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STIMULATION OF HAIR REGROWTH USING LOW LEVEL LASER TREATMENT IN A RAT MODEL OF ALOPECIA

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Low-level laser therapy (LLLT) may induce *in vivo* biostimulation and photobiomodulation. The study aimed to evaluate the hair regrowth effect of different LLLT devices on a rat model of alopecia, applied alone or associated with Neoptide topical treatment as a hair growth stimulator. Laser treatments were performed 3 times a week. Group I and group II were treated with Laser I (HairMax Professional 12) for 1 minute 30 seconds, respectively 3 minutes. Laser II (Laser D68-1 Marp) was applied for 1 minute 40 seconds in group III. Group IV received topical Neoptide 0,3ml daily and group V got Laser I for 3 minutes plus Neoptide. The hair regrowth process was evaluated using clinical macroscopic aspect, grown hair weight and histopathological examination for skin thickness, follicle count and % anagen induction. Results revealed that treatment with Laser I had a time depending stimulatory effect on hair regrowth, the longer exposure having a better effect. Laser II had a negative effect by inducing hair loss. Combined treatment Laser I plus topic Neoptide application induced better hair regrowth than laser or Neoptide alone. Conclusion: In experimental rat alopecia the combination of LLLT with Neoptide had a better hair regrowth effect than each alone.

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Keywords: Low-level laser therapy; hair growth; alopecia; rat

1. Introduction

Light therapy is one of the oldest therapeutic methods used by humans. The use of lasers and light emitting diode (LED) was an important forward step in the technological development of light therapy. Lately a great number of new devices have been created specifically to improve and help the medical aesthetic field. Intense pulsed light (IPL) systems are high-intensity light sources that emit polychromatic, non-coherent light over a broad wavelength spectrum of 550 nm to 1200 nm. Cut-off filters limit the range of wave-lengths, making these devices extremely effective in treating vascular and pigmented lesions of the skin [**1**]. Low-level laser therapy (LLLT), known as cold laser or soft laser, induces biostimulation and photobiomodulation. The term „laser" refers to the fact that monochromatic and coherent light is used to treat injuries and to stimulate the healing of different lesions [**2**]. Non-coherent light emitting devices, such as the LED, are marketed under the name of SLD. In early times they emitted low-intensity red light, but modern versions are available across the visible ultraviolet and infrared wavelengths with very high brightness [**3**].

There are unknown mechanisms regarding how energy from therapeutic lasers and LED works at cellular and organism levels. For LLLT the therapeutic window ranges roughly from 600

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to less than 1400nm and it is close to the absorption spectrum of hemoglobin and water. Furthermore, respiratory chain components (mainly cytochrome c) have similar absorption spectrum [**4**]. The final enzyme in ATP production pathway in the mitochondria, cytochrome c oxidase, seems to accept energy from laser-level lights, making it a possible candidate for mediating the properties of laser therapy [**5**].

LLLT has been first tested in mice in 1967, when Dr. Endre Mester from Semmelweis University, Budapest, Hungary, thought that laser radiation might cause cancer in black and white mice [**6**]. He studied the effect of laser radiation on mice with shaved backs by exposing them to 694 mm ruby laser (1 cm^2 of shaven skin, 1 J of pulsed light, every week for up to 11 weeks). Instead of producing cancer, hair grew back quicker on the treated area than on the rest of the body. Furthermore, he found differences between the black and white mice: increased hair growth on the irradiated area was observed in all black animals after 5th-7th treatments, while in the white mice the hair growth effect was noticed only after the 8th irradiation. This was the first demonstration and assessment of „laser biostimulation" [**7**].

The effects of LLLT appeared to be limited to a specified set of laser wavelengths although more research is required to determine the ideal wavelengths, treatment time, dose and location [**2, 8**]. LLLT devices are widely marketed and used for hair re-growth, however, there have been only a few literature reports with observations on LLLT-induced hair growth effect in patients or evidence of improvement in alopecia [**7**]. Most experts agree that LLLT is safe for the treatment of hair loss (alopecia), but they claim that more studies are needed to confirm its therapeutic effect [**9**].

Multiple studies performed on cell culture, animal models and patients, mentioned the biphasic dose response and correlated it with the fluence (the total delivered light energy density) [**7**]. There is an optimal dose of light for any particular application, meaning that a lower dose will have a diminished therapeutic outcome and a high dose might have a harmful effect [6].

A study performed in 2011, analyzed the effects of the Hair Max Laser applied for 20 sec daily, three times per week for a total of 6 weeks, on C3H/HeJ mouse model of alopecia areata. Their conclusion was that the laser therapy induced a much longer growth phase because hair re-growth was first observed 2 weeks later and the follicles were either still in anagen or in catagen [**10**].

This study aimed to determine, on a rat model of alopecia, the hair growth effect of two different laser devices, Hair Max Professional 12 (HMP 12) and Laser D68-1, applied alone or associated with a topical treatment with Neoptide.

2. Material and method

2.1. Experimental design

The experiments were performed on 40 adult female Wistar-Bratislava albino rats (mean age 17 weeks), weighing 200-250 g, in early telogen hair cycle, bred in the Animal Facility of "Iuliu Hatieganu" University of Medicine and Pharmacy. The animals were randomly assigned to five groups (n=8), were housed under controlled conditions (12 h light/dark cycle, at an average temperature of 21-22 ^oC and humidity of 50-55%), and had free access to standard pellets as basal diet and water ad libitum.

The experiments were performed under general anesthesia with a combination of Ketamine (i.p. 50mg/kg b.w.) and Xylazine (20 mg/kg b. w.) [**11**]. On the first day, animals were shaved on the back on two rectangular areas of 2cm width /4 cm length each, situated on both sides of the spine. The right area was used for laser treatment, and the left area as negative control area (fig.1).

The HMP 12 (Lexington, International LLC) used as Laser I was tested at 655nm, < 5mW, continuous emission, three times per week (Monday, Wednesday, Friday) on two groups: group I was exposed for 1 minute 30 seconds, and group II for 3 minutes. The Laser D68-1(Marp Electronic) used as Laser II was tested on Wistar rats group III at 685nm, 5J, for 1 minute and 40 seconds, continuous emission, applied three times per week (Monday, Wednesday, Friday). Topic

Neoptide solution (0,3 ml) was applied daily, at group IV alone, and at group V associated with exposure to laser HMP 12, applied for 3 minutes, 3 times per week (Monday, Wednesday, Friday). Neoptide (Ducray, Pierre Fabre, Paris) is a hair growth promoter which contains a peptide complex that stimulates the regrowth process (Tetrapeptid + Neoruscine + Nicotinamid + GP4G). After the experiment animals were sacrificed by cervical dislocation.

The study protocol was approved by the Institutional Animal Ethical Committee (IAEC) of Iuliu Hatieganu University of Medicine and Pharmacy Cluj-Napoca. Experiments were performed in triplicate.

2.2. Hair growth evaluation

The assessment of hair growth was performed on day 0 and 28, by clinical macroscopic examination, by measuring the weight of the removed hair from a 1/1cm area, and by histological examination of a skin biopsy. The morphometric parameters recorded histopathologically were skin thickness, follicle count and % anagen induction.

Clinical macroscopic evaluation scoring system based on the comparison with the control area was as follows:

Type 1: Uneven hair growth on the test area
Type 2: Similar hair growth on both areas
Type 3: Moderately increased hair growth on the test area
Type 4: Markedly increased hair growth on the test area.

For the histopathological evaluation the skin biopsies were immediately fixed in 10% phosphate-buffered formalin (Chempur, Poland) for 24 h, embedded in paraffin wax (Histowax, Histo-Lab. Ltd, Gothenburg, Sweden), cut into 3–5-μm sections, and stained with hematoxylin and eosin (HE). Using conventional light microscopy (Olympus BX 51 microscope equipped with Olympus SP 350 digital camera and OLYMPUS Stream Start software), we measured skin thickness from epidermis to panniculus carnosus, and hair follicles were counted manually in all skin layers by an observer blinded to the experiment [**12**]. The percentage of anagen induction was calculated using the following formula: (follicle in subcutis)x100/(follicle count).

2.3. Statistical analysis

All results were expressed as the mean ± standard error (SE). For the clinical evaluation and hair weight statistical comparisons between two independent groups were performed using the Student's *t*-test. The morphometry data analysis was performed using Shapiro-Wilk normality test followed by the two-sample t-test. Values of $P<0.05$ were considered to be statistically significant. Analyses were performed using SPSS (Statistical Package for the Social Sciences) 16.0 for Windows (SPSS Inc, USA) and R' software [**13**]

3. Results

3.1. The clinical macroscopic evaluation

Comparing group I (Laser I, 1 minute 30 seconds) with group II (Laser I, 3 minutes), we found that with laser HMP 12 a longer exposure had a significantly better effect ($p<0.001$). By comparing the effects of Laser I (HMP 12) in group I with those of the Laser II (D68-1) in group III, we found that for the same exposure time the first was more efficient ($p<0,001$) (Fig.1 and 2).

The comparative assessment of the experimental groups proved that the lowest hair grow effect was noticed in group III ($p<0,001$). Group III was treated with Laser II (D68-1) for 1 minute 40 seconds. The rats from this group had type 1 (uneven hair grow on tested area) and type 2 pattern of hair grow (similar hair growth with the control area) (Fig.1 and 2).

Group IV treated only with Neoptide had a comparable effect with group II ($p>0,05$), and better that groups I and III ($p<0,001$) (Fig.1 and 2).

On the other hand, the best hair growth effect has been identified in group V (Laser I for 3 minutes combined with topic Neoptide). Almost all the rats from group V presented type 3 and

type 4 pattern of hair growth (increased and markedly increased hair growth on tested area). The group V effects were significantly better than those from group IV ($p<0.01$) treated only with Neoptide (Fig.1 and 2).

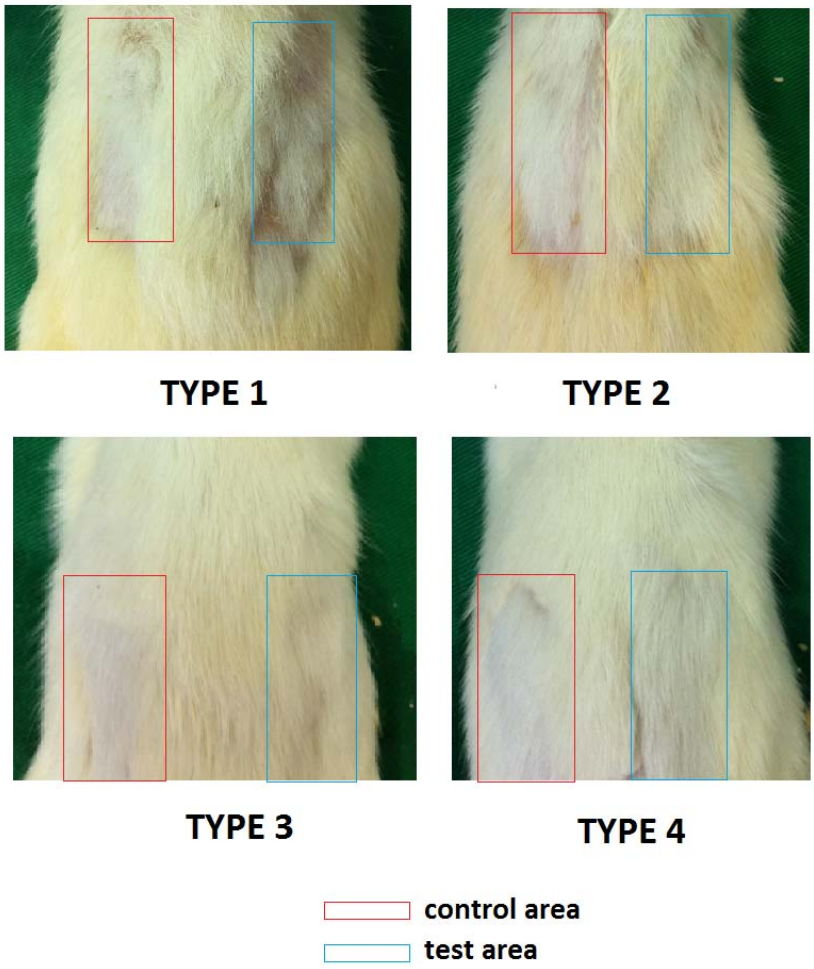

Fig 1. Classification of the hair growth effect: type 1; type 2; type 3; type 4.

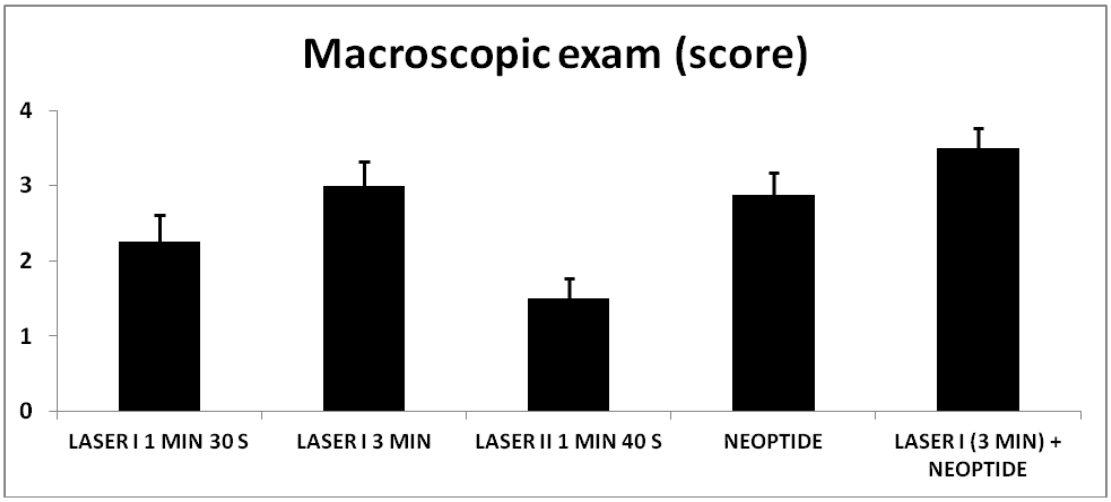

Fig. 2: Clinical macroscopic examination scoring expressed as mean±standard error.
LASER I = HMP 12*; LASER II = Laser D68-1;*

3.2. The hair weight evaluation

Compared to the negative control area from the same animal, hair weight increased significantly in group II (Laser I applied for 3 minute), group IV (with topic application of Neoptide) and group V (combined treatment with Neoptide + Laser I 3 minutes) ($p<0,05$). Comparing the three groups, it was found that the stimulating effect were almost similar ($p>0.05$) (Table 1).
Laser II treatment (group III) proved to have the lower regrowth effect as compared to all other experimental groups ($p<0,001$) (Table 1).

*Table 1. The hair weight removed upon one cm^2 from the treated area and the control area, expressed in mg/cm^2. Results are expressed as mean ± SE of the mean. * = $p<0,05$.*

Treatment	Hair weight (mg/cm^2)	*P value*
Laser I (1'30'')	41.4 ± 1.9	*0.187*
Control	37.5 ± 2.2	
Laser I (3')	46.0 ± 1.7	*0.031 **
Control	39.8 ± 1.9	
Laser II (1'40'')	35.8 ± 1.6	*0.829*
Control	36.2 ± 1.2	
Neoptide	48.8 ± 1.7	*0.012 **
Control	41.6 ± 1.9	
Neoptide+Laser I (3')	45.2 ± 1.93	*0.026 **
Control	8.3 ± 2	

Stimulation with HMP 12 laser for 3 minutes (group II) has a significantly better effect when compared to stimulation for 1 minute (group I) ($p<0.001$) (Table 1).

3.3. The histopathological examination

In our experiment, we used increase in thickness and presence of follicles in the subcutis layer as evidence for transition of follicles from telogen to anagen phase of hair growth.

The transition of telogen phase to anagen phase of hair growth in the treated area in group I was of 27.1%, in group II - 22.6%, in group III - 23.3%, in group IV - 73.1% and in group V - 50.6% (Table 2). In each case, these results were compared with the percentage of anagen induction from the untreated side of the animal in each group that served as control. After 4 weeks of the HMP 12 laser treatment in Groups I and II, an increase in number of hair follicles ($p>0.05$ as compared to control area) was observed in the subcutaneous layer, the majority of which were in the anagen phase, though some had entered the catagen phase (Table 2, Fig. 3). In the rats treated with Laser II (group III) the follicle count in subcutis was very low, suggesting that the laser did not induce hair growth. The anagen induction was decreased in the testing area so we assume that the Laser had hair depilation effect (Table 2, Fig. 3). The anagen induction was also

significantly increased ($p < 0.05$ as compared to control area) in group IV (with topic application of Neoptide) and in group V (combined treatment with Neoptide + Laser) (Table 2, Fig. 3).

In the representative histopathological sections (Fig. 3) the anagen phase of hair growth, which is associated with increase in follicle size, was located in the deep subcutis when compared to telogen phase skin where follicle lies in the dermis only.

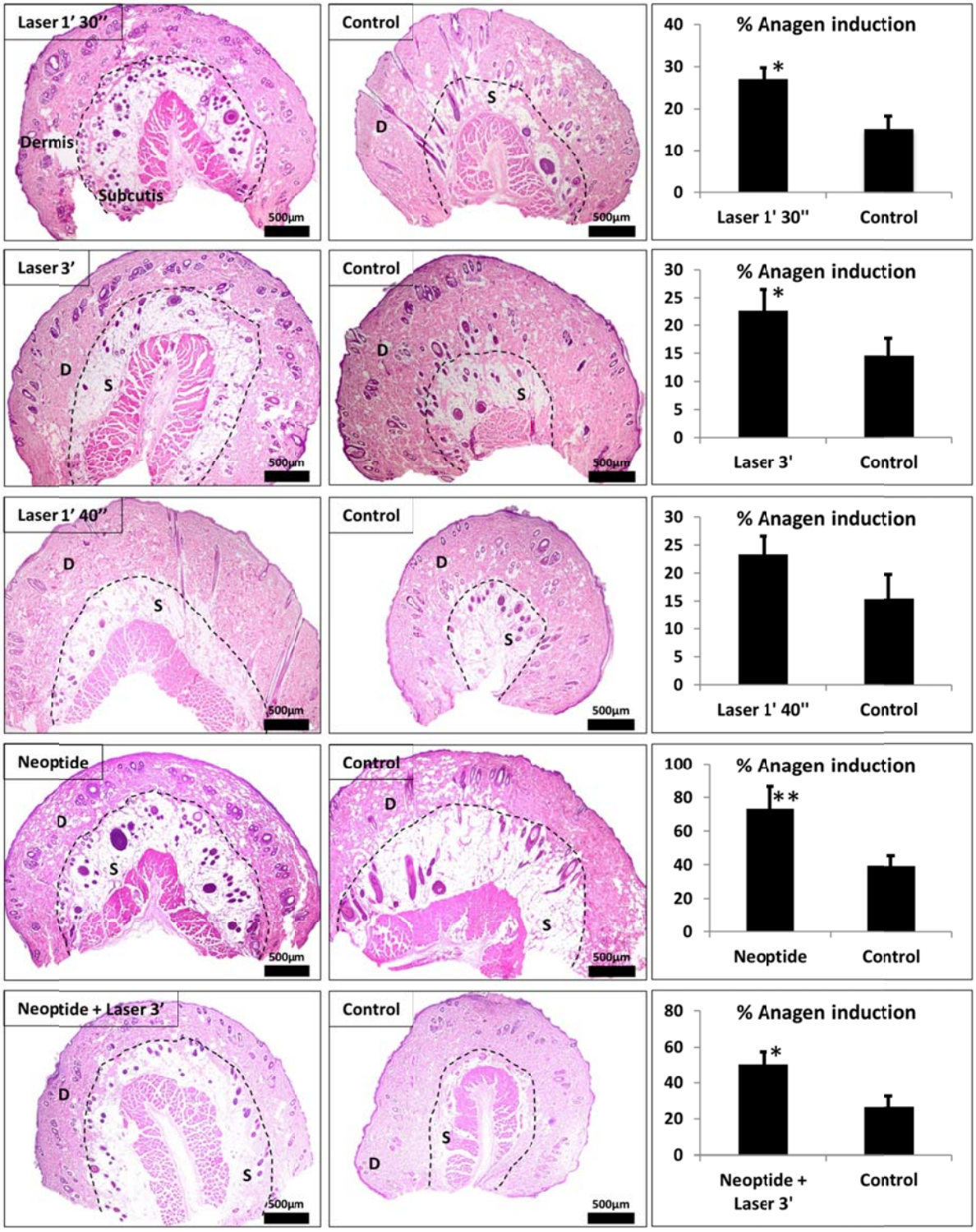

*Fig. 3: The effect of the different types of treatment (left column) on the hair growth activity in Wistar rats, compared with the negative control area. The dotted lines indicate the junction between dermis (D) and subcutis (S). The columns on the right illustrate the percentage of anagen phase induction for each type of treatment. (*p<0.05; **p<0.01)*

Regarding the skin thickness, the results showed that there were no significant variations among the control area and the treated area in none of the five experimental groups (table 2).

Table 2. Histopathologic evaluation: skin thickness (µm), number of follicles, follicle count in the subcutis, the percentage of anagen induction. P-value compared to the negative control area. Results are expressed as mean ± SE of the mean.

Treatment	Skin thickness (µm)	Follicle count	Follicle count in subcutis	Anagen induction (%)	*P value*
Laser I (1'30'')	1426.8 ± 64.3	97.1 ± 24.3	26.3 ± 9.8	27.1 ± 2.7	*0.01**
Control	1354.0 ± 74.8	74.8 ± 20.2	11.4 ± 5.7	15.2 ± 3.1	
Laser I (3')	1344.5 ± 68.5	79.0 ± 11	17.8 ± 6.5	22.6 ± 3.9	*0.033**
Control	1632.3 ± 98	44.8 ± 12.6	6.5 ± 5.1	14.6 ± 3.2	
Laser II (1'40'')	1506.8 ± 62.5	41.4 ± 4.5	9.62 ± 1.6	23.3 ± 3.2	*0.33*
Control	1532.3 ± 86.9	55.3 ± 11.7	8.51 ± 5.9	15.4 ± 4.2	
Neoptide	1185.7 ± 48.7	64.3 ± 14.6	47.0 ± 22.5	73.1 ± 13.3	*0.005 **
Control	1477.3 ± 59	32.4 ± 5.3	12.8 ± 3.4	39.5 ± 6	
Neoptide+Laser I (3')	1328.6 ± 95.9	56.3 ± 14.8	28.5 ± 11.3	50.6 ± 6.8	*0.018 **
Control	1534.9 ± 49.7	43.0 ± 17.1	11.4 ± 9.6	26.5 ± 6.1	

4. Discussions

Our study explored the efficacy of different laser devices and different time exposures upon hair growth induction in animal model. We analyzed and compared two laser devices, HMP 12 and D68-1 for possible hair growth effects.

The usually used LLLT wavelengths are between a range of 600 – 1000nm, with a power of 5-500 mW [**14**]. Evans and Abrahamse studied the effect of different light wavelengths, and found the most stimulatory effect on wounded fibroblasts using 5J/cm2 of 632,8 nm light. They also found the dose of 16J/cm2 to cause DNA damage and reversible cell damage [**15**].

Hair Max (Lexington, International LLC) is safe regarding the adverse events and is FDA Class 2, cleared for marketing since 2001. It works at 655nm, < 5mW, continuous emission. For human male or female patients, a treatment session lasts 8 minutes and the indication is for 3 treatments per week [**16**]. The HMP 12 laser is indicated for the treatment of androgenetic alopecia in males (Norwood Hamilton Classification IIa to V) and females who have Ludwig (Savin) I-1, II- 1, II-2 or frontal pattern of hair loss [**17,18**].

In our study the HMP 12 laser was efficient because it induced hair growth effect on the treated area, both in group I (exposure to laser lasted 1 minute 30 seconds, 3 times/week) and group II (exposure lasted 3 minutes, 3 times/week). This device works via the principle of PhotoBio Stimulation, a process through which laser energy is delivered to the tissue. Increase in ATP synthesis, proton electrochemical potential, and oxygen uptake have all been shown in rat liver mitochondria. LLLT has been shown to increase procollagen synthesis in fibroblasts [**19,20**]. It is an accepted theory that low level laser energy is able to stimulate the production of ATP by releasing bound nitric oxide in the cellular respiratory chain, which increases metabolic energy, promotes microcirculation, contributes to regulation of cellular apoptosis, and stimulates cell proliferation. Clinical evidence demonstrates reverse miniaturization, increase in number of hairs per follicular unit, and induction and prolongation of the anagen phase [**5,21**].

Laser D68-1 (Marp Electronic) is an IPL device, 50mW, at 685nm designed to perform laser therapy. Choice of a suitable laser probe allows application of laser radiation at different wavelengths with pulsed or continuous emission. The unit is capable of connecting two different probes simultaneously, the unit's memory consists of 46 programs for 685nm and 808 nm radiation and it is possible to store commonly used therapy parameters in 40 memory cells [**22**].

Wikramanayake et al (2012) showed that HMP 12 laser seems to be an effective and convenient device for the treatment of alopecia areata in the C3H/HeJ mouse model. They noticed that the sham-treated skin demonstrated reduced skin thickness and reduced number of hair follicles [**10**].

For Laser D68-1, applied at similar parameters: 685nm and 50mW, with 1 minute 40 seconds time of exposure, 3 times/week, the tested area did not show more hair than in the control area after the 4 weeks of the study. Furthermore, it was not a hair growth promoter as it was responsible for inducing a depilatory effect.

The study also evaluated the hypothesis that a combined treatment, laser plus topic application of a hair growth inducer would have a better effect than each therapy alone. There is evidence in the scientific literature of the use of LLLT in combination with topic applications or oral medication [**23**]. The two medications approved by the United States FDA for hair loss pattern are Minoxidil 2% or 5% for males or females and Finasterid for males only [**24,25**]. The HMP 12 laser (Lexington International LLC) producers claim that significant improvements have been observed when it was used in combination with oral Finasteride.

In 2008, Dr. Maria Muricy conducted a study in Brazil to evaluate hair growth with HairMax alone and in combination with topic Minoxidil [**16**]. Her results demonstrated a reversal of follicular apoptosis using BCL2 markers.

As the effect of LLLT combined with Minoxidil has already been studied, we chose another substance used as hair grow promoter: Neoptide. The key ingredient of Neoptide is "acetyl tetrapeptide-2". It is marketed as a "youth hormone". It is responsible for collagen synthesis and commonly used as skin conditioner. In literature, it is reported to compensate for the loss of thymopoietin, a protein involved in the thymus gland, which plays a role in the immune system. Neoptide contains "Ruscus Aculeatus" (Butcher's Broom) with anti-inflammatory effects and "Niacinamide", which is used to prevent wrinkles and UV damage. "Artemia extract" boosts the effects of other anti-aging ingredients, protects DNA against UV and free radicals [**26**].

For human use, Neoptide dosage is 12 sprays (1 ml), once a day and massaged until fully absorbed. Only a study has been performed on 28 women for 6 months and the results showed good tolerance and +75% hair growth [**26**]. The lotion works directly on the hair follicle to stimulate the anagen phase (hair growth phase) as the amino acids concentration promotes the proliferation of the hair growth phase. Moreover, through cutaneous microcirculation, the entire hair shaft is stimulated; this results in an increased hair length. Neoptide restarts and stimulates growth. All of the hair is thus stimulated, hair loss slows down and hair mass is boosted [**27**].

The hair follicle cycle is influenced by numerous growth factors, cytokines, hormones, neuropeptides and pharmaceutical products, which can induce the cyclic transformations from phases of rapid growth (anagen), via apoptosis-driven regression (catagen) to relative quiescence (telogen) [**28**]. The decision of including Neoptide into the study groups was taken by acknowledging that the hair cycle is influenced also by peptides.

The main three phases of the hair follicle cycle have been described and investigated in most pigmented mouse strains, albino mice and in some mouse mutants [**29**]. As previously reported in the literature, spontaneous hair cycles or those provoked by plucking have been studied in Wistar SM male albino rats and each cycle (G) lasted approximately 32 days: anagen about 18 days, catagen 3 to 4 days, telogen about 10 days [**30**].

The rats used in our experiment were 119-122 days old. Being approximately 17 weeks old, the rats were in early telogen phase [**30**]. The experiment lasted 4 weeks and 3 days that were necessary for the assessment of results. In this period and under these circumstances, the rats passed through an almost complete hair cycle. The hair growing effect has been confirmed for both groups, one treated only with Neoptide and the other one with Neoptide and HMP 12 laser therapy, but the best results were obtained after the combined treatment plan.

In Dr. Mester's experiment design, before each successive treatment the skin was again depilated by shaving. This can induce mechanic stimulation of hair growth, as previously reported in the literature. An interesting study from 2008, observed the system of linear loops formed by the regrowing hairs on rat skin. The adult rats' hairs were dyed and then shaved and then observed;

regrowth indicated that the linear hair regrowth was closely correlated with the shaving process [**31**]. In order to avoid this effect, we did not shave the tested area of the rats before each daily therapy.

We have noticed during our experiment uneven hair growth in some animals, on the tested area: some animals did not experience hair growth and some had diffuse hair regrowth. We checked the literature and found out that a similar situation has been previously reported [**6**]. No further hair growth was observed on half of the control animals (both among black and white mice). At the same time, a diffuse hair growth appeared on some animals, while in other animals an uncharacteristic, sometimes diagonal strip appeared [**6**]. Furthermore, in our experiment on the tested area the hair appeared to make some specific linear loops that we observed macroscopically. Li-Yaun Liu et al (2008) detailed linear hair regrowth patterns observed in rats. The authors stated that hair-lines coincided with the previously observed sympathetic-substance lines (SSLs) in the rat's skin. Some waves of regrowth were cranio-caudally-oriented lines, 2-15 mm wide, symmetrically from both sides of the body, running from the head through the torso to the limbs. The four main patterns of the hair-loop-lines were: the Dorsal Loop and the Lateral Dorsal Loop (running along the dorsum and hind limb), the Ventral Loop and Lateral Ventral Loop travel along the thorax, abdomen, and forelimb. The histological observation indicated that rat hair follicles along the hair-lines were in anagen phase [**31**].

To further investigate the hair promoting effect, we used the histopathological examination of skin thickness, number of follicles, number of follicles to be found in the subcutis, the percentage of catagen induction. As previously reported by Liu et al. (2008) in Wistar rats, following shaving new hair-lines appear and the hair follicles in the new hair-lines are always in anagen phase. Similar to our findings, the same authors found that the bulb of the hair follicles was enlarged and deeply inserted into the subcutis. However, the study also revealed that the hair follicles in the shaved bare areas were short, small, and in the phases of telogen, proanagen, or catagen (Liu et al., 2008) [**31**].

Previous microscopical studies on pigmented C57/BL6 mice [**32**] revealed that there is an association of increasing skin thickness, follicle count and the macroscopic development of skin pigmentation with anagen induction. In our study on Wistar rats, we showed that although in most treated groups there was significant anagen induction, this was not paralleled by the increase in skin thickness.

Our results show that LLLT treatment with HMP 12 laser for 1 minute and 30 seconds but especially for 3 minutes as well as the 3 minutes stimulation combined with daily Neoptide, affects the normal hair cycle by inducing the resting follicles to enter into the anagen phase of hair growth. The follicle count and skin thickness data were complemented by results of hair weight measurement which showed that the 3 minutes laser treatment both with and without Neoptide had stimulating effects on the hair growth.

5. Conclusions

The results of our study revealed that the treatment with low level laser therapy (LLLT) performed with HMP 12 laser had a stimulatory effect on hair growth. This effect was time dependent, the longer exposure to the laser diodes (3 minutes) induced a significantly better hair growth on the treated area than the shorter one (1minute and 30 seconds). The Laser D68-1 applied for 1 minute and 40 seconds induced a depilatory effect. The study pointed out that combined treatment with HMP 12 laser for 3 minutes and daily topic Neoptide application induced better results than any of the two used separately.

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