Effect of Combined Application of Dressing Films Based on Mucous Secretion of Achatinafulica and Low Level Laser Therapy on Wound

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Abstract

The secretion of Achatinafulica which has recently been related to antibacterial and antifungal healing is influenced by the type of food offered. Therefore, this study investigates the potential for developing healing dressing films based on secretion of mucus of Achatinafulica. Thus, surgical wounds in Wistar rats were dressed with back films based on mucus secretion of A. fulica fed with Lactuca sativa while strips served as control wounds. Besides the treatment with films, rats were associated with daily transcutaneous irradiation using a semiconductor diode, calibrated, GaAlAs laser with continuous emission at 660 nm wavelength of 100s (25s point each). The output power used was 40 mW, with a focal point of 0.04 cm^2, and power density of 1 W/cm^2. The total energy per session was estimated at 4 J and energy density 20 J/cm^2 was divided into four different equidistant points (5 J/cm^2 each) over 7 days. The first irradiation was performed immediately after the surgical procedures. After 3, 7, 14 and 21 days the animals were sacrificed and the wounds were evaluated microscopically. On days 3 and 7mucus was put from films based on the accelerating maturation of granulation tissue formation, epithelialization and on the best rates of faster replacement from type III to type I collagen fibers. On days 14 and 21, these dressings induced deposition with more intense rate and better architectural arrangement of collagen fibers type I, and hastened the regeneration of skin phaneros. The most significant and effective results on the dressings were obtained when A. fulica fed with Lactuca sativa was used. This study suggests that the films produced with the secretion of mucus of A. fulica can successfully be used as a dressing, especially if the snails are fed with Lactuca sativa. Observation of the treatment with laser irradiation combined with dressing films based on mucus secretion of A. fulica, gave us an insight on how we can accelerate the remodeling phase of scar healing, providing a better architectural arrangement of collagen fibers.

Keywords: *Achatinafulica*, mucus, films, lasertherapy, skin repair

Resumo

A secreção de *Achatina fulica* tem sido relacionado com propriedades antibacteriana, antifúngica e cicatricial, e é influenciado pelo tipo de alimento oferecido. Portanto, este estudo investigou o potencial de cura de filmes preparados a partir secreção de muco de *Achatina fulica*. Assim, feridas cirúrgicas foram realizadas em ratos *Wistar* e tratadas com filmes de muco de *A. fulica* alimentados com *Lactuca sativa*. Paralelamente ao tratamento com filmes, outro grupo de ratos receberam tratamento com os filmes associados a laserterapia, transcutânea, utilizando um semicondutor de diodo, previamente calibrado, laser GaAlAs, com emissão contínua (CW) a 660 nm de comprimento de onda de 100 s (25 s ponto cada) A potência de saída utilizada foi de 40 mW, com um ponto focal de 0,04 cm^2, e densidade de potência de 1 W/cm^2. A energia total por sessão foi estimado em 4 J e a densidade de energia foi de 20 J/cm^2 distribuídos em 4 diferentes pontos equidistantes (5 J/cm^2 cada) ao longo de 7 dias com intervalos de 48h. A primeira irradiação realizada imediatamente após os procedimentos cirúrgicos. Após 3, 7, 14 e 21 dias os animais foram sacrificados, e as feridas foram avaliados microscopicamente. Nos dias 3 e 7 as feridas tratadas com filmes a partir do muco mostraram aceleração da maturação e formação de tecido de granulação, melhores taxas de epitelização e substituição mais rápida do tipo III para fibras de colágeno tipo I. Nos dias 14 e 21, esses curativos induziram deposição mais intensa e melhor disposição arquitetônica das fibras de colágeno tipo I, e mostrou a regeneração da fônoros cutâneos. Esta expressiva maturação sugere que os filmes produzidos com a secreção de muco de *A. fulica* pode ser empregada com sucesso como curativo, especialmente se os caracóis são alimentados com *Lactuca sativa*.

A partir da secreção de muco de *A. fulica* a combinação de filmes e laserterapia acelerou a fase de remodelação da ferida, proporcionando melhor arranjo arquitetônico das fibras de colágeno.

Palavras- chaves: *Achatinafulica*, muco, filmes,laserterapia, reparo tecidual.

Introduction

Whenever tissue loss occurs, it can be restored either by regeneration or cicatrization. Regeneration is the replacement of damaged cells or tissue by tissue similar to the original, re-establishing the function, whereas cicatrization also called repair is characterized by the formation of new connective tissue with substitution of the damaged cells and alteration of the architecture of the tissue. Although this results in a satisfactory functional and structural condition of the tissue,it's primary condition before the injurycannot be fully regained (Li et al, 2007).

The analysis of the kinetic of this biological process in response to different forms of dermal substitution is important for the development of efficient therapeutic products capable of stimulating the wound healing (Werner, Grose, 2003; Diegelman, Evans, 2004). In recent years, there have been many advances in wound healing and dermal substitution research, but no product described so far in experimental studies,is able to fully substitute the natural living skin (Shakespeare, 2001). In this vein, the use of natural polymers has been employed in healing of dermal wounds, owing to their biocompatibility, nontoxic properties, and to their ease and safety application on dermis (Bae et al, 2004; Allahverdian et al, 2006.).

Achatinafulica is a land snail able to produce a glycoproteic secretion which has been demonstrated to present some biological effects, such as antibacterial properties against *gram* positive and negative microorganisms (Iguchi et al, 1982; Kubota et al, 1985; Fuchino et al, 1992) and it is involved in the dynamics of the healing process. Furthermore, mucus produced by the snail has been tested in surgical wounds of experimental animals and was found to improve the dermal cicatricial repair (Martins et al, 2003; Sírio, 2005). More recently, it has been proposed that the type of plant offered as food to snails can influence the composition of the mucus by incorporating the secondary metabolities of plants after digestion (Lorenzi, 2008).

Lasers emit a highly concentrated, non-invasive, non-ionising radiation that promote thermal, photochemical and nonlinear effects when in contact with different tissues (Hamblin Demidova, 2006) by increasing mitochondrial respiration and adenosine triphosphate (ATP) synthesis (Morimoto et al, 1994; Ninomiya et al, 2007). Several studies have indicated that laser arrays at low frequencies are quite helpful in modulating different biological activities, such as analgesic (Nes, Poso, 2005; Turhani et al, 2006), anti-inflammatory (Pugliesi et al, 2003; Correa et al, 2007; Boschi et al, 2008; Medrado et al, 2010) and healing effects (Byrnes et al, 2004; Demidova-Rice et al, 2007; Ribeiro et al, 2009a; Chung et al, 2010a).

The use of low-level laser irradiation to speed wound healing first appeared in literature in 1971 (Master et al, 1971).

Since then, many studies have been reported attesting the beneficial effects of laser irradiation in different steps of wound healing, such as improvement of collagen synthesis (Conlan et al, 1996), fibroblast proliferation (Pourzarandian et al, 2005), blood vessels formation (Corazza et al, 2006; Melo et al, 2011), and myofibroblasts differentiation (Medrado et al, 2003; Ribeiro et al, 2009b).

The goal of this study was to investigate by histological analysis on how the application of dressing films based on mucous secretion of *Achatinafuclica* combined with low level laser irradiation affects the wound healing in rodent model.

Materials and Methods

Snail Samples

Thirty non-infected land snails *Achatinafulica* proceeded from the Snail Research Station (SRS) of the Science and Technology Institute (STI/Aracaju/SE, Brazil), weighing 45±5 g, were selected for this study. The animals were housed in wood timber boxes (35 cm width x 30 cm height), with land bedding added to calcium carbonate (to provide calcium levels required for shell development), containing 10 snails each. The boxes were daily sprinkled with water in order to maintain the humidity. The ration developed for the snails was formulated according to Pacheco, Martins (1996). Food and water were offered *ad libitum*.

Obtention of Mucous Secretion

In order to avoid contamination of the biological material when collected, the snails were housed individually in plastic cages (75 x 40 x30 cm) and kept under abstinence from food for 3 days. Subsequently, approximately 5 mL of mucus secretion was collected by manual stimulation of the podal glands of the snails. Euthanasia of the snails was not necessary to obtain the mucus, so that the animals were redelivered to the SRS/STI thereafter.

Preparation of the Films

The film dispersion was prepared using 20 mL of mucus (3.975g) and polyethylene glycol 400 (PEG400), which was employed as plasticizer. The proportion of plasticizer used was 20%w/w of dry mucus. This dispersion was homogeneizated and the films were obtained by casting process. The films obtained in this step of the experiment were cut off in square shape (2x2cm).

Surgical Procedurse and Groups Formation

Eighty adults male *Rattusnorvegicusalbinus*, *Wistar* lineage (250-300 g) were used in this study under an approved by the Ethics research Committee of Tiradents University (protocol n° 101107). The rats were housed in clear plastic cages with solid floors and loose hardwood chip bedding, and supplied with food and water *ad libitum* in a temperature and humidity-controlled environment. The animals were anesthetized with intraperitonealketamine-xylazine (100 mg/kg – 5 mg/kg) and 1cm^2 standard-sized square-shaped wounds were performed in the back of the animals. Animals were handled in accordance with the principles of aseptic chain in order to avoid any possibility of exogen bacterial contamination. Subsequently, rats were randomly assigned into four groups of twenty animals each, according to the wound dressing: CTR – untreated control group; ACH – dressed with films obtained from mucus of *A. fulica*; LT – laser-irradiated undressed; ACHLT – mucus of *A. fulica* fed with *Lactuca sativa* dressed and laser-irradiated. Five animals of each group were euthanized at 3, 7, 14 and 21 days after surgical.

Photoirradiation Protocol

Animals were submitted to daily transcutaneous irradiation using a previously calibrated semi-conductor diode laser GaAlAs (Twin Laser , MMOptics, São Paulo, Brazil) with continuous emission (CW) at 660 nm wavelength for 100 s (25 s each point) with interval of 48h for seven days. The output power used was 40 mW, with a focal spot of 0.04 cm^2, and power density of 1 W/cm^2. The total energy per session was estimated in 4 J and the energy density was 20 J/cm^2 distributed in 4 different equidistant points (5 J/cm^2 each) over the course of 7 days. The first irradiation was performed immediately after the surgical procedures.

Histological Procedures

After death certification, the area corresponding to the wound region in the back of the animals was surgically removed and the specimens were formalin-fixed and paraffin-embedded according to routine laboratorial techniques.

Subsequently, serial 5µm thick sections were obtained and stained in hematoxylin-eosin to perform the analysis of the inflammatory reaction, and picrosírius, analyzed under polarized light, to study the deposition of collagen fibers, and toluidin blue, to assess the mast cells population. Histopathologic analysis was performed and photographs were obtained with an Olympus light microscope.

Assessment of the Inflammatory Profile (IP)

Histological sections stained in hematoxylin-eosin were used to the descriptive analysis of the inflammatory profile (IP) and epithelization rates (ER). The intensity of the inflammatory response was assessed as follows: + (inflammatory cells representing less than 10% of the cell population observed within the wound area), ++ (inflammatory cells representing between 10 and 50% of the cell population observed within the wound area), and +++ (inflammatory cells representing more than 50% of the cell population observed within the wound area). Moreover, the profile inflammatory (IP) was classified as acute (predominance of polymorphonuclear cells) and chronic (predominance of mononuclear cells), and graded as slighter/absent, moderate or severe.

Assessment of the Ephitelization Rates (ER)

In order to assess the epithelization rates (ER), photomicrographs of the wounds were taken from all the samples (40x) and processed in a software (ImageLab®, Sof- tium, São Paulo, SP, Brasil). The total wound surface extent (TWE) was assessed, as well as the extent of the epidermal migration from the normal wound margin to the point where the migrating epithelium stopped processing (ME). ER (%) was determined as follows:

ER(%) = (ME X 100)/TWE

Assessement of the Collagen Deposition

Histological sections stained in picrosirius and analyzed under polarized light were used to the descriptive analysis of the collagen deposition. Collagen fibers were analyzed according to their birefringence pattern (greenish/yellow-greenish or orange, orange-reddish), morphological appearance (wavy or stretched, thin or thick, short or long) and disposition (reticularly arranged or interlaced). The quantitative analysis of the area occupied by collagen fibers in the healing area was determined by optical density in the image analysis system in different randomly selected fields. The system used, consists of a CCD Sony DXC-101 camera, applied to an Olympus CX31 microscope, from which the images were sent to a monitor (Trinitron Sony). The digitizing system was by (Olympus C-7070 WIDEZOOM) the images were inserted into a computer (Pentium 133 MHz), and processed by a software (ImageTool). A total of ten fields per case were analyzed at a magnification of 100x. The thresholds for collagen fibers were established for each slide, after enhancing the contrast up to a point at which the fibers were easily identified as birrefringent (collagen) bands. The area occupied by the fibers was determined by digital densitometric recognition, by adjusting the threshold level of measurement up to the different color densities of the collagen fibers. The area occupied by the fibers was divided by the total area of the field. The results were expressed in percentage of the skin area fraction occupied by the collagen fibers.

Statistical Analysis

Statistical significant difference in the severity of the inflammatory reaction was assessed by chi-squared test, whereas the significances of the differences in the ER, and collagenization rates were verified by analysis of variance (one-way ANOVA) and Tukey test. Each time point was analyzed separately, and two-tailed α-level of $P < 0.05$ was significant.

Results and Discussion

As shown in table 1, the inflammatory content decreased over the experimental time in all groups. However, in 07 and 14 days, the irradiated groups (LT and ACHLT) showed significant decrease in the inflammatory content in relation to both non-irradiated ones (CTR and ACH) ($p<0.05$). The profile of the inflammatory infiltrate presented some differences among the groups. In three days, the polymorphonuclear neutrophils were predominant in all groups. However, in seven days, the content of such neutrophils was less expressive in LT and ACHLT when compared to the mononuclear infiltrate (macrophages and lymphocytes). Lymphocytes and some few macrophages, constituted the inflammatory infiltrate, evidenced in all groups in 14 and, occasionally in 21 days, irrespective to the treatment applied to the wounds. These findings point at an important photobiomodulatory role played by laser rays on the dynamics of the inflammatory response during wound healing, as reported in previous studies (Freitas et al, 2001, Ribeiro et al, 2004, Correia et al, 2007, Boschi et al, 2008) .

Such activity might be related to the inhibitory effect of laser energy on the release of prostaglandin and TNF-α (Sakurai et al, 2000), mast cell degranulation (Pereira et al, 2010) and on lymphocytic proliferation (Ribeiro et al, 2009a).

Table I. Assessment of the Intensity of the Inflammatory Response in the Experimental Groups, in 3, 7, 14 And 21 Days after the Surgical Procedures

Days	Animals	Intensity of the inflammatory response			
		CTR	ACH	LT	ACHLT
3days	R1	+++	+++	+++	+++
	R2	+++	+++	+++	+++
	R3	+++	+++	+++	+++
	R4	+++	+++	+++	+++
	R5	+++	+++	+++	+++
7days	R1	+++	+++	++	++
	R2	+++	++	++	++
	R3	+++	++	++	++
	R4	+++	+++	++	++
	R5	+++	++	++	++
14 days	R1	+	+	-	+
	R2	++	+	+	-
	R3	++	+	-	-
	R4	+	++	+	-
	R5	++	++	-	-
21 days	R1	+	-	-	+
	R2	-	-	-	-
	R3	-	-	-	-
	R4	-	-	-	-
	R5	+	-	-	-

(-) – inflammatory infiltrate is absent; (+) – mild inflammatory infiltrate corresponding to less than 10% of stromal cells; (++) moderate inflammatory infiltrate corresponding to more than 10 and less than 50% of stromal cells; (+++) intense inflammatory infiltrate corresponding to more than 50% of stromal cells.

The granulation tissue was clearly more exuberant in LT and ACHLT than in the other groups in seven days, but inconspicuous in 14 days. In ACH in seven days, it was conspicuous, although less expressive than in the irradiated groups, whereas in 14 days the vascular content was substantially reduced. In CTR, the granulation tissue was more vascular in seven days and less conspicuous, but clearly distinguishable, in 14 days (figure 1). These results suggest that laser irradiation provided more rapid onset of the vascular steps of wound healing. Similar findings were previously reported in other studies (Medrado et al, 2003, Bayat et al, 2006, Ribeiro et al, 2009a) and they are likely related to direct stimulation of angiogenesis induced by laser irradiation (Corazza et al, 2007, Pereira et al, 2010; Melo et al, 2010). Besides, the contents of blood vessel were substantially reduced in the irradiated groups in 14 days, suggesting that laser irradiation improved the angiogenesis in the earlier phases of wound healing without providing undesirable longer-term maintenance of the vascular supply, which could impair the final scar formation (Ribeiro et al, 2009a).

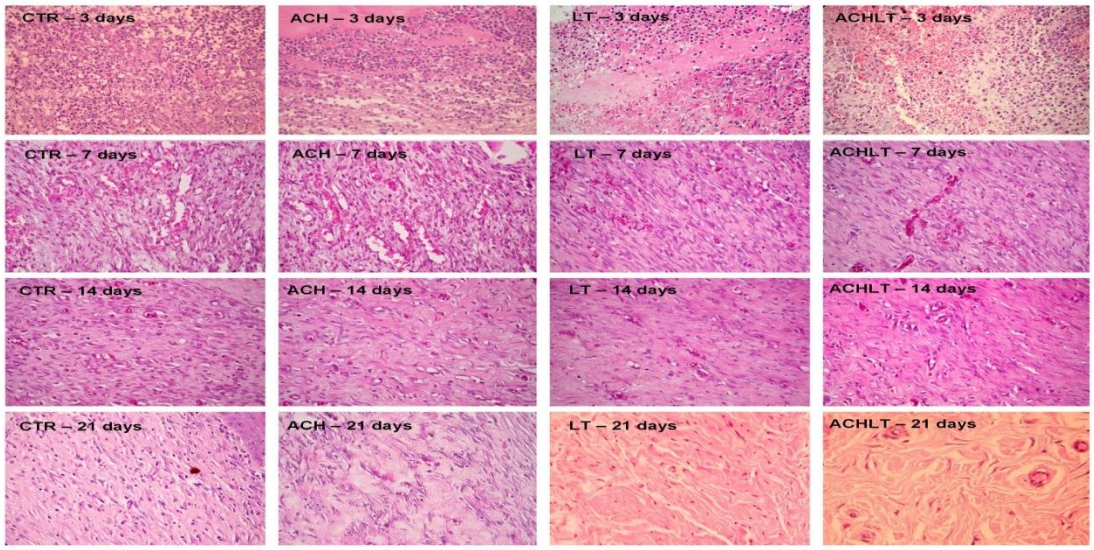

Figure 1. Histological sections showing evolution of the healing process in the experimental groups over the time. In 3 days, acute inflammatory response (predominance of neutrophils) is observed in all groups. In 7 days, the granulation tissue is more vascular in CTR and ACH and more fibroblastic in LT and ACHLT. In 14 days, CTR showed distinguishable residual granulation tissue, in opposition to ACH, Lt and ACHLT. In 21 days, the inflammatory or vascular content are inconspicuous in all groups, but the cicatricial scar is composed of denser and grosser interlaced fibers in LT and ACHLT (HE, 200X).

The epithelization rates (ER) are presented in figure 2. In seven and 14 days, the ER observed in LT and ACHLT were significantly higher than in CTR and ACH ($p<0.05$), but there was no significant difference in the other experimental times ($p>0.05$). It has been demonstrated that low level laser irradiation is able to stimulate keratinocyte proliferation *in vitro* (Eduardo et al, 2007), which could explain the more rapid epithelial lining reconstitution in both irradiated groups. In addition, laser-induced improved epithelization during wound healing has also been reported in previous investigations (Ribeiro et al, 2009a, Chung et al, 2010a, Chung et al, 2010b), supporting the histological findings presented in this study.

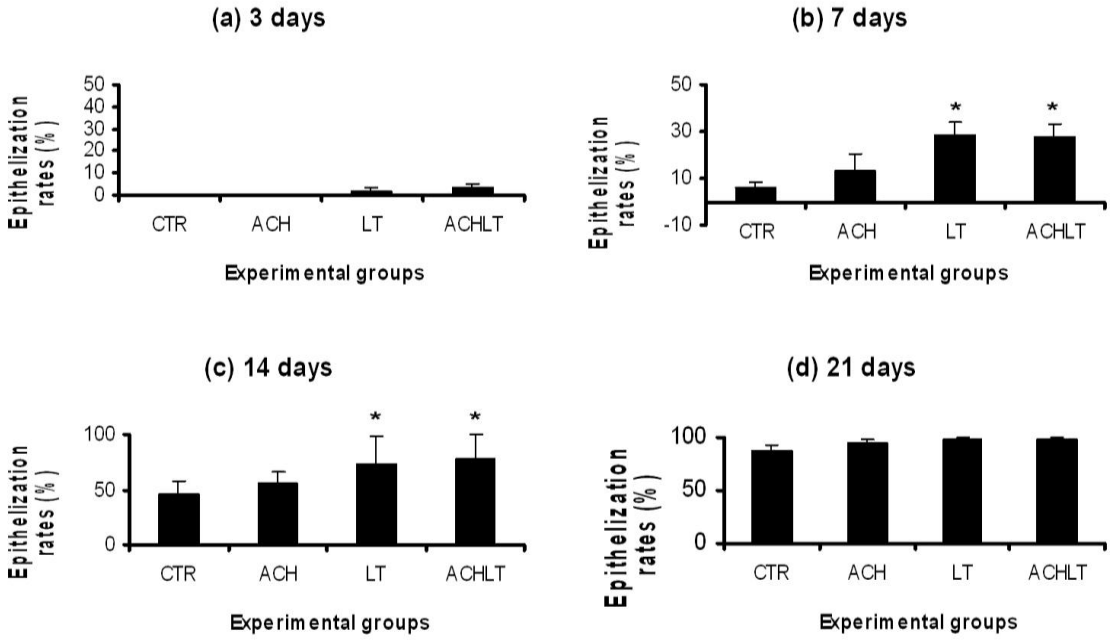

Figure 2: Assessment of the Epithelization Rates in the Experimental Groups over the Experimental time. (*) Significantly different from CTR and ACH ($p<0.05$)

As showed in figure 3, scanty deposition of thin delicate reticular-arranged collagen fibrils, exhibiting yellow-greenish birefringence (type III collagen) was observed in all groups after three days. In seven days, the fibers were thicker, but the type III collagen were still predominant in CTR and ACH, whereas in LT and ACHLT, these fibers were predominantly gold or reddish (type I collagen). Moreover, the fibers were more compacted disposed in these last groups. In 14 days days, there was a remarkable improvement of the collagenization and type I collagen fibers were more abundant in all groups. It was evidenced intense deposition of gross thick parallel-arranged collagen bundles, less densely deposited in the top of the scars, with apparent complete replacement of type III for type I collagen fibers, in CTR. In ACH, LT and ACHLT deposition of gross wavy parallel-arranged collagen bundles was also observed, but the density of the collagen disposition in these groups was clearly more intense and compacted than in CTR. In 21 days, CTR and ACH showed dense deposition of type I collagen fibers, but they were still parallel-arranged in CTR and densely interlaced in ACH. On the other hand, LT and ACHLT exhibited deposition of interlaced long delicate collagen fibers, composed of both type I and type III collagen, densely compacted, resembling the normal dermal connective tissue.

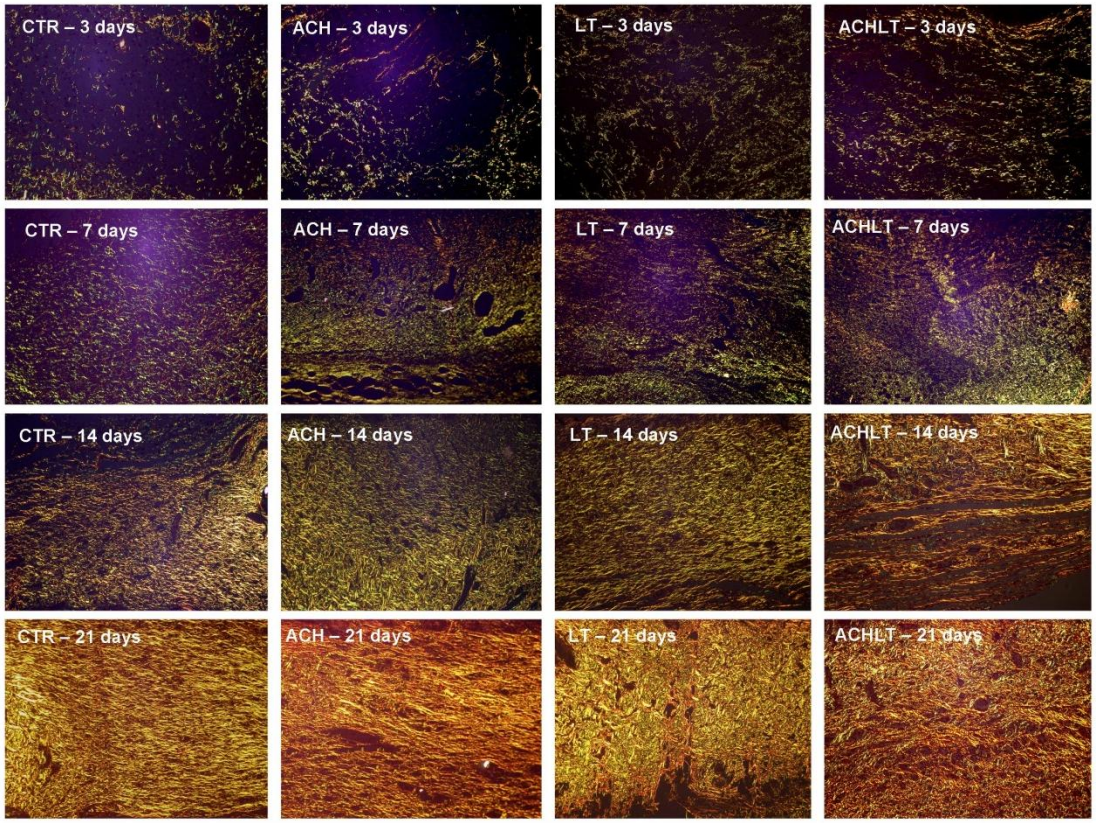

Figure 3. Histological sections showing the evolution of the collagenization in the experimental groups over the time. In 3 days, predominance of reticular-arranged thin delicate fibrils of type III collagen is seen in all groups. In 7 days, partial replacement of type III for type I collagen is observed in the treated groups. In 14 days, the type I collagen fibers are predominantly parallel-arranged and more compacted disposed in LT and ACHLT. In 21 days, the densest organization of type I collagen is evidenced in the groups; the highest density of collagen deposition is seen in LT, whereas in ACHLT, the interlaced fibers are less compacted and composed of both types of collagen (Sirius Red/Polarization, 200x).

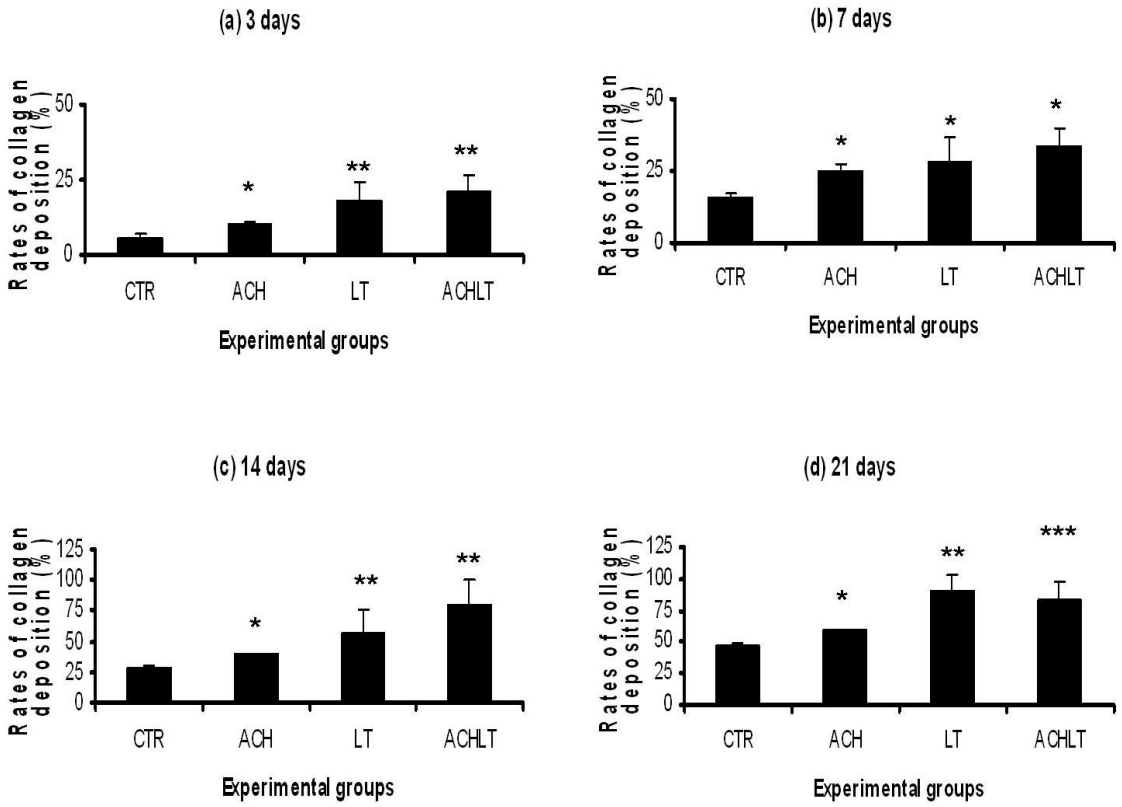

Figure 4: Assessment of the Collagen Deposition rates in the Experimental Groups over the time. (*) Significantly different from CTR (p<0.05); (**) Significantly different from CTR (p<0.01) and ACH (P<0.05); (***) Significantly Different from CTR, ACH and LT (p<0.05)

Type III collagen is produced in early stages of wound healing, likely to orientate the migration of endothelial cells during the granulation tissue formation, whereas type I collagen is ultimately deposited to provide tensile strength to the tissue, so that the scar acquires mechanical stability (Albuquerque-Júnior et al, 2009; Nunes et al, 2011). Therefore, the replacement of a substantial part of the content of type III collagen for type I molecules during the healing process is an absolutely expected phenomenon. Both dressing films and low level laser irradiation applied in this study induced rapid replacement of type III for type I collagen and improved the spatial organization of the collagen fibers from parallel disposition to interlaced arrangement, characteristic of normal dermis. Regarding the morphometrical analysis of the collagen deposition rates (figure 4), it was observed that all treated groups showed significantly increased rates of collagen deposition in relation to the control group (CTR) over the time, but in seven and 14 days, the irradiated groups presented significantly higher rates compared to CTR and ACH. In 21 days, LT and ACHLT demonstrated the highest collagenization rates, but surprisingly the rates observed in ACHLT were lower than in LT.

The presence of high contents of mucin in the mucous secretion of A. fulica (not shown data), a protein rich in aminoacid residues closely related to the biochemical synthesis of collagen molecules, such as serine asparagine, hydroxylysine and/or threonine (Adikwu, 2006), could support the results observed in this study. Besides, other studies showing the improvement of wound healing after treatment with mucous secretion of A. fulica has been previously reported (Martins et al, 2003; Sirio et al, 2005), supporting the attesting of the healing potential of this biological material derived from giant snails. However, the irradiated groups showed the most distinguished effects on the collagenization. It has been demonstrated that LLLT, not only improves the collagen deposition (Ribeiro et al, 2009a), but also provides better architectural organization of the collagen fibers (Medrado et al, 2008). It is likely that such modulatory properties are associated with the laser capability to stimulate fibroblast, as previously demonstrated in experimental *in vitro* assays (Pereira et al, 2002).

However, the excessive production of type I collagen, as observed in the treated groups, might favor the formation of undesirable hypertrophic scars and keloids (Verhaegen et al, 2009). Therefore, the moderate content of less thick collagen fibers in ACHLT appears to provide low probability of forming hypertrophic scars. Moreover, the balance in the content of both type I and type III collagen fibers as seen in ACHLT, suggests that the remodeling phase of the scar represented by degradation of the gross previously formed connective matrix, followed by gradual deposition of a newly depurated matrix rich in both collagen molecules (Albuquerque-Júnioir et al, 2009, Nunes et al 2011), has already been installed, which would justify the clear resemblance with the normal histological appearance of the dermal collagen. It must also be stressed that despite the biological effects of the combination of this singular dressing film and low level laser irradiation on the fibroplasia dynamics seem to be likely related to a possible increase in the fibroblast metabolism, as long as both synthesis and degradation of the collagen molecules were apparently stimulated in this study, further investigations are required in order to fully clarify the precise mechanism underlying these healing modulation pathways.

In conclusion, despite low level laser therapy itself was able to improve many steps of wound healing, the combination of dressing films based on mucous secretion of *A. fulica*and laser irradiation seemed to hasten the remodeling phase of the healing scar, providing better architectural arrangement of the collagen fibers.

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References

Adikwu, M. M. Mucins and their potentials (editorial). Trop. J. Pharm. Res. 5: 581-582, 2006.

Albuquerque-Júnior, R. L. C.; Barreto, S. A. L.; Pires, A. J.; Reis, P. F.; Lima, O. S.; Ribeiro, G. M. A. ; Cardoso, C. J. Effect of bovine type-I collagen-based films containing red propolis on dermal wound healing in rodent model. Int. J. Morphol., 27(4):1105-1110, 2009.

Allahverdian S, Carbohydrates and epithelial repair - more than just post-translational modification. Curr Drug Targets 2006; 7(5):597-606.

Bae JS, The effect of polysaccharides and carboxylcellulose combination to prevent intraperitoneal adhesion and abscess formation in a rat peritonitis model. J Vet Med Sci 2004; 66:1205-1211.

Bayat M, Vasheghani MM, Razavi N.Effect of low-level helium-neon laser therapy on the healing of third-degree burns in rats.J PhotochemPhotobiol B. 2006 May 1;83(2):87-93.

Boschi ES, Leite CE, Saciura VC, Caberlon E, Lunardelli A, Bitencourt S, Melo DA, Oliveira JR.Anti-Inflammatory effects of low-level laser therapy (660 nm) in the early phase in carrageenan-induced pleurisy in rat.Lasers Surg Med. 2008 Sep;40(7):500-8.

Byrnes KR, Barna L, Chenault VM, Waynant RW, Ilev IK, Longo L, Miracco C, Johnson B, Anders JJ. Photobiomodulation improves cutaneous wound healing in an animal model of type II diabetes. Photomed Laser Surg 2004;22(4):281–290.

Chung TY, Peplow PV, Baxter GD. Laser photobiomodulation of wound healing in diabetic and non-diabetic mice: effects in splinted and unsplinted wounds. Photomed Laser Surg. 2010 Apr;28(2):251-61.

Chung TY, Peplow PV, Baxter GD. Laser photobiostimulation of wound healing: defining a dose response for splinted wounds in diabetic mice. Lasers Surg Med. 2010 Nov;42(9):656-64

Conlan MJ, Rapley JW, Cobb CM. Biostimulation of wound healing by low-energy laser irradiation. J Clin Periodontol 1996; 23:492–496.

Corazza AV, Jorge J, Kurachi C, Bagnato VS. Photobiomodulation on the angiogenesis of skin wounds in rats using different light sources.Photomed Laser Surg. 2007 Apr;25(2):102-6.

Demidova-Rice TN, Salomatina EV, Yaroslavsky AN, Herman IM, Hamblin MR. Low-level light stimulates excisional wound healing in mice. Lasers Surg Med 2007;39(9):706–715.

Diegelmann RF, Evans MC. Wound healing: an overview of acute, fibrotic and delayed healing.Front Biosci 2004; 1(9):283-9.

Eduardo FP, Mehnert DU, Monezi TA, Zezell DM, Schubert MM. Eduardo CP et al. Cultured epithelial cells response to phototherapy with low intensity Laser.Lasers Surg Med 2007; 39: 365-72.

Freitas AC, Pinheiro AL, Miranda P, Thiers FA, Vieira AL. Assessment of inflammatory effect of 830 Laser light using C-reactive protein levels. Bras Dent J 2001; 12: 187-190.

Fuchino HO, Bactericidal action of a glycoprotein from the body surface mucus of giant african snail. Comparative biochemical Physiology 1992; 101(3):607-613.

Guerino MR, Baranauskas V, Guerino AC, Parizotto N. Laser treatment of experimentally induced chronic arthritis. Appl Surf Sci 2000;154–155:561–564.

Iguchi SMM, Aikawa T, Matsumoto JJ. Antibacterial activity of snail mucus mucin. Comparative Biochemistry and Physiology 1982; 72A(3): 571-574.

Kubota Y, et al. Purificação e caracterização do fator antibacteriano do muco de escargots. Comp. Biochem. Physiol 1985; 82(2): 345-348.

Li J, Chen J, Kirsner R. Pathophysiology of acute wound healing. Clin Dermatol 2007; 25:9–18.

Lorenzi AT, Martins MF. Análise colorimétrica e espectroscópica do muco de caracóis terrestres Achatina sp alimentados com ração diferenciada.R. Bras. Zootec 2008; 37(3): 572-579.

M.R. Hamblin, T.N. Demidova, Mechanisms of low-level light therapy, Proc.

Martins MF. Avaliação macro e microscopica da cicatrização de lesões experimentalmente provocadas em pele de coelhos tratadas com secreção mucoglicoproteica do escargot Achatina fulica. Brazilian Journal of Veterinary Research and Animal science 2003; 40(3):213-218.

Medrado AP, Soares AP, Santos ET, Reis SR, Andrade ZA.Influence of laser photobiomodulation upon connective tissue remodeling during wound healing. 2008 Sep 18;92(3):144-52.

Medrado, A.P., Pugliese, L.S., Reis, S.R., and Andrade, Z.A. (2003). Influence of low level laser therapy on wound healing and its biological action upon myofibroblasts. Lasers Surg. Med. 32, 239–244.

Medrado, A. R. A. P. Pugliese, L.S., Reis, S.R. Effect of low-power Laser irradiation on cell growth and procollagen synthesis of cultured fibroblasts. Lasers in Surgery and Medicine, v. 31, n. 4, p. 263-267, 2010.

Melo VA, Anjos DCS, ; Albuquerque Júnior RLC, Melo DB, Carvalho FUR. Effect of low level laser on sutured wound healing in rats. Acta Cir. Bras. [serial on the Internet]. 2011 Apr [cited 2011 June 27] ; 26(2): 129-134.

Mester E, Spiry T, Szende B, Tota JG. Effect of laser rays on wound healing. Am J Surg 1971;122(4):532–535.

Morimoto Y, Arai T, Kikuchi M, Nakajima S, Nakamura H. Effect of low-intensity argon laser irradiation on mitochondrial respiration. Lasers Surg Med 1994; 15:191–199.

Nes AG, Posso MB. Patients with moderate chemotherapy-induced mucositis: Pain therapy using low intensity Lasers. IntNurs Rev 2005;52:68-72.

Ninomiya T, Hosoya A, Nakamura H, Sano K, Nishisaka T, Ozawa H. Increase of bone volume by a nanosecond pulsed laser irradiation is caused by a decreased osteoclast number and an activated osteoblasts. Bone 2007; 40:140–148.

Nunes, P. S. Albuquerque-Junior, R.L.C., Cavalcante, D. R. R. ; Dantas M. D. M, Cardoso J. C., Bezerra M. S., Souza J. C. C. Serafini M. R, Quitans-jr, L. J., Bonjardim L. R., Collagen-Based Films Containing Liposome-Loaded Usnic Acid as Dressing for Dermal Burn Healing Journal of Biomedicine and Biotechnology, Article ID 761593, 9 pagesdoi:10.1155/2011/761593, 2011.

Pereira MC, de Pinho CB, Medrado AR, Andrade Zde A, Reis SR.Influence of 670 nm low-level laser therapy on mast cells and vascular response of cutaneous injuries. J PhotochemPhotobiol B. 2010 Mar 8;98(3):188-92.

Pereira, A.N., Eduardo, C.D.E.P., Matson, E., and Marques, M.M. (2002). Effect of low-power laser irradiation on cell growth and procollagen synthesis of cultured fibroblasts.vLasers Surg. Med. 31, 263–267.

Pourzarandian A, Watanabe H, Ruwanpura SM, Aoki A, Ishikawa I. Effect of low-level Er:YAG laser irradiation on cultured human gingival fibroblasts. J Periodontol 2005; 76:187–193.

Pugliese A.M., Latini S., Corradetti R., Pedata F. Brief, repeated, oxygen–glucose deprivation episodes protect neurotransmission from a longer ischemic episode in the in vitro hippocampus: role of adenosine receptors. Br. J. Pharmacol. 2003.

Ribeiro M.A.G, Cavalcanti de Albuquerque RL, Santos Barreto AL, Moreno de Oliveira VG, Santos TB, Freitas Dantas CD.Morphological analysis of second-intention wound healing in rats submitted to 16 J/cm^2 lambda 660-nm laser irradiation.Indian J Dent Res. 2009a Jul-Sep;20(3):390.

Ribeiro MAG,Albuquerque Jr RLC, Ramalho LMP, Pinheiro ALB, Bonjardim LR, Cunha SSC . Immunohistochemical Assessment of Myofibroblasts and Lymphoid Cells During Wound Healing in Rats Subjected to Laser Photobiomodulation at 660 nm. Photo. and laser sugery 2009b; 27(1):49-55.

Ribeiro MS, Silva F, Araújo CE, Oliveira SF, Pelegrini CM, Zorn TM, *et al*. Effect of low-intensity polarized visible Laser radiation on skin burns: A light microscopy study. J Chin Laser Med Surg 2004;22:59-66.

Ribeiro MS, Silva F, Araújo CE, Oliveira SF, Pelegrini CM, Zorn TM et al. Effect of low-intensity polarized visible Laser radiation on skin burns: a light microscopy study. J Chin Laser Med Surg 2004; 22: 59-66.

Ribeiro, M.A.; Albuquerque, R.L.; Ramalho, L.M.; Pinheiro, A.L.; Bonjardim, L.R. & Da Cunha, S.S., 2009b. Immunohistochemical Assessment of Myofibroblasts and Lymphoid Cells During Wound Healing in Rats Subjected to Laser Photobiomodulation at 660 nm. Photomed. Laser Surg., 27, 49-55.

Sakurai Y, Tamaguchi M, Abiko Y. Inhibitory effect of low-level Laser irradiation on LPS-stimulated prostaIGandin E2 production and cyclooxygenase-2 in human gingival fibroblasts. Eur J Oral Sci 2000; 108: 29-34.

Shakespeare P. Burn wound healing and skin substitutes. Burns 2001; 27:517-522.

Sírio O J (2005). Verificação da potencialização do efeito cicatrizante do muco de caracóis do gênero *Achatina* promovida por dieta à base de Confrei (*Symphytum officinale L*.). Dissertação, Mestrado. Programa de Pós-Graduação em Nutrição e Produção Animal. Faculdade de Medicina Veterinária. Universidade de São Paulo (USP). Pirassununga 2005; 87.

SPIE. 6140 (2006) 1-11.

Turhani D, Scheriau M, Kapral D, Benesch T, Jonke E, Bantleon HP. Pain relief by single low-level laser irradiation in orthodontic patients undergoing fixed appliance therapy. Am J OrthodDentofacialOrthop 2006;130(3):371–377.

Verhaegen, P.D., Van Zuijlen, P.P., Pennings N.M., Van Marle J., Niessen, F.B., Van Der Horst, C.M., Middelkoop, E., 2009. Differences in collagen architecture between keloid, hypertrophic scar, normotrophic scar, and normal skin: An objective histopathological analysis. Wound Repair Regen, 17, 649-56.

Werner S, Grose R. Regulation of wound healing by growth factors and cytokines. Physiol Rev 2003; 83: 835-870.