Kinetics of Tissue Repair In A Experimental Regeneration Model Under The Influence Of Laser Therapy

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Abstract: Over time, the animals lose organ regeneration capacity, which remains in invertebrates and vertebrates turbellarians such as fish, amphibians and some reptiles. However, understanding of the molecular and cellular mechanisms of regeneration on these groups is interesting because this knowledge can be applied in the future treatment of diseases in mammals, including humans. Laser therapy is used in the medical clinic for varying purposes, including repair of damaged tissues. Are scarce in the literature, studies have examined the action of the laser on the regeneration processes of animal organs which are characterized by the formation of blastema. This study evaluated the possible actions of the visible laser diode in the regeneration of tail gecko (Hemidactylus flaviviridis) considering the kinetics of the process. In Methodology two groups of 14 adult animals were submitted to tail induced amputation, one of them received laser treatment and the other does not. The treated and not treated groups were divided in two subgroups according to the day of sacrifice, being killed on the 11th and 21st day. It was evaluated parameters as the measure of the macroscopic length of the regenerated at different times after amputation of tail. With the obtained data was calculated: the average variation of the regenerated length in gecko, 11 and 22 days after the amputation of the tail, the reduction percentage of the regenerated, the moment that there were the different stages of regeneration in each group and subgroup and the percentage of subjects who achieved the growth phase of the blastema. It was also documented by photographs each stage of the regeneration process of the tail in the experimental groups. The laser therapy model used in this experiment had inhibitory action on the growth and differentiation of epimorphic regeneration of the tail gecko, Hemidactylus flaviviridis, similar to the action of other anti-inflammatory therapies.

Key words: regeneration, laser therapy, autotomy.

I- INTRODUCTION

Regeneration is the ability to reproduce organs or structures after traumatic loss or other causes [1]. Among the invertebrates, many are able to regenerate whole body, from fragments through reorganization of cell known as morphallaxis [2]. Since vertebrates, such as amphibians and gecko, perform epimorphic regeneration, which consists
in regeneration of new cells, either by the proliferation of preexisting stem cells or by dedifferentiation of adult cells in order to form the lost appendix of the animals was being lost as they became more complex despite being well accepted in the scientific world, is not fully clarified, requiring greater understanding of the basic mechanisms involved in this event [5].

The idea that the regenerative capacity of the animals was being lost as they became more complex despite being well accepted in the scientific world, is not fully clarified, requiring greater understanding of the basic mechanisms involved in this event [5].

Its main indication are all pathological situations where is required better quality and faster repair process (frames after surgery, soft tissue repair, bone and nervous), edema condition installed (where is searched a measurement inflammatory process), or in cases of chronic and acute pain. [6]

Studies that aims to understand the possible low-power laser action in tissue repair and even organs in animals that have the ability to promote tissue regeneration instead of healing are very scarce. After search for papers with deal with laser therapy and its effect on regeneration, it was possible to find only one work by Lopes et al. [7]. This paper used Girardia tigerina and verified the effect of low power laser (660 nm) as a regenerative process after amputation and treatment for twenty-one days. These flatworms have high regeneration capacity, which does not involve the formation of blastema. Then concluded that the low power laser improves the quality of regenerating planarians.

According to Brooks et al. [8], it is fundamental to understand the cellular and molecular mechanisms that promote regeneration in animals like planarians, amphibians and reptiles. Knowledge of the events that occur in cells that compose the blastema, if was understood in the light of cell and molecular biology and genetic engineering, could lead to the production of knowledge that would be applied in mammals, giving this class of animals the power of enlargement regeneration of its organs, limited so far by cicatrization.

Thus, it becomes important to develop work which using animals with large regenerative power, has as objective to clarify the kinetics of cellular events that occur after the amputation of a limb. Furthermore, the use of known therapies that are involved in stimulation of proliferation and tissue repair can make bigger its importance.

II- OBJECTIVES

1.1. General objective:

Evaluate the effects of laser therapy in different stages of tail regeneration in an experimental model, considering its kinetics.

2.2 Specific objectives:

- Describe from macroscopic parameters, the different phases of the regeneration process of the tail geckos (Hemidactylus flaviviridis);

- Compare the potential gross alterations on gecko’s tail in regeneration (Hemidactylus flaviviridis) according receive or not treatment with laser in each phase considered.

III- MATERIALS AND METHODS

4.1 Experimental groups:

It were collected 29 geckos of the species Hemidactylus flaviviridis, adult, weighing 5.0 ± 0.76 grams, with an average size of 12.3 ± 1.23 cm and of both sexes. They were then divided into two groups, one treated with laser and the other not. Each of these groups was organized in two subgroups according to the time it was removed material for histological analysis, which occurred on the 11th and on the 21st day of treatment. The animals were kept in boxes adapted for their maintenance during the months from May to July. Due to the lower temperatures in these weeks the boxes were warmed with yellow light bulb of 40 Watt, reaching an average temperature of 28 to 30 ° C. They were also freely and daily fed with water and larvae Tenebrio. In each group, half of animals were anesthetized by freezing on day 11 and half at day 21 for obtaining the material for histological analysis.

4.2 amputation of the tail and the visible diode laser treatment:
The autotomy of the tail was stimulated by the use of bilateral pressure thumbs 5 mm from the cloaca, where 5 to 8 vertebral plans remained rostral to the amputation site. For laser therapy was used a visible laser diode Dentolflex brand, quasar model, wavelength 632.8 nm, 90 J, lasting 1 minute and at a distance of 15 cm from the amputated region five days a week until the twenty-second day of the experiment.

4.3 Parameters for evaluation of the laser effect from macroscopic data:

Using a graph caliper was measured the length of the regenerated at different times after amputation of the tail. From those data, several parameters were calculated [8]. Thus, it were calculated the average length in the regenerated geckos, Hemidactylus flavivirides of 4 to 22 days after amputation of tail. It is also averaged the variation of the length of the regenerated stump in each group, considering the value obtained from subtracting the length of the regenerated stump on the 11th or the 22nd day of that observed in the first days after amputation, in each treatment group. It was also determined the percentage of reduction of regenerated considering the difference between the mean change in the regenerated stump treated group and the same measurement obtained for the control group after 11 and 22 days. Furthermore, the percentage of individuals of each group and subgroup reached the stage of growth and differentiation has been identified, considering the time at which this occurred.

I were also documented through photographs, growth stages and differentiation of the tail’s regeneration process in the experimental groups considering macroscopic aspects established for the different stages of tail regeneration in a kind of gecko, such as color, shape and other aspects of wound, as the regularity of surface and the relationship between length and width as established by Mclean & Vickaryous [9].

4.4 Statistical analysis:

Data were presented as mean ± standard deviation and differences were considered significant at P <0.05. It was carried out variance test and applied tests of medium Mann-Whitney rank sum test, after verifying that the samples didn’t show normal distribution.

4.5 Ethical considerations:

The animals were treated within the rules governing the ethical principles of animal research, fed freely and did not undergo any suffering or pain. At the end of the experiment all animals were returned to nature. The project was submitted to the Research Ethics Committee and approved with the protocol number 088/11 (annex).

IV- RESULTS

The experimental model used in this study suggests inhibitory effects on the repair process of Hemidactylus flaviviridis tail due to laser therapy, as observed in Figure 1.
Figure 1. Effect of laser therapy on the regenerative process in *Hemidactylus flaviviridis* tail at 11 and 22 days after the amputation of the tail. A- Animal Control on day 11 of the experiment: Observe that the regenerated is characterized by having a diameter greater than the thickness and light pink color; Animal B- control the 22nd day of the experiment: the regenerated stump appear as a tapered filament, wherein the ratio of length and breadth is greater than 1; Animals subjected to laseterapia C on day 11 of treatment: note that the region in regenerative process is characterized by having very irregular surface and reddish color and the presence of exudate; D Animal treated with laser after 22 days: the presence of a stump with smoother surface and light pink color.

**Source:** author

Laser inhibitory action on the regenerated stump growth is also perceived when assessing the length of this on the 11th and after 22 days of treatment, as recorded in Figure 2.

![Figure 2](image.png)

**Figure 2:** Tail regeneration length in geckos, *Hemidactylus flaviviridis* at different times after amputation and submitted (n = 7) or not (n = 6) to the laser treatment and the twenty-two days after the beginning of the experiment.

* P = 0.002 for tail regenerated on the 4th day length between the two groups;
○ P = <0.001 for the regenerated tail length in the comparison between the 4th and the 22nd day of the experiment in the control group;
** P = 0.039 for tail regenerated length on day 7 between the two groups;

Test: Mann-Whitney U Statistic;

**Source:** author

By observing the length of the tail’s regenerated in geckos that were subjected to laser treatment, it appears that the 4th to the 22nd day of the experiment, there was wide variation in their length, which increased from 6 ± 1.5 mm to 7, 5 ± 1.8 mm, whereas in untreated animals, this same period, there was a variation of ± 3.3 8.9 ± 0.55 mm to 2.4 mm, a length reaching in the end almost three times higher than the initial value (Figure 2). Still as recorded in Figure 2 shows that the 4th to the 7th day, the group of animals treated with laser exhibited regenerated stump significantly higher than that seen in untreated animals (laser Group - Day 4: 6 ± 1 25mm, day 7: 6.2 ± 1.5 mm; control group - day 4: 3.3 ± 0.55mm, day 7: 4.6 ± 0.75 mm).

It was analyzed also variation in the average length of the tail regenerated considering the time of 11 days and 22 days and the type of treatment received, namely, if it was or not subjected to laser therapy. The results obtained are reported in Figure 3 and suggested significant difference for this parameter at the end of the experiment by the treatments.
The reading of Figure 3 suggests that when comparing the length of the regenerated on both occasions, 11 days and 22 days and laser effects, you can once again realize the inhibitory effect of laser therapy. Thus on the 11th day, the geckos treated with laser (0.47 ± 0.66mm) had significantly lower regenerated stump than that observed in the group that received no treatment (3.03 ± 0.93mm). This same effect was observed on the 22nd day after phototherapy, with untreated animals (5.6 ± 2.1mm) presented significantly higher stump than those treated (1.5 ± 0.8 mm).

To understand if the inhibitory effect of the laser action is different in the two tail regeneration moments considered here, it was also evaluated the percentage of decrease of the residual limb compared with the control animals. Results were recorded in Figure 4.

Thus, it is observed that both in 11 and in 22 days of the experiment, laser therapy induces decreased regenerated when compared to the control values, however, this inhibitory action occurred at levels similar percentages in these two moments. Therefore, after 11 days the laser induced the development of a stump with the length of 83.7 ±
21.6% lower than that observed in control, while at 22 days the percentage reduction in the treated group was equivalent to 73.4 ± 7.4.

In the evaluation of effects of laser considering the three stages of regeneration after tail amputation, established by Susan et al. [10] was confirmed once again the laser inhibitory action and the results are shown in Table 1.

<table>
<thead>
<tr>
<th>Tratamento/fase da Regeneração</th>
<th>Fechamento da ferida</th>
<th>Inicio do crescimento do blastema (2-12mm)</th>
<th>Diferenciação (13-24mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grupo Controle (n=15)</td>
<td>1º dia</td>
<td>6º e 11º dia</td>
<td>21º dia</td>
</tr>
<tr>
<td>Grupo tratado com laser (n= 14)</td>
<td>1º dia</td>
<td>6º, 8º, 11º, 15º, 18º e 22º dia</td>
<td>Não foi observado</td>
</tr>
</tbody>
</table>

**FIGURE 5** - Regeneration phases after the amputation of the gecko’s tail, *Hemidactylus flaviviridis*, and time in days when it was observed that have been achieved and its relation to the type of treatment.

**Source:** author

Obeserving Table 1 it can be concluded that laser interferes on the phases of growth and differentiation. Among the controls the start of the blastema growth phase took place on the 6th and 11th day, whereas, among the treated some animals entered this phase later in the 11th, 15, 18 and 22 days. Regarding the differentiation stage, between the control animals, this was recorded in 21 days and among those treated with laser, no animals entered at this stage.

**Figure 6** - Percentage of individuals * who have reached the blastema growth phase according to time after amputation of the tail and type of treatment (n = 29).

* Data were considered on the 11th day of the experiment.

**Source:** author

Figure 6 is reported the percentage of individuals who entered the stages of growth and differentiation according time after amputation, thereby complementing the information of Figure 3.

Therefore, it is observed that the 6-8 days the majority (73%) of the control group Geckos reached the growth phase, while this same time, only 29% of treated animals reach this stage. In the period from 9 to 11 days, 27% of control animals start phase blastema growth, while only 7% of those treated also achieve growth. Among the treated animals it is also possible to register individuals initiating the growth over the period of 12 to 15 days (21%), 16 to 18 days (14%) and 19 more days (7%). It is also highlighted that 36% of geckos undergoing laser therapy did not reach the growth phase.
V- DISCUSSION

The results of this experiment indicate the possible inhibitory role of laser therapy both in growth and in tissue differentiation involved in epimorphic regeneration of Hemidactylus flavivirides tail.

The similarity between the characteristics observed in theregeneration process at this present study and its framework on criteria described by Susan et al. [10] and Mclean & Vickaryous [9] to describe the staging of epimorphic regeneration in lacertílios, established that the control animals whose regenerated stump was removed on day 11 after autotomy, had reached the regeneration phase characterized as growth, while those whose material was analyzed after 22 days showed anatomical features consistent with the differentiation stage. In this way, the methodology proposed here is valid as a tool for understanding the laser therapy's shares through temporal comparison of macroscopic events that occur in the tail regeneration in the different experimental groups of geckos used herein.

The therapeutic lasers or low power are useful for accelerating the soft tissue repair processes or hard, especially through their biomodulators effects on cells and tissues, activating or inhibiting physiological, biochemical and metabolic processes through their photophysical or photochemical effects [11] [12].

The effect of laser therapy, specifically low-power laser, in vitro and in vivo studies are presented also with conflicting results. However, many studies in animal models and humans have suggested its role as an important tool in the treatment of various diseases by his biostimulator role in wound healing and collagen synthesis in skeletal muscle regeneration after injury, promoting decreased inflammatory response and stimulating angiogenesis [13].

The important action of the laser on the healing process is attributed to increased cell proliferation through mechanisms not yet fully understood [14], but that seems to be based on the acceleration of the mitotic cycle, favoring neovascularization and formation of granulomatous tissue that are involved in [11] repair process.

The visible low power laser has biostimulator effect on mitochondria when absorbed by the various components of the respiratory chain such as cytochrome C and porphyrins, which promote the production of free radicals or reactive oxygen species [15] [16], which has been related to increased proliferation of keratinocytes [12].

After the photoreception laser light, there will be amplification and signal transduction which results in cell proliferation, differentiation or increase protein synthesis and growth factors that stimulate further proliferative process [16] [17]. There will be also change in the mitochondrial oxidation reduction activity, which may be stimulatory for cell proliferation when response bind to oxidation. It may be yet inhibiting to the growth process when to occur stimulus for reduction reactions in the mitochondrion [18].

There are few papers that have the action associated with the therapeutic laser regeneration processes in animals whose regenerative capacity is greater than that observed in mammals. Thus, it was possible to find only one work of Lopes, Old Fields and Munin [7] evaluated the effect of low power laser in planarian regeneration. They concluded that beneficially influences laser therapy in regenerative response in these invertebrates according the dosage used in the treatment and intensity.

The results presented here are contradictory to the findings Lopes, Old Fields and Munin [7], since the animals subjected to the laser treatment used herein, showed a decrease in growth and differentiation of the tail. This inconsistency may relate to differences in tissue regeneration mechanism between these groups of animals, because among planarians the tissue repair is promoted by cellular proliferation in a formed blastema, but also for regeneration morfolática type, which involves the remodeling of tissues to restore pre-existing symmetry, and the proportion [19]. Among geckos, regeneration is characterized as epimorphic involving only the formation of a blastema, which is a source of stem cells through various processes such as dedifferentiation, transdifferentiation and activation of resident stem cells.

The literature has pointed to the anti-inflammatory effect of low power laser in both animal [20] [21]
and humans models[22]. And this anti-inflammatory action may have been decisive for the inhibitory action on the tail regeneration of geckos here observed.

The inhibitory effect of an anti-inflammatory therapy in regenerative processes Hemidactylus flaviviridis tail was also observed by Sharma and Suresh [9], which aimed to understand the role of prostaglandina2 (PGE2) in the regulation of regeneration epimorphic in this reptile. Observed then that blockade of PGE2 by administering various cycloxygenase inhibitors promoted a reduction continues at all stages of regeneration.

Thus, it is believed that the laser treatment model used in this work may have contributed to the inhibitory effects of regeneration of the tail of geckos, from the 7th day and also to diminish the inflammatory response that accompanies the early stages of the repair process. The presence of an inflammatory infiltrate relates release and synthesis of growth factors, chemotactic substances, and fundamental cytokine for the modulation of cell proliferation and tissue repair process.

Moreover, the effects of laser in mitochondria of porphyrins could have stimulated release of reactive oxygen species, which stimulate the proliferation and differentiation of keratinocytes, producing differentiation and faster growth of a mature epithelium, already differentiated, rather than allowing maintaining for longer AEC. As already reviewed, the AEC is essential for activation of cells that form the blastema and the rapid differentiation of the undifferentiated epithelium could prevent or delay the regeneration process which delays to reach the second phase of regeneration, characterized by the formation and growth of blastema.

This same argument related to the rapid differentiation of the epithelium lining the site of the wound promoted by an anti-inflammatory agent was also used by Sharma and Suresh [9] to explain the inhibition of geckos tail regeneration in their studies and also probably related the presence of most regenerated stump between animals treated with laser on the fourth and seventh day of this experiment.

However, the place of appearance of the wound of animals treated with laser 22 days after autotomy observed here showing aspect of closure woundand blastema growth beginning, register that other sources of stem cells such as dermal and muscle may have contributed to the formation of this late blastema, as described by Susan et al. [10]. According to these authors, besides of AEC, there are other sources of stem cells forming blastema, so the dermis are responsible for 43% of them and musculature with the percentage of 17%.

Another factor that could have stimulated the lock in the regenerating tail growth process, from the seventh day of the experiment, could be the favoring of reducing reactions in the mitochondria of cells, which is defended by Mognato et al. [18]. However, to establish the exact mechanisms of the inhibitory action of the laser on the regeneration of geckos tail present the results of this work, the development is needed are other experiments to examine tissue sections, and the use of cellular and molecular markers.

VI- CONCLUSION
The laser therapy model used in this experiment had inhibitory action on the growth and differentiation of epimorphic regeneration of the tail of geckos, Nemidactylus flaviviridis, from day 7, similar to the action of other anti-inflammatory therapies.

REFERENCES


Mestrado. Faculdade Odontologia de Bauru, Universidade de São Paulo, São Paulo.


[69] Wills, A. A.; Holdway, J. E.; Major, R. J.; Poss, K.D.; Regulated addition of new myocardial and epicardial cells fosters homeostatic cardiac growth and maintenance in adult zebrafish. Development. 2008a;

