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Abstract

This study determined the effects of gallium-aluminum-arsenide laser (GaAlAs), gallium-arsenide laser (GaAs) and Dersani[®] healing ointment on skin wounds in Wistar rats. The parameters analyzed were: type I and III collagen fiber concentrations as well as the rate of wound closure. Five wounds, 12 mm in diameter, were made on the animals' backs. The depth of the surgical incision was controlled by removing the epithelial tissue until the dorsal muscular fascia was exposed. The animals were anesthetized with ketamine and xylazine via intraperitoneal injection. The rats were randomly divided into five groups of 6 animals each, according to the treatment received. Group 1 (L4): GaAs laser (4 J/cm²); group 2 (L30): GaAlAs laser (30 J/cm²); group 3 (L60): GaAlAs laser (60 J/cm²); group 4 (D): Dersani[®] ointment; group 5 (control): 0.9% saline. The applications were made daily over a period of 20 days. Tissue fragments were stained with picosirius to distinguish type I collagen from type III collagen. The collagen fibers were photo-documented and analyzed using the Quantum software based on the primary color spectrum (red, yellow and blue). Significant results for wound closing rate were obtained for group 1 (L4), 7.37 mm/day. The highest concentration of type III collagen fibers was observed in group 2 (L30; 37.80 ± 7.10%), which differed from control (29.86 ± 5.15%) on the 20th day of treatment. The type I collagen fibers of group 1 (L4; 2.67 ± 2.23%) and group 2 (L30; 2.87 ± 2.40%) differed significantly from control (1.77 ± 2.97%) on the 20th day of the experiment.

Key words: Cicatrization; Laser; Healing ointment; Collagen I and III; Cutaneous wound

Introduction

Skin wounds are present in approximately 10% of hospitalized patients and 20% of bedridden patients treated at home, with consequent high financial costs and ranking second as the most common cause of work absenteeism (1). The healing of skin wounds is a highly organized dynamic process that works to restore the structure and function of the damaged tissue. In this process, a complex interaction occurs between cells, extracellular matrix, blood vessels, proteases, cytokines, and chemokines (2). The healing process involves inflammatory cell migration, granulation tissue synthesis, collagen and proteoglycan deposition, and scar maturation, being associated with intense remodeling (3).

The main component of a mature scar is type I collagen, and in the healing wounds, fibroblasts are recruited

and induced to synthesize collagen in a process called fibroplasia, initiated by the granulation tissue in the wound, and rich in type III collagen (4). This tissue is made up of loose collagen matrix, fibronectin and hyaluronic acid, also containing macrophages, fibroblasts, and new vessels. As the tissue recovery process advances, the granulation tissue is replaced by a less vascularized and more resistant tissue rich in type I collagen (5).

Most therapeutic interventions are designed to facilitate the wound healing process, with emphasis on the importance of protecting wounds to prevent complications (6). Essential fatty acids such as those derived from linoleic acid are among the resources supporting the healing process (7). Dersani[®] ointment belongs to this group, being used

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in any type of cutaneous lesion as a bactericide during the different phases of the process (3).

The laser technique is one of the methods used to hasten the recovery of tissue functionality in the treatment of wounded tissues. Due to the efficacy of laser procedures in the healing process, they are recommended for the recovery of the tissue by inducing vessel dilatation, collagen synthesis and by increasing tissue oxygenation (8). According to Enwemeka et al. (9), all phases of the healing process are positively affected by laser treatment, with wavelength and energy density being crucial factors for successful treatment (10).

The results of a study conducted by Reddy (11) indicated that one of the devices used, gallium-arsenide (GaAs) laser, was effective in healing wounds of diabetic rats by promoting a significant increase in the amount of collagen. This device is extensively used in procedures aimed at treating skin wounds and also the reason it was selected for the present study. Gallium-aluminum-arsenide (GaAlAs) laser (904 nm), when applied to the microcirculation of live rats, promotes dilatation of the irradiated arterioles, followed by an increase in arteriolar blood flow. A good tissue vascularization and an efficient collagen deposition are determining factors for successful cicatrization (6).

Since the identification of adequate healing-promoting techniques is highly beneficial for patient care and immediate cost reduction, investigating the healing effect of different laser modalities and determining their action on collagen fibers throughout the healing process are of fundamental importance to establish mechanisms to promote faster, safer and infection-free cicatrization, reducing the healing and recovery time, and improving the quality of life of the patients at a lower cost.

The aim of the present study was to determine the effect of GaAs laser (4 J/cm²), GaAlAs laser (30 and 60 J/cm²) and Dersani[®] healing ointment on the skin healing process by determining the amount of type I and type III collagen fibers, and the rate of wound closure.

Material and Methods

Ten-week-old male Wistar rats (*Rattus norvegicus*) weighing on average 324 g, from the Central Animal House of the Health and Biological Sciences Center, Federal University of Viçosa (UFV), were used. The animals were maintained under natural light and humidity conditions (temperature 22°C; 12-h light/dark cycles) in individual cages cleaned daily, fed once a day and offered water *ad libitum*. The experiment was conducted according to the Guiding Principles in the Use of Animals Ethics Committee of the Faculty of Physiotherapy of Minas Gerais (FAMINAS; registration #005/2008), taking the proper care of the animals into consideration.

The animals were anesthetized with ketamine and xylazine via intraperitoneal injection (60 mg/kg body weight).

Trichotomy was then performed on the dorsolateral region of all animals and five circular secondary intention wounds of 12 mm in diameter were made in the dorsolateral region of the animals by performing a surgical incision in the skin and subcutaneous cell tissue using a scalpel. The area of the wounds was previously marked using crystal violet and checked using an analogical pachymeter (Mitutoyo Sul Americana Ltda[®], Brazil). The depth of the surgical incision was controlled by removing the epithelial tissue until the dorsal muscular fascia was exposed. All therapies were initiated 6 h after surgery and repeated daily for the 20 days of the experiment. Fragments of the different wounds containing edges and center were collected every 4 days to compare with the unwounded tissue. The size of the fifth wound was measured every 4 days with the use of a caliper, and this wound was used as control to verify how fast the wounds closed.

The rats were divided randomly into five groups of 6 animals each. Their wounds were cleaned with 0.9% saline once a day in the morning. In group 1 (L4), the animals were submitted to GaAs laser radiation at a wavelength of 904 nm and energy density of 4 J/cm² (0.8 J/mm² per point). Group 2 (L30) was treated with GaAlAs laser at a wavelength of 830 nm and energy density of 30 J/cm² (6 J/mm² per point). In group 3 (L60), the animals were treated with GaAlAs laser at a wavelength of 830 nm and energy density of 60 J/cm² (12 J/mm² per point). The scanning technique was used and the duration of radiation application was automatically adjusted. The Quasar laser device (Dentoflex[®], Brazil) was used. Group 4 (D) was treated with Dersani[®] ointment (caprylic acid, capric acid, lauric acid, soy lecithin, vitamin A, vitamin E, caproic acid, and sunflower oil - linoleic acid), with 0.1 g of the ointment being applied once a day. Group 5 (control group) consisted of animals whose wounds were only cleaned with 0.9% saline once a day.

The animals were weighed every 4 days, an interval coinciding with the removal of the wound fragments. The fragments removed for histological analysis were preserved in 10% buffered formaldehyde for 24 h. The material was processed for paraffin embedding by ethanol dehydration, diaphanization with xylene, infiltration and embedding in paraffin wax. Cuts of 4 µm were obtained with a rotary microtome (Reichert-Jung 2045 Multicut[®], Germany) and stained with picosirius.

Images of histological sections were captured with a polarization microscope (Olympus AX-70[®], Brazil) and the amount of type I and III collagen fibers was evaluated. Four random images of different parts of the field were obtained from each sample. The images were analyzed using the Quantum software program (Department of Soil Science, Federal University of Viçosa, Viçosa, MG, Brazil) that differentiates fibers by the colors red, green and blue in each image. The intervals defining the identification of the colors red, green and black were previously standardized and

then used for all images obtained. Red represented type I collagen fibers, green type III collagen fibers and black the regions where no fiber was found. The program provides the percentage of the area corresponding to each color.

Statistical analysis

The data were analyzed statistically by the Kruskal-Wallis test, with the level of significance set at 5%.

Results

The most significant results regarding the extent of wound reduction were obtained for group 1 (L4; Table 1), which also presented the fastest rate of wound closure (7.37 mm/day; Table 2).

Analysis of the percentage of type III collagen fibers showed that, on the fourth day of the experiment, groups 2 (L30) and 3 (L60) presented the highest percentage of fibers ($P < 0.05$). On the 8th day of treatment, no statistical differences were found among the groups. On the 12th day, all groups presented a statistically significant difference compared with the control group ($P < 0.05$) except group 3. Moreover, group 4 (D) presented a lower percentage of

collagen fibers than group 2 (L30) ($P < 0.05$). On the 20th day, group 2 (L30) presented a significantly higher percentage of type III collagen fibers than the other groups ($P < 0.05$), whereas group 1 (L4), treated with 4 J/cm² laser, had a significantly lower percentage of type III collagen fibers than the other groups ($P < 0.05$). These data are shown in Table 3.

Table 1. Extent of wound diameter reduction after 20 days of different treatments.

Treatment	Reduction of extent of wound (%)
Group 1 (4 J/cm ² , 904 nm)	94.76 ± 0.16*
Group 2 (30 J/cm ² , 830 nm)	77.6 ± 0.45
Group 3 (60 J/cm ² , 830 nm)	83.43 ± 0.21
Group 4 (Dersani®)	81 ± 0.14
Group 5 (0.9% saline)	81 ± 0.21

Data are reported as means ± SD for 6 rats in each group. Group 1 = gallium-arsenide laser (GaAs); group 2 = gallium-aluminum-arsenide laser (GaAlAs); group 3 = GaAlAs laser; group 4 = Dersani® ointment; group 5 = control cleaned with saline daily. * $P < 0.05$, group 1 compared to all other groups (Kruskal-Wallis test).

Table 2. Partial rate and average rate of extent of wound closure in relation to time and treatments.

Treatment	Rate of wound closure (mm)					Average rate (mm/day)
	4 days	8 days	12 days	16 days	20 days	
Group 1* (4 J/cm ² , 904 nm)	7.9 ± 6.8	13.8 ± 1.5	5.2 ± 1.2	6.8 ± 5.8	2.96 ± 1.6	7.37 ± 2.6
Group 2 (30 J/cm ² , 830 nm)	9.75 ± 3.6	17.84 ± 4.5	12.08 ± 1.9	8.83 ± 2.1	6.08 ± 1.1	7.01 ± 1.1
Group 3 (60 J/cm ² , 830 nm)	6.75 ± 2.4	12.70 ± 1.8	5.66 ± 3.1	5.33 ± 0.36	4.54 ± 2.64	7.00 ± 1.4
Group 4 (Dersani®)	1.96 ± 0.36	16.58 ± 6.07	12.70 ± 2.4	3.08 ± 1.04	4.87 ± 0.9	7.05 ± 1.6
Group 5 (0.9% saline)	7.1 ± 6.8	8.2 ± 1.5	9.6 ± 3.1	4.8 ± 4.0	4.41 ± 1.3	6.80 ± 2.6

Data are reported as means ± SD for 6 rats in each group. Group 1 = gallium-arsenide laser (GaAs); group 2 = gallium-aluminum-arsenide laser (GaAlAs); group 3 = GaAlAs laser; group 4 = Dersani® ointment; group 5 = control cleaned with saline daily. * $P < 0.05$, group 1 compared to all other groups (Kruskal-Wallis test).

Table 3. Average values of type III collagen fiber count related to time and treatment with saline, Dersani® ointment and different laser types and intensities.

Group	Concentration of collagen fiber III (%)					
	0 days	4 days	8 days	12 days	16 days	20 days
Group 1 (4 J/cm ² , 904 nm)	12.43 ± 4.70	3.12 ± 1.86	17.92 ± 12.57	27.50 ± 11.89	19.89 ± 13.28	16.22 ± 5.44
Group 2 (30 J/cm ² , 830 nm)	16.18 ± 0.58	4.21 ± 2.20	18.36 ± 16.03	33.32 ± 17.05	25.24 ± 8.33	37.80 ± 7.10*
Group 3 (60 J/cm ² , 830 nm)	10.40 ± 4.95	3.30 ± 1.92	18.84 ± 13.85	10.40 ± 4.95	12.35 ± 6.79	32.12 ± 11.17
Group 4 (Dersani®)	15.54 ± 8.28	2.55 ± 1.29	15.93 ± 11.82	21.60 ± 14.53	18.48 ± 8.53	25.90 ± 4.56
Group 5 (saline)	10.19 ± 5.62	3.01 ± 2.69	13.015 ± 12.69	12.96 ± 12.84	30.73 ± 7.01	29.86 ± 5.15

Data are reported as means ± SD for 6 rats in each group. Group 1 = gallium-arsenide laser (GaAs); group 2 = gallium-aluminum-arsenide laser (GaAlAs); group 3 = GaAlAs laser; group 4 = Dersani® ointment; group 5 = control cleaned with saline daily. * $P < 0.05$, group 2 compared to all other groups on the 20th day of treatment (Kruskal-Wallis test).

Analysis of type I collagen fibers (Table 4) showed that on the fourth day of the experiment, group 2 (L30) had a significantly higher percentage ($P < 0.05$) compared with the other groups, except group 4 (D). Also, a significant

Table 4. Average values of type I collagen fiber count related to time and treatment with saline, Dersani[®] ointment and different laser types and intensities.

Treatment	Concentration of collagen fiber I (%)					
	0 days	4 days	8 days	12 days	16 days	20 days
Group 1 (4 J/cm ² , 904 nm)	22.38 ± 15.72	0.69 ± 0.58	0.23 ± 0.73	3.57 ± 12.74	1.05 ± 0.46	2.67 ± 2.23
Group 2* (30 J/cm ² , 830 nm)	18.24 ± 6.58	2.83 ± 1.02	0.19 ± 0.40	2.91 ± 3.32	1.26 ± 2.23	2.87 ± 2.40
Group 3 (60 J/cm ² , 830 nm)	22.76 ± 12.93	1.16 ± 0.77	0.36 ± 1.04	2.50 ± 2.90	3.17 ± 3.77	2.50 ± 2.81
Group 4 (Dersani [®])	18.75 ± 12.26	2.08 ± 1.97	0.29 ± 1.27	2.51 ± 1.83	2.61 ± 4.61	0.60 ± 1.61
Group 5 (0.9% saline)	21.75 ± 10.06	1.59 ± 0.86	0.51 ± 0.48	3.02 ± 1.65	0.14 ± 0.35	1.77 ± 2.97

Data are reported as means ± SD for 6 rats in each group. Group 1 = gallium-arsenide laser (GaAs); group 2 = gallium-aluminum-arsenide laser (GaAlAs); group 3 = GaAlAs laser; group 4 = Dersani[®] ointment; group 5 = control cleaned with saline daily. * $P < 0.05$, group 2 compared to all other groups, except group 4 (Kruskal-Wallis).

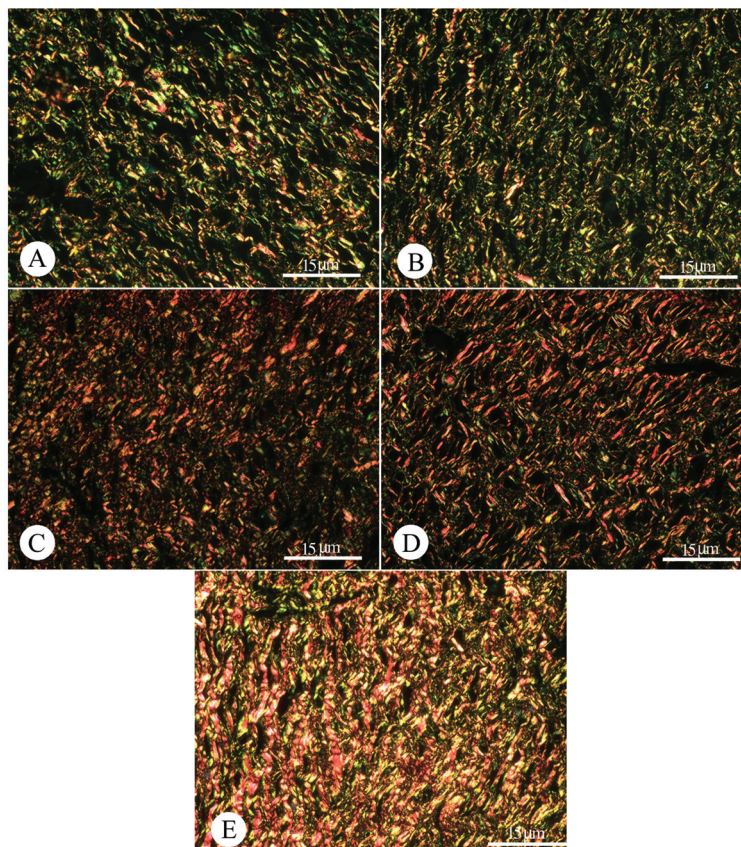


Figure 1. Histological sections of Wistar rat skin on the 20th day of the experiment stained with picrosirius and observed under a polarization microscope. A, Group 5 = control cleaned with 0.9% saline daily; B, group 4 treated with Dersani[®] ointment; C, group 1 treated with gallium-arsenide laser (GaAs, 4 J/cm²); D, group 3 treated with gallium-aluminum-arsenide laser (GaAlAs, 60 J/cm²); E, group 2 treated with GaAlAs laser, 30 J/cm². Groups 2 (L30) and 1 (L4) had a significant increase in collagen fibers compared with group 4 (D) and the control group ($P < 0.05$). Group 3 (L60) showed a significantly higher proportion of type I collagen fibers than group 4 (D; $P < 0.05$).

proportional increase in type I collagen fibers was observed in group 4 (D) compared with group 1 (L4) ($P < 0.05$). On the 8th and 12th days of treatment, no significant difference was found among the different groups. On the 16th day, group 1 (L4) and group 3 (L60) had a significant increase compared with the control group ($P < 0.05$). On the 20th day, groups 2 (L30) and 1 (L4) had a significant increase compared with group 4 (D) and the control group ($P < 0.05$). Group 3 (L60) showed a significantly higher proportion of type I collagen fibers than group 4 (D) ($P < 0.05$). Figure 1 shows the presence of type I and type III collagen fibers and their distribution on the 20th day of the experiment.

Discussion

In the present study, different wavelengths as well as different energy densities were used to determine the efficiency of the collagen synthesis treatments in promoting a faster and higher quality cicatrization. *In vitro* studies on fibroblast laser irradiation have reported a proliferative effect on protein synthesis depending on the characteristics and parameters of the type of laser used, such as wavelength, form of emission and energy density (12,13).

The present study shows that, based on the rate of wound closure, the best results were obtained for group 1 (L4) on the 8th day of the experiment. The closing of the wounds treated with GaAs laser, at a wavelength of 904 nm and energy density of 4 J/cm², was also faster compared with the other groups. According to Medrado et al. (14) and Pugliese et al. (15), the use of GaAIs laser at lower energy densities had a significant effect on the cicatrization of tissues, accelerating tissue repair through decreased edema, improved granulation tissue and fibroblast proliferation stimulation. In contrast to our results, they showed that 4 J/cm² laser is less effective for early wound closure during every experiment (15). According to Enwemeka et al. (9) and Woodruff et al. (16), the use of 632.8 and 780 nm laser therapy positively influences several tissue repair parameters, including reduced wound area and healing time, while the best energy density results range between 19 and 24 J/cm² and densities below 8.25 J/cm² and above 130 J/cm² do not produce good wound closure results.

Considering the constitution of the wound related to type III collagen fibers, group 2 (L30; $\lambda = 830$ nm and 30 J/cm²) was the most effective in producing these fibers. It is interesting to note that low densities would be important for early wound closure, but not to promote type III collagen synthesis. Similar results were obtained by Mendez et al. (17), who investigated the use of 830-nm GaAIs laser at

energy densities of 20 and 50 J/cm² for tissue repair in cutaneous wounds of rats. The most effective results were obtained for the group treated at a density of 50 J/cm².

Regarding type I collagen fibers, groups 2 (L30; $\lambda = 830$ nm, 30 J/cm²) and 1 (L4; $\lambda = 830$ nm, 4 J/cm²) presented a significant increase compared to control and to group 4 (D). This important point indicates that scars reached maturity early in the groups treated with these two types of laser and seems to indicate the efficiency of laser radiation in the replacement of type III collagen fibers with type I collagen fibers. One reason could be that in the 4 J/cm² laser-treated group, although a lower type III collagen fiber count was observed, replacement was faster and more efficient, providing this group with a significantly higher amount of type I fibers at the end of the experiment compared to the other groups. According to Reddy (11), type I collagen fibers are thicker, supplying the scar with greater traction strength and resistance.

The proliferation of collagen fibers promoted by laser radiation was also observed by Fung et al. (18) when analyzing the therapeutic effect of GaAIs 63.2 J/cm² laser on the repair structure of the medial collateral ligament of rats. These investigators found that the animals receiving laser radiation presented wider fibers than those in the control group, and that the distribution of those fibers was similar in the groups receiving irradiation for 3 to 6 weeks. In the groups irradiated with 63.2 J/cm² GaAIs laser, a larger number of collagen fibers was also found per area of transverse section. According to Ng et al. (19), multiple laser applications to the lesion may improve collagen morphology and healing.

In the present study, at the end of the experiment, the group treated with GaAIs 60 J/cm² laser did not differ significantly from the control group, but only differed from the Dersani® group (group 4), demonstrating that high energy densities do not present good results for type I collagen fibers. After analyzing different wavelengths and energy doses applied to fibroblast cells, it was found that the highest number of mitoses was reached with a dose of 15 J/cm² and that above 60 J/cm² there was a reduction in cell division (20,21). According to Meirelles et al. (2), marked maturation of granulation tissue is found on the 21st day of treatment using the energy density of 20 J/cm² at 660 and 780 nm.

We conclude that an energy density of 30 J/cm² was the most efficient in stimulating the production of type I and type III collagens, while the use of 4 J/cm² was more effective in the synthesis of type I collagen, and mainly in speeding up the rate of wound closing.

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