was studied in the different groups by comparing inflammatory and proliferative processes and tissue reorganization. For this purpose, samples were collected from the wound area two, six, ten and 14 days after experimentally induced injury. Temporal differences in tissue repair were observed between the different treatments.

All samples obtained from control animals (group A) and animals treated with the *J. curcas* seed oil (group C) were similar in terms of the parameters studied. In these groups, the inflammatory phase was already established by day 2 after injury and the structural features of the proliferative and remodeling phases were observed between day 6 and day 14 (Figure 1). Differences were observed for animals submitted to microcurrent stimulation (group B) and microcurrent stimulation plus *J. curcas* seed oil (group D). These animals presented an increase in the total number of cells in the wound area by day 6 when compared to groups A and C. On day 10, this area was already reepithelialized and the collagen fibers were compacted and reorganized.

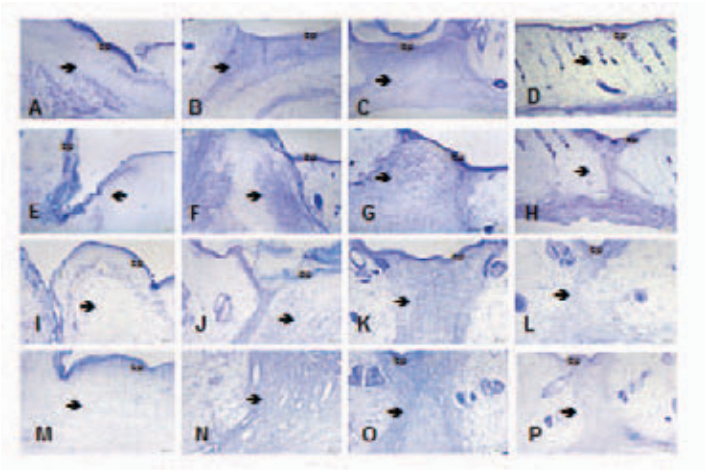

FIGURE 1 – Photomicrographs of cross-sections of wounds experimentally induced in Wistar rats. Group A, control (**A-D**); group B treated with microcurrent (10 μA/2 min/day) (**E-H**); group C treated with *Jatropha curcas* seed oil (**I-L**), and group D treated with *Jatropha curcas* seed oil and microcurrent (10 μA/2 min/day) (**M-P**). Tissue samples were collected on day 2 (**A, E, I** and **M**), day 6 (**B, F, J** and **N**), day 10 (**C**, **G, K** and **O**), and day 14 (**D**, **H, L** and **P**) after injury. The sections were stained with Toluidine blue. (Ep) Epithelium; (➔) repair area in the dermis. Bar = 100 μm.

Tissue repair was significantly higher in groups B and D between day 6 and day 10 when compared to groups A and C, but remained stable between days 10 and 14 in all groups (Figure 2). An expressive increase in the total number of cells in the repair area was observed between days 2 and 6 after surgically induced injury in all groups. However, this number was higher in groups B and D after 10 and 14 days of treatment (Figure 3). These same parameters were seen for the formation of newly formed blood vessels, with the observation of significantly larger numbers in groups B and D (Figure 4). Reorganization of the wound area was similar in the different groups (Figures 1 and 5).

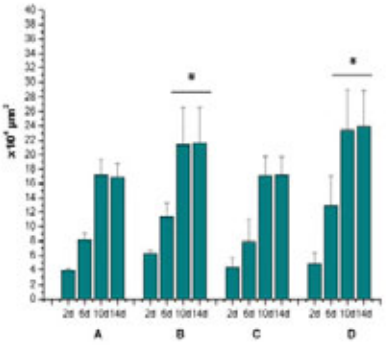

FIGURE 2 – Size of the tissue repair area (x 10^4 μm^2) in wounds experimentally induced in Wistar rats. Group A, control; group B treated with microcurrent (10 μA/2 min/day); group C treated with *Jatropha curcas* seed oil, and group D treated with *Jatropha curcas* seed oil and microcurrent (10 μA/2 min/day). Tissue samples were collected on day 2 (2d), 6 (6d), 10 (10d), and 14 (14d) after injury. Results are reported as the mean and standard deviation of each group and were compared by ANOVA and Tukey's post-hoc test ($p<0.05$). The asterisk indicates significant differences between time points.

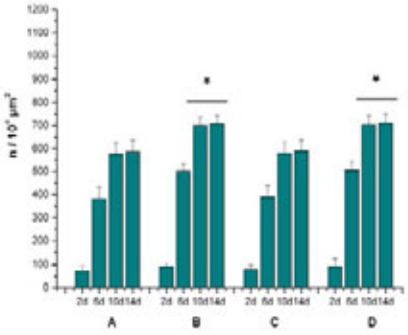

FIGURE 3 – Total number of cells ($n/10^4$ μm^2) in wounds experimentally induced in Wistar rats. Group A, control; group B treated with microcurrent (10 μA/2 min/day); group C treated with *Jatropha curcas* seed oil, and group D treated with *Jatropha curcas* seed oil and microcurrent (10 μA/2 min/day). Tissue samples were collected on day 2 (2d), 6 (6d), 10 (10d), and 14 (14d) after injury. Results are reported as the mean and standard deviation of each group and were compared by ANOVA and Tukey's post-hoc test ($p<0.05$). The asterisk indicates significant differences between time points.

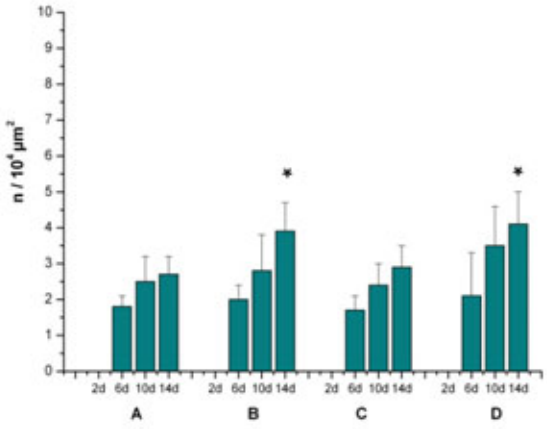

FIGURE 4 – Number of newly formed blood vessels ($n/10^4$ μm^2) in wounds experimentally induced in Wistar rats. Group A, control; group B treated with microcurrent (10 μA/2 min/day); group C treated with *Jatropha curcas* seed oil, and group D treated with *Jatropha curcas* seed oil and microcurrent (10 μA/2 min/day). Tissue samples were collected on day 2 (2d), 6 (6d), 10 (10d), and 14 (14d) after injury. Results are reported as the mean and standard deviation of each group and were compared by ANOVA and Tukey's post-hoc test ($p<0.05$). The asterisk indicates significant differences between time points.

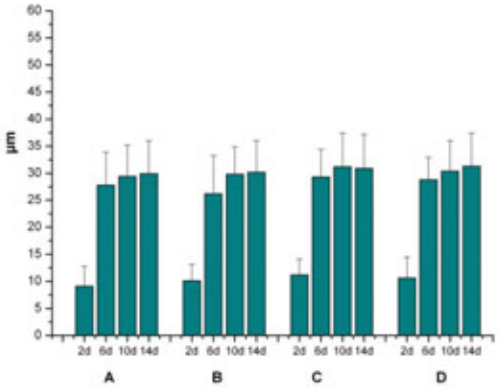

FIGURE 5 – Epithelial thickness (μm) in wounds experimentally induced in Wistar rats. Group A, control; group B treated with microcurrent (10 μA/2 min/day); group C treated with *Jatropha curcas* seed oil, and group D treated with *Jatropha curcas* seed oil and microcurrent (10 μA/2 min/day). Tissue samples were collected on day 2 (2d), 6 (6d), 10 (10d), and 14 (14d) after injury. Results are reported as the mean and standard deviation of each group and were compared by ANOVA and Tukey's post-hoc test ($p<0.05$). No significant differences were observed between time points.

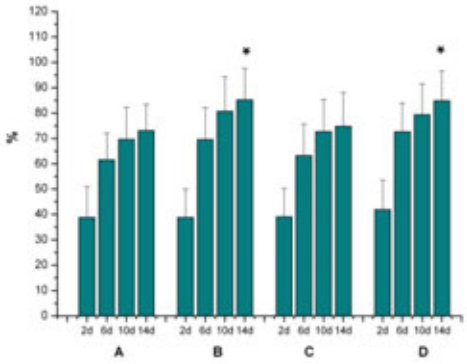

FIGURE 6 – Percent area occupied by mature collagen fibers in relation to total repair area in wounds experimentally induced in Wistar rats. Group A, control; group B treated with microcurrent (10 μA/2 min/day); group C treated with *Jatropha curcas* seed oil, and group D treated with *Jatropha curcas* seed oil and microcurrent (10 μA/2 min/day). Tissue samples were collected on day 2 (2d), 6 (6d), 10 (10d), and 14 (14d) after injury. Results are reported as the mean and standard deviation of each group and were compared by ANOVA and Tukey's post-hoc test ($p<0.05$). The asterisk indicates significant differences between time points.

Analysis of the dermal extracellular matrix showed intense deposition of collagen fibers in all groups in the first days after experimentally induced injury. Marked fiber remodeling and an increase in the degree of fiber compaction were observed between days 10 and 14. In all groups, this remodeling of new fibers first became evident at the periphery of the wound area and then extended into the center of the newly formed tissue (Figure 1). These data were confirmed by differences in the area occupied by birefringent fibers in relation to the total repair area (Figure 6), with animals of groups B and D presenting significantly higher values than those of groups A and C.

No elastic system fibers were detected in the repair area until day 10 of treatment in any of the groups. However, elastic fibers were seen close to the epidermis on day 14 after experimentally induced injury (Figure 1).

Discussion

In the present study, application of *Jatropha curcas* L. seed oil alone had no beneficial effects on the healing time of wounds experimentally induced in Wistar rats. However, significant effects were observed when the oil was applied simultaneously with microcurrent stimulation. In this respect, microcurrent application alone or in combination with the seed oil promoted an increase in the total number of cells, number of newly formed blood vessels, epithelial thickness, and compaction of mature collagen fibers in the wound area of animals on days 10 and 14 after experimentally induced injury.

In a phytochemical study of ethanolic leaf extract of *J. curcas*, Ebuehi and Okorie[27] detected the presence of alkaloids, cardiac and cyanogenic glycosides, tannins, flavonoids, and saponins. Tannins and flavonoids are known to be angiogenic and to block necrosis[28]. The antioxidant properties of phytotherapeutic agents have been suggested to be related to the presence of compounds such as tannins, flavonoids, isoflavones, and other phenolic compounds[29,30].

Analysis of the composition of *J. curcas* oil showed the presence of 42% oleic acid, 35% linoleic acid, 14% palmitic acid, and 6% stearic acid[31], compounds also conferring antioxidant properties to this oil. *In vitro* studies have demonstrated the efficacy of conjugated linoleic acid in reducing free radicals peroxidants[32,33]. The antioxidant activity of this acid is comparable to that of synthetic antioxidants[34-36]. In a phytochemical study of different parts of *J. curcas*, Nwokocha *et al*.[37] detected phenolic compounds such as tannins, flavonoids, alkaloids and saponins in the leaves, stem and seeds of this plant. Numerous studies have investigated the effect of plant extracts with antioxidant properties on tissue healing[23,38-40].

Although antioxidant compounds have been identified in the seed oil of *J. curcas*, in the present study application of the oil alone was not effective in accelerating wound healing in treated animals. On the other hand, microcurrent application to the wound area elicited positive responses, including an increase in the repair area, total number of cells, number of newly formed blood vessels, and percentage of mature collagen fibers at the site of injury[19,41-43]. According to Mendonça *et al*.[23], microcurrent application to wounds might be used as a coadjuvant to accelerate the healing process, increasing fibroblast proliferation, angiogenesis, and collagen deposition at the site of injury.

Similar effects were observed when microcurrent and *J. curcas* seed oil were applied simultaneously to the wounds. These findings agree with experimental studies combining microcurrent stimulation and plant extracts[23,39,40]. However, analysis of the findings indicates that the positive effects of this combination were the result of microcurrent stimulation and not of the *J. curcas* seed oil since the latter alone did not promote significant wound healing in treated animals. Therefore, the efficacy of the seed oil in wound healing does not seem to be the same as that reported for the crude leaf extract of this plant[13], probably because different parts of *J. curcas* contain different concentrations of antioxidant compounds. According to the phytochemical analysis conducted by Nwokocha *et al*.[37], the concentration of tannins, flavonoids, saponins, and phenols is much lower in the seeds of this species

compared to its leaves.

Conclusion

The *J. curcas* seed oil alone was not effective in promoting the healing of wounds experimentally induced in the back skin of Wistar rats, whereas microcurrent application alone or combined with the oil exerted significant effects on the different parameters studied when compared to the control group.

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Correspondence:
Fernanda Aparecida Sampaio Mendonça
UNIARARAS – Centro Universitário Hermínio Ometto
Av. Maximiliano Barutto, 500
13.607-339 Araras – SP Brasil
Tel.: (55 19)3543-1474
fernandamendonca@uniararas.br

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