Laser Phys. Lett. **00** (2015) 000000 (7pp) UNCORRECTED PROOF

Letter

Effects of He–Ne laser on *Daphnia magna* Straus manifested in subsequent generations

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Received 1 July 2015, revised 8 August 2015
Accepted for publication 10 August 2015
Published

CrossMark

Abstract
The effects of He–Ne laser irradiation (including visible laser light 632.8 nm in wavelength and an attendant electromagnetic field from the laser apparatus) on the fertility, newborn quality and linear dimensions of the body of Cladocera *Daphnia magna* Straus were determined. It is shown that the attendant electromagnetic field generated by the laser apparatus can have significant impact on the results of low-intensity laser therapy. The laser light exposure of daphnia to a dose range of 1.7–1300 mJ cm^{-2} stimulates the integral functions of the daphnia's body. At the same time, the attendant electromagnetic field (72 mA m^{-1} in the frequency range of 0.02–20 kHz and 400 mA m^{-1} in the frequency range of 3–4 MHz) causes the appearance of quickly dying and/or abnormal newborn (up to a small percent) that has never been observed in the control. Anomalies of daphnia mainly consisted of swimming antenna pathologies. Moreover, under the influence of the attendant electromagnetic field on parental daphnia only, these anomalies appear even in several subsequent generations of newborn. The negative impact on daphnia disappears completely after the suppression of the attendant electromagnetic field to laboratory background level. It is shown that laser light can partially compensate the negative impact of the attendant electromagnetic field on Daphnia.

Keywords: laser therapy, daphnia, side effects

AQ3 (Some figures may appear in colour only in the online journal)

1. Introduction

Laser therapy (phototherapy), which involves exposing biological objects or human organisms to monochromatic (laser) or nonmonochromatic (light-emitting diodes, LEDs) visible or near-infrared light up to 100 mW in power, has been actively developed over the past few decades and is nowadays widely used in medical practice in many areas of modern medicine [1–8] and veterinary medicine [9–11]. There are a number of positive effects of low-level laser therapy, such as anti-inflammatory and analgesic effects, increased general and local immunity, reduced blood viscosity, increased blood supply and many others [3–8, 12]. To reveal the mechanism of action, develop treatment methods, and find out safe treatment regimens, numerous investigations have been conducted at the level of both cells [13–18] and various organisms [9, 11, 19–21]. In particular, it was demonstrated that laser therapy has a positive, stimulating effect on both prokaryotic and eukaryotic

JNL:LPL PIPS: AA01D0 TYPE: LET TS: NEWGEN DATE:26/8/15 EDITOR: JP SPELLING: US

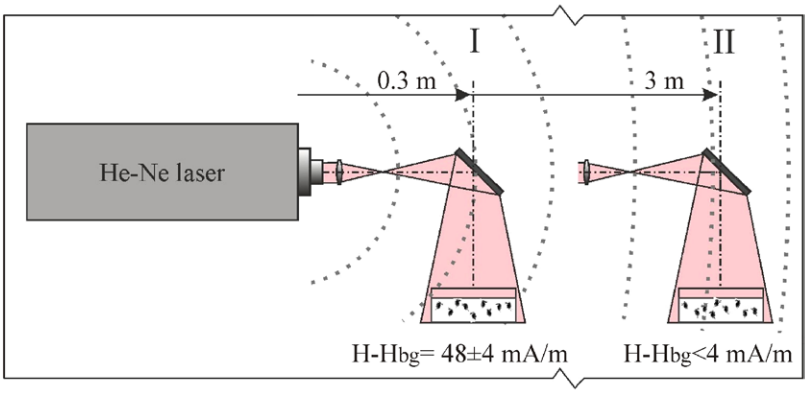

Figure 1. Schemes of laser irradiation of daphnia in a petri dish in the proximal (I) and distal (II) positions. (*H*- attendant EMF from laser apparatus; H_{bg}—laboratory background level of EMF). Each item's integral excess levels of EMF are shown in the range of 0.02–20 kHz *H* above the laboratory background value H_{bg} (field lines are conditionally shown by dashed lines). AQ4

organisms [22, 23]. As for the mechanism of low-intensity laser therapy, it is still debatable (see, for example [13–15, 24, 25]). Some authors suggest that the activation of cell metabolism is caused by the increase of mitochondrial redox potential, mitochondrial functional activity and ATP synthesis [26]. Others believe that the common link that explains the numerous biological effects of laser therapy is biological fluids, as a structural matrix, in which the most important metabolic processes take place [11].

Plenty of studies on the effects of low-intensity laser on biological objects have been conducted using helium–neon (He–Ne) laser 632.8 nm in wavelength. Exposure to He–Ne laser radiation was known to lead to an increase in the survival rate of the embryos of the loach *Misgurnus fossilis* and in the number of hatched larvae, and also to a more rapid growth of the juvenile fish [4]; to have a positive effect on the viability of the spermatozoa of mollusks and echinoderms by prolonging their activity by a factor of 1.5 on average [11, 27]; to improve the condition of the offspring of the crustaceans *Daphnia magna* [28]. Irradiation of fertilized spawn led to a substantial increase in the vitality of fish and their size and weight parameters and improved their temperature tolerance and resistance to poisoning [11, 19].

But against the background of the generally recognized positive therapeutic effect of low-intensity laser therapy there appear in the literature, reports on the absence of any positive effect and even the development of side effects [29–31]. We drew attention to the fact that laser is an electrical apparatus, which may be not only a source of laser light, but also of other attendant electromagnetic fields (EMF) which can potentially make an impact on the overall effect of laser therapy. For example, a typical He–Ne laser comprises a high-voltage pulse power supply and discharge tube; both of these elements are potential sources of laser EMF effects which on biological objects can't be ignored. Indirect evidence for the existence of such effects has been recently obtained [29–31]. Therefore, a comprehensive study of the effects of all types of electromagnetic radiation from the laser apparatus used on living organisms and also the identification of the possible long-term effects of such exposure, particularly as potentially manifested in subsequent generations, become very topical.

Table 1. Laser radiation doses used in the experiments, mJ cm^{-2}.

Intensity, mW cm^{-2} Exposure time, s	13	2 (with filter)	1	0.17 (with filter)
10	130	20	10	1.7
100	1300	200	100	17

For these purposes, it is very convenient to use freshwater crustaceans that are widely used as test-organisms with short life cycles [32].

In the present study the effects of He–Ne laser on the fertility, the quality of the offspring and the linear dimensions of the body of *Daphnia magna* Straus (daphnia) are revealed. We show directly that a distinct addition to the stimulating action of laser light at a wavelength of 632.8 nm on daphnia causes certain fetal abnormalities in juveniles born to them. It was found that these violations also occur in subsequent generations, and their cause is the effect on daphnia of the attendant electromagnetic field generated by the laser apparatus.

2. Materials and methods

Used as a test object in this work was a laboratory culture of freshwater crustaceans *Daphnia magna* Straus (Crustacea, Cladocera) that find wide application in bioassay and aquatic toxicology for the evaluation of anthropogenic impacts. The crustaceans exist in culture in the form of parthenogenetic females; they multiply the whole year round and have a short lifespan. The onset of sexual maturity occurs at 7–9 d of age, and the first offspring appears at 9–11 d. These traits of the animals make them convenient subjects for studying the remote effects of various factors in a series of generations, because several successive generations can be obtained within a relatively short time.

The crustaceans were cultivated in accordance with the standard instructions in a Model B3 climatic chamber (Energolab, Russia) at a constant temperature and under controlled day/night illumination conditions.

Irradiation was carried out with a He–Ne laser (wavelength 632.8 nm, output power 20 mW) Model GNL 111 (Polyaron,

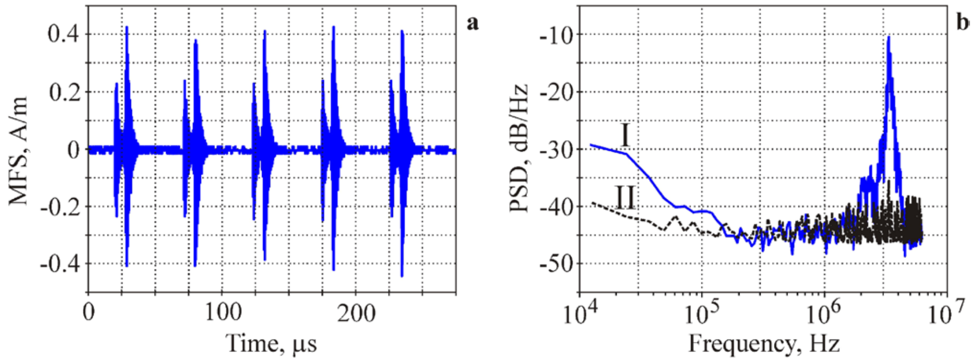

Figure 2. (a) Magnetic field strength (MFS) of attendant EMF signal, generated by GNL 111 laser apparatus in the proximal position (I). (b) Power spectral density (PSD) of attendant EMF signal in the proximal (I) and distal (II) positions.

USSR) that during 3 decades was widely used in experimental biology and clinical practice (see [5, 11, 31]). The irradiation of ~one-day-old daphnia in a petri dish was made in two positions: the proximal, at a distance of 0.3 m from the laser device (position I), and the distal, at a distance of 2 m from the laser device (figure 1) (position II).

The laser light intensity measured at the location of the petri dishes with the crustaceans was 1 or 13 mW cm^{-2}, respectively. Additionally to lower the radiation intensity, a CC2 blue light filter was used. The exposure time was 10 and 100 s for each of the radiation intensity values. Thus, the irradiation doses studied ranged between 1.7 and 1300 mJ cm^{-2} (see table 1).The radiation intensity was measured with a power meter (FieldMaster, Coherent) equipped with a measuring head (LM10HTD, Coherent).

As already noted, some light-emitting devices have generated significant levels of EMF, which may have an impact on the irradiated organism. The value of EMF generated by our GNL 111 laser apparatus was detected over a wide spectral range up to 25 MHz, with a measuring coil and digital oscilloscope (AFG 3021, Tektronix). Figure 2 shows that the GNL 111 laser apparatus generates high-frequency electromagnetic pulses, with energy concentrated mostly in two ranges: in low frequency (30 kHz) and high frequency (3 MHz).

The integral values of the attendant (side) magnetic field of the GNL 111 laser apparatus in the low-frequency range (0.02–20 kHz) were measured using a G79 Microteslameter. In the proximal (I) position it was 72 ± 4 mA m^{-1}, while in the distal (II) position it was 24 ± 4 mA m^{-1}, which was comparable with the background laboratory level of the magnetic field H_{bg}.

To evaluate the possible effects of this attendant EMF on daphnia, an experiment was conducted in which daphnia were randomly divided into three equal groups of twenty animals each. The daphnia of the first group were exposed to He–Ne laser light for only 3 h with a dose of 14.4 J cm^{-2}. In this case the daphnia were placed at position II (figure 1), where the effect of the attendant EMF was reduced to the laboratory background value. The second daphnia group was exposed to the attendant EMF generated by the laser apparatus for 3 h by placing them directly near the operating device (position I, figure 2), but not exposing them to the laser (the laser beam was blocked by a metal screen). The third, control daphnia group was not subjected to the action of either laser light or attendant EMF from the laser apparatus. The experiment evaluated the effects of such exposure on the fertility and quality of offspring daphnia.

One-day-old crustaceans were irradiated once in petri dishes containing water 0.5 cm in height. The irradiated daphnia were transferred to test glasses, five animals per 250 ml of water each, in four-fold replication. The control, unexposed crustaceans were selected from the same brood as the test specimens. The water in the test glasses was replaced and the daphnia fed with the chlorococcous algae *Chlorella vulgaris* every other day.

To reveal the remote effects of irradiation, three consecutive generations (F_1–F_3) of the irradiated and control daphnia were observed. To obtain generation F_1 from the first brood of the irradiated and control daphnia, 20 individuals were randomly selected from each group and transferred to separate test glasses, 5 daphnia per 250 ml of water each, in four-fold replication. To obtain generations F_2 and F_3, 20 daphnia were selected in the same way from the first brood of generations F_1 and F_2, respectively. Generations F_1 through F_3 were left intact (unexposed). All the water replacement and feeding manipulations were performed in the same way as for the parent generation P. Each generation was observed for 21 d. The survival rate, fertility, and quality of the offspring were registered regularly. The linear body size, from the head to the base of the apical spine, was measured at 21 d. The results obtained were checked for the statistical significance of the difference between the experimental and control data by Student's *t*-test (significance level 0.05). The correlations between the irradiation dose and the variation of the parameters under study were checked by means of Spearman's rank correlation.

3. Results and discussion

Irradiation in position I (figure 1) had no effect on the survival rate and the onset time of sexual maturity. No crustacean deaths were observed in either the control or the test samples; the onset of sexual maturity occurred at 7–9 d.

The irradiation of crustaceans in position I resulted in some cases in a significant change in linear size (measured for each generation for 21 h of daphnia life), compared with that of

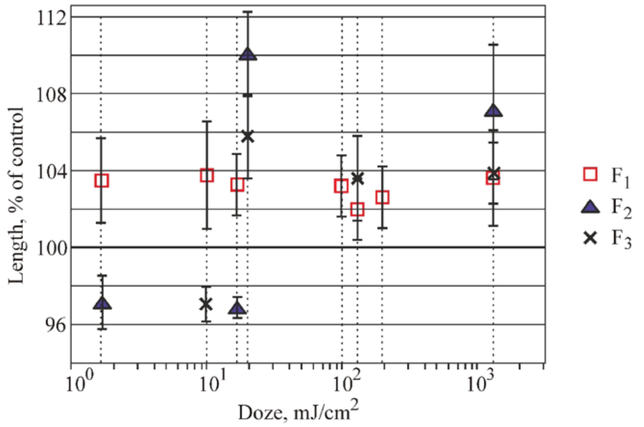

Figure 3. Linear body size of daphnia (in percent of the control) in the series of generations from P through F_3 following the exposure of the parent individuals P to He–Ne laser radiation. The vertical dotted lines indicate the irradiation doses used. Shown are only the results that differed statistically significantly from those of the controls ($p < 0.05$).

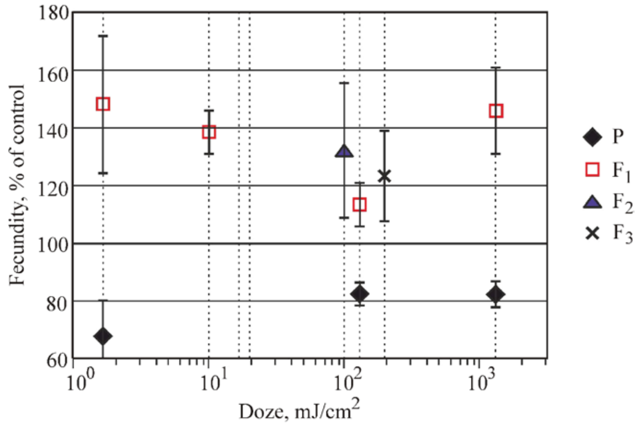

Figure 4. Fertility of daphnia (in percent of the control) in the series of generations from P through F_3 following exposure of the parent population P to He–Ne laser radiation. The vertical dotted lines indicate the irradiation doses used. Shown are only the results that differed statistically significantly from those of the controls ($p < 0.05$).

the control (figure 3). In the irradiated parental generation P, a tendency was observed towards a slight decrease in body size. No statistically significant differences from the controls were found, no matter what laser irradiation dose was used. For this reason, in figure 3 data for the parental generation are not listed. In the first filial generation F_1, a statistically significant increase of body size by 2–4% was observed at all the laser irradiation doses used, except at 20 mJ cm^{-2} (irradiation at a dose of 20 mJ cm^{-2} showed an insignificant 2-percent growth stimulation). Fixed in the second generation F_2 was a significant reduction of body size at some low doses (1.7 and 17 mJ cm^{-2}) and significant growth stimulation at individual high doses (20 and 1300 mJ cm^{-2}). In the third generation F_3, a significant decrease, by more than 3%, was observed in the linear body size of the daphnia at a relatively low dose of 10 m mJ cm^{-2}, while at doses of 130 and 1300 mJ cm^{-2} the linear body size of the crustaceans was observed to increase by almost 4%.

Figure 3 is noteworthy in that for the F_2 generation similar doses, 17 and 20 mJ cm^{-2}, cause opposite effects: with 17 mJ cm^{-2}, a significant decrease in linear dimensions is observed, and at 20 mJ cm^{-2}, a significant increase takes place. We believe that such behavior can be associated with the different laser intensities, which differ by more than an order of magnitude (table 1) (0.17 and 2 mW cm^{-2}, respectively).

The effect of laser radiation on the linear body size of daphnia was described earlier [26]. The one-time exposure of isolated daphnia eggs in the early- and late-blastula stages of development to He–Ne laser radiation (exposure time 60 s, intensity 0.16 mW cm^{-2}, dose 9.6 mJ cm^{-2}) was shown to give rise to larger-sized offspring in the first generation F_1. We demonstrated that such a pattern of relationship was characteristic of a broad dose range (1.7–1300 mJ cm^{-2}) and remained after the onset of sexual maturity.

The laser irradiation of the daphnia also had an effect on their fertility (figure 4). In the parental irradiated generation P, irradiation in doses of 130 and 1300 mJ cm^{-2} resulted in an 18% reduction of fertility in comparison with that of the crustaceans from the control sample. At the same time, the other irradiation doses used exerted no significant effect on the fertility of the parental daphnia generation P. Fixed in the first unexposed generation F_1 was an increase of fertility for the five irradiation doses used, with the maximum, an almost 50% increase, noted at the lowest (1.7 mJ cm^{-2}) and the highest (1300 mJ cm^{-2}) of the doses. In the second generation F_2, a statistically significant 32% fertility stimulation was observed at an irradiation dose of 100 mJ cm^{-2}, while in the third daphnia generation F_3 a statistically significant stimulation of fertility by 23% was revealed at a dose of 200 mJ cm^{-2}.

The coefficients of Spearman's rank correlation between the daphnia irradiation dose and fertility, as well as between the daphnia irradiation dose and linear body size, proved lower than the critical values for the pertinent samples, which can point to the absence of any statistically significant relationship between these parameters.

It is important to note that found among the juveniles from all the brood of the initially irradiated parents (P) were short-lived and/or abnormal individuals never observed in the control. The abnormalities were associated with the defects of the swimming antennae (figure 5).

The normal swimming antennae of daphnia are biramous; they consist of a base, the basipodite, and two branches—the four-segment exopodite and the three-segment endopodite. Both branches of each antenna have setae; their number and arrangement on each segment are described by formulas 0-0-1–3 and 1-1-3 (figure 5(a), black arrow). Observed in abnormal daphnia offspring of parents exposed to He–Ne laser radiation is the underdevelopment or reduction of bristles on the swimming antennae (figures 5(b) and (c), black arrows): the setae are substantially shorter and thinner than the setae in normal individuals and are frequently but barely discernible under a light microscope. Such a structural abnormality could be accompanied by a spiral deformation of the antennae (figure 5(b), black arrow). In some cases, only one antenna was defective; the other antenna could have a normal structure with clearly visible long setae. The apical spine in such individuals

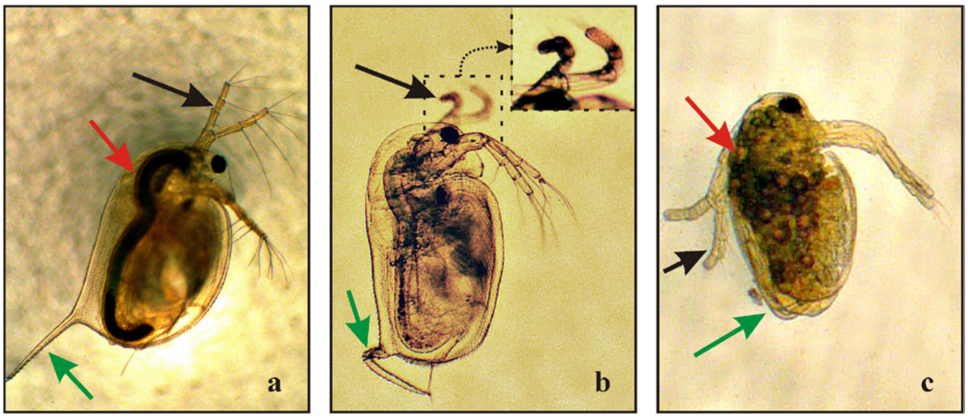

Figure 5. (a) Normal daphnia, (b) abnormal daphnia with a defective swimming antenna, and (c) underdeveloped daphnia embryo born after a single exposure to He–Ne laser radiation. The black arrows indicate the swimming antennae, the red ones the intestines, and the green ones the apical spine.

was frequently folded under the ventral edge of the carapace (figure 5(b), green arrow). Individuals with defective antennae could not move freely in the water column and swam mainly near the bottom. As a rule, they died within 1–3 d of birth. In rare cases molts took place, after which the daphnia regenerated the normal structure of the swimming antennae.

In rare cases, individuals with underdeveloped intestines and numerous droplets of fat (red arrow in figure 5(c)) could be found among abnormal daphnia. Apparently such individuals did not undergo the first juvenile molt. They were characterized by elongated antennae, reaching the rear of the body, and a folded apical spine (figure 5(c), black and green arrows).
AQ5 A few hours after birth such individuals died. To all appearances such individuals bear witness to the embryotropic effect of radiation that prevented embryonal development from completion.

Note that such abnormalities appeared not only in the brood of the parent generation (P), irradiated in position I (figure 1), but also in those of the subsequent generations (F_1–F_3) that were not exposed. No abnormal or short-lived individuals were found in the control samples, which can point to the existence of some relationship between irradiation and the defects observed. No statistically significant correlation was revealed in all the generations (P–F_3) between the irradiation dose the parent generation P was exposed to and the quantity of abnormal juveniles. The percentage of abnormal juveniles in the parent generation P, averaged over all the radiation doses used, amounted to $0.54 \pm 0.36\%$. The percentages of abnormal juveniles, averaged over all the radiation doses used, did not differ significantly between the different generations P–F_3; on average, this percentage averaged over all the irradiation doses and over all the generations came to $0.43 \pm 0.31\%$. The maximum quantity of defective juveniles, equal to 1.7%, was fixed in the parent generation (P) exposed to the minimal laser light dose of 1.7 mJ cm^{-2}. Apart from the control, no anomalies were also found in generations F_1 and F_3 at a laser radiation dose of 100 mJ cm^{-2}.

Such anomalies of the swimming antennae were fixed when exposing the daphnia to the 650 nm incoherent red light of a LED matrix in a dose range from 0.09 to 270 mJ cm^{-2} [29], and subsequent to their exposure to various chemicals, particularly pesticides—such as atrazine [34].

The fact that the percentage of abnormal juveniles irradiated in position I (figure 1) did not depend on the laser radiation dose used, allowed us to assume that the appearance of anomalies in our experiments was associated with the effect of the attendant EMF generated by the laser apparatus, rather than that of the laser radiation itself. To verify this assumption, a separate experiment was performed in which the first group of daphnia was exposed to the visible light of the He–Ne laser only (position II in figure 1) for 3 h with a dose of 14.4 J cm^{-2}, while the second group was affected for 3 h by the attendant EMF generated by the laser apparatus only (with no laser light).

The experiment showed that the effect of radiation from visible He–Ne laser light in a dose of 14.4 J cm^{-2} (which is an order of magnitude higher than the maximum dose used during daphnia exposure near position I) but without the attendant EMF of the laser apparatus, does not result in any anomalies. In contrast, the irradiation of daphnia by the attendant EMF of the He–Ne laser apparatus has led to the appearance of abnormal juveniles in an amount of 0.2%. This value is not significantly different from the previously determined average value of anomalies ($P = 0.54 \pm 0.36\%$) determined for all doses in position I for young abnormal individuals of the parental generation. Abnormal juveniles had a coiled antenna without bristles and underdeveloped intestines.

The appearance of anomalies that manifest themselves in a series of generations can be explained by the damage caused to the DNA of the daphnia. The effect of phototherapy on DNA is inconsistent [21, 35, 36]. The He–Ne laser, while increasing the activity of enzymes, induced substantial changes in the germination of wheat [37]. The exposure of fibroblasts to He–Ne laser radiation in doses of 10 and 16 J cm^{-2} decreased their survival and proliferation rates and caused damage to the cell membranes and DNA [38]. The exposure of mammalian cells to incoherent red light (660 nm) in doses of 2 and 20 J cm^{-2} did not reveal any increase in the frequency of mutations and DNA damage [39]. On the other hand, the preliminary exposure of HeLa cells to He–Ne laser light in a dose of 100 J

m^{-2} promotes the restoration of oxidative DNA damage [40] and causes a protective effect against gamma radiation [41].

Also well known is the effect of electromagnetic fields on daphnia. To illustrate, the exposure of daphnia to an electromagnetic field with a frequency of 42.25 GHz decreased their fertility and increased their sensitivity to the effect of toxicants [42]. Exposure to an electromagnetic field 240 Hz in frequency led to a decrease in the linear body size and body mass of the crustaceans throughout five generations [43].

We believe that organisms in position I (figure 1) experience two opposite effects from the laser apparatus: (1) the negative effect of the attendant EMF that provokes alteration of the normal embryonic development of the organisms and (2) the compensatory effect of visible red light that can partially level out the effect of the attendant EMF.

So the maximum value for young abnormal individuals (1.7%) in the parental generation (P) was observed at the minimal laser dose of 1.7 mJ cm^{-2}, which can indirectly testify to the protective effect of red laser light.

Since for all control groups and after the long-term exposure of the daphnia to a visible-light He–Ne laser only (position II in figure 1) with a dose of 14.4 J cm^{-2} such anomalies were not observed, there is reason to conclude that the emergence of abnormalities is caused by the attendant EMF generated by the laser apparatus. The experiment on the direct effects of the background EMF of the laser apparatus on daphnia showed that such exposure leads to abnormal juveniles. Considering that in our previously described experiments abnormal individuals were detected in all generations of juveniles born from initially irradiated parental individuals, we can conclude definitely that the attendant EMF of the laser apparatus is responsible for the abnormalities in the offspring of not only exposed daphnia but also of their descendants up to the third generation.

4. Conclusions

The effects of He–Ne laser irradiation (including visible laser light 632.8 nm in wavelength and the attendant electromagnetic field from the laser apparatus) on the fertility, newborn quality and linear dimensions of the body of Cladocera *Daphnia magna* Straus were determined. It is directly shown that the attendant electromagnetic field (EMF) generated by the laser apparatus can have a significant impact on the results of low-intensity laser therapy produced by laser light 632.8 nm in wavelength (from the He–Ne laser). It has been established that the exposure of Daphnia to optical radiation in the dose range 1.7–1300 mJ cm^{-2} can affect the integral function of the daphnia's body. At the same time, the attendant EMF (72 ± 4 mA m^{-1} in the frequency range of 0.02–20 kHz and 400 ± 25 mA m^{-1} in the frequency range of 3–4 MHz) causes the appearance of quickly dying and/or abnormal newborn (up to a small percent) that has never been observed in the control. The anomalies of the daphnia mainly consisted of swimming antenna pathologies. Moreover, under the influence of the attendant EMF of the laser apparatus on individual parental daphnia only, these anomalies appear even in several subsequent generations of newborn. The negative impact on the daphnia disappears completely after the suppression of the EMF acting on the Daphnia to laboratory background level. It was also found that the laser light is able to compensate the somewhat negative effect of the EMF on the Daphnia.

Thus, when using laser devices in experimental biology and medicine one should take into account the possible side effects of the attendant electromagnetic fields generated by the laser apparatus.

Acknowledgments

This work was supported by the Russian Science Foundation (project 14-25-00055).

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