Repeated Exposure To 650 Nm Diode Laser Induced Congenital Malformations In Chick Embryos. A Morphological Study.

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Abstract: The widespread use of diode laser devices has increased the concern about its potential effects on the human body and embryo development. The aim of this study is to investigate the effects of diode laser on chick embryo development. Fertilized chicken eggs were divided into three groups: control, treated and sham. Treated embryos were exposed to diode laser 650nm three times a day five days a week for one minute each time. Embryos were extracted on day 7, 10 and 14 of incubation. Incidence of congenital malformations increased in treated embryos compared to the sham and control groups of all experimental ages. The congenital malformations seen were growth retardation, subcutaneous bleeding, limb malformation, abdominal hernia, beak malformations and decreased feather formation around the body. It was concluded that diode laser at 650nm caused congenital malformations in chick embryos.

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1. Introduction

Laser is used nowadays in many applications, such as, telecommunications, research, medicine, graphics, grocery stores, military, agricultural, industrial, and commercial applications (Svelto 2009, Hitz *et al.,* 2001).). Many persons are daily using many devices with laser light emission such as library scanners, supermarket scanners, mice, pointers and Blu-ray disk players (Coldren *et al*., 2012; Ito *et al*., 2012).

The word "laser" is an acronym for " Light Amplification By Stimulated Emission Of Radiation" (Hitz et al., 2001; Silfvast, 2004). Lasers are classified according to the physical state of the active material, solid, liquid or gas, giving different types of laser: gas lasers, diode laser, Solid-state laser and dye laser (Silfvast 2004; Svelto 2009). Diode laser has been shown to increase cell proliferation *in vitro* (Eduardo *et al.,* 2007). Nowadays many females that might be pregnant work in places where they are exposed to diode laser rays several times during their work such as check-outs, libraries, as medical personnel during applying medical laser treatment, or in beauty salons.

Embryonic development involves different cell activities such as cell migration, cell multiplication and cell differentiation (Wolpert *et al*., 2002; Slack 2006; Gilbert 2007; Karim and Al-Quds 2008). Many congenital malformations with unknown causes were seen in recent years. These might be due to several environmental factors that could affect cellular activities during development (Gilbert and Epel 2015).

Chicken embryo is a good model organism for the scientific experiments in developmental biology, as it is available all year around. It has a cheap price and rapid development as it needs only 21 days for hatching. All stages of the development of the embryo can be dealt with. It has a relatively large size. It also seems to have considerable similarity to the human embryo at the molecular, cellular and anatomical level (Wolpert *et al*., 2002; Vergara and Canto-soler 2012).

This study aims to investigate the potential teratogenic hazards that could result from the daily exposure of chick embryos to diode laser scanner device used in different places such as check out cashier or library book scanning, mimicking the exposure of pregnant mothers working daily for several hours with these devices.

2. Material and Methods

All experimental procedures were approved by the Biology Department at King Abdul-Aziz University. Fertilized chicken eggs were obtained from Al-Jamom chicken farm in Jeddah / Saudi Arabia. Egg Incubators were purchased from Al-Hakeem Foundation, model number WQ –(56 egg incubators) incubation Specification, automatic temperature, automatic egg turning (every 2 hours), 220 V, power 80 watt. The diode laser device used in the present study was a barcode scanner with wavelength of 650 nm, power 100 mw, 2.4 GHz, purchased from Al Ghamdi Technology Barcode systems.

Experimental design:

Fertilized chicken eggs were divided into three groups control, sham and treated. All groups were incubated under identical standard conditions temperature 37.5 C° and humidity 80%. Treated embryos were exposed to diode laser three times a day (8:30, 10:30 am and 12:30 pm) five days a week for one minute each time, the diode laser was positioned vertically at a distance of 15 cm from the eggs (see figure 1). During laser exposure the cover of the incubator needed to be removed, which caused a change in temperature and humidity for the fertilized eggs. This caused the introduction of temperature and humidity factors in the experiment. To study the effect of laser alone, the sham group cover was also removed during the exposure of the treated group to eliminate the effect of temperature and humidity variation. Embryos were extracted on day 7, 10 and 14 of incubation, weighed, photographed and fixed in Bouin's solution. To detect hatchability rate and any congenital malformations after hatching a batch of eight fertilized eggs of each group was left until hatching time; the treated group was treated for 13 days only.

Figure 1; Showing the method of exposure of eggs to diode laser during the experiment. The blue arrow shows the laser light on the egg.

Photographing:

Each embryo was photographed using an iPhone 6S Plus camera (12 Megapixel) attached to a holder. A ruler was put near the embryo to be used as a scale when preforming morphometric studies using the photo. The camera zooming and the distance between the camera and specimen was the same for all whole body photos. Photographs were used for detecting congenital malformations and for morphometric studies.

Morphometric studies:

The computer software (Image tool) downloaded free from (http://cme.msu.edu/cmeias/) was used to measure full embryonic length and beak length.

Statistical analysis:

Data was analyzed using SPSS 22. The test used with normal distribution was Anova, Student-Neuman Keul test. In case of abnormal distribution Man-Whiteney U test was used from the non parametric tests. Significance was at p <0.05.

3. Results

Morphological studies

Seven days chick embryo:

The control chick embryos on the 7th day of development had a normal body size and head size. The eyes were large and located on either side of the head separated by the forebrain anteriorly and the beak ventrally. There was clear appearance of neck and upper and lower beak. The three major segments of wing and leg were seen but still in the original paddle-shaped with no detectable digits, (figure 2 A). The sham embryo seemed similar to the control embryo with slight decrease in body size (figure 2B).

The defects seen in 7 day treated embryos were beak malformation, growth retardation and bleeding. The head and the eyes were small with the absence of beak and neck. The limbs appeared as buds (figure 2 C).

Ten-day chick embryo

The control chick embryos on the 10th day of development had large and bulky eyes compared to the head size. The nictitating membrane that covers the sclera was seen. The tip of the upper beak had white scales. The limbs started to be elongated. The three parts of each forelimb became clear. The nasal opening in the beak was seen. Hind limb toes seemed to be developed, but were not separated yet. There was a visible appearance of short feathers covering the entire body (figure 2 D). The sham embryo seemed similar to the control embryo but with less feather distribution (figure 2 E). Almost all 10 day treated embryos had less feather distribution. One embryo was smaller than the control and the sham embryo with no visible feathers. It had abnormal upper beak without white scales and no lower beak. The forelimbs were under developed. The hind limbs and tail were absent (figure 2 F).

Fourteen-day chick embryo:

The control chick embryos on the 14th day of development had eyelids. The size of the eyes became proportional to the size of the head. The hind and front limbs are clearly developed. The four toes of the

hind limbs showed the formation of the claws. The white scales were present in the upper and lower beak. The body was covered with long thick feathers (figure 2G). The sham embryos were similar to the control embryo but seemed smaller. The feathers distribution in the sham embryo were slightly lower compared the control (figure 2H). The treated embryos were smaller than the control and the sham. The feathers were scarce compared to the control and the sham embryos (figure 2I).

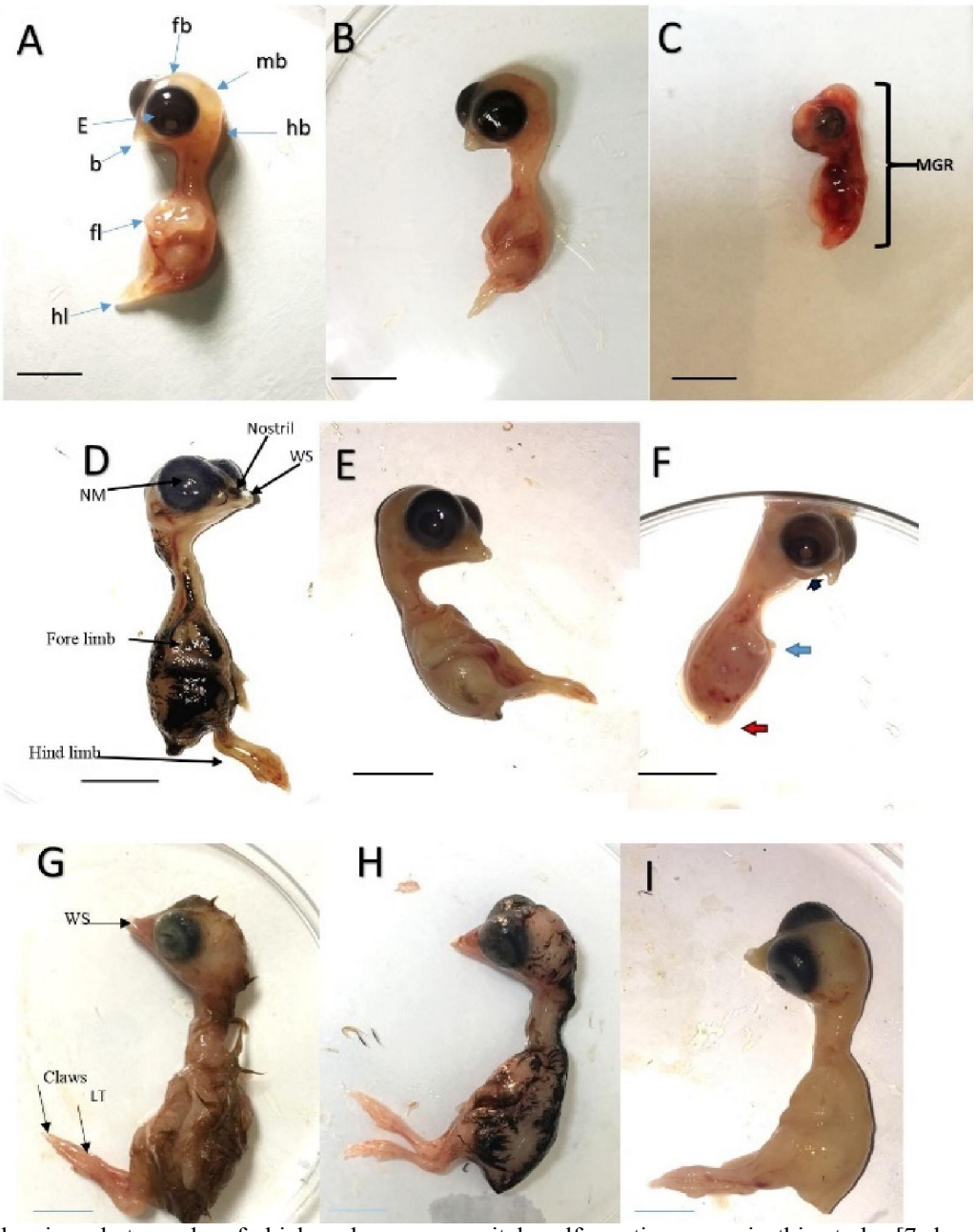

Figure 2. Showing photographs of chick embryos congenital malformations seen in this study. [7 day old chick embryos (A) control, (B) sham, (C) treated]. [10 day old chick embryos (D) Control, (E) sham, (F) treated.] [14 day old chick embryos (G) Control, (H) sham, (I) treated.] Note reduced feather distribution in (E), and complete lack of feathers in (F), malformation of fore and hind limbs (blue arrowhead), tail (red arrowhead) and lower beak (Black arrowhead) in (F), the reduced feather distribution in (H), and scarce of feathers in (I). (MGR) Major Growth Retardation, (fb) forebrain, (mb) mid-brain (hb) hind brain, (E) eye, (b) beak, (fl) fore limb, (hl) hind limb,. (NM) Nictitating Membrane, (WS) White Scales, (LT) Limb Toes, scale bar = 1 cm.

Congenital malformations caused by diode laser

Congenital malformations were seen in 7, 10 and 14-day embryos in sham and treated groups. However, they occurred more in the treated group compared to the sham and control groups. These malformations included subcutaneous bleeding, growth retardation, beak malformations such as absence of beak and decrease in feather formation around the body (see figure 2). The frequency of malformations are shown in table 1. Moreover, on day 10 of incubation, one case of major congenital malformation in the treated group was seen. Showing an embryo with three eyes, two beaks and a non-closed upper spinal cord and brain, (see figure 3) no other cases were seen in the control or sham groups throughout the experiment.

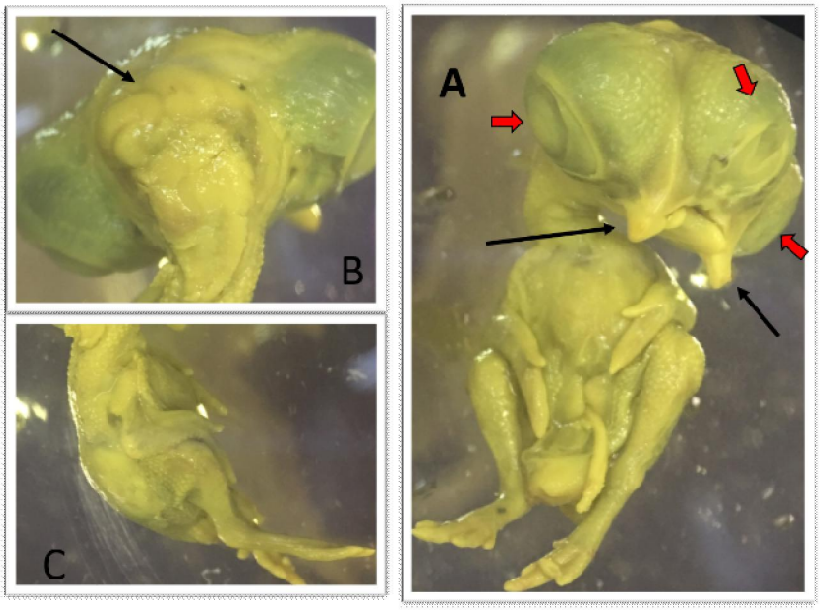

Figure 3. Showing a major congenital malformation in a 10-day embryo of the treated group. Photos were taken after fixation in Bouin's. (A) Front view, (B) dorsal head view, (C) lateral body view. Note the presence of three eyes (red arrows) and two beaks (black arrows) in (A), the non-closed upper spinal cord and brain in (B).

Table 1. Showing the percentage and types of malformations seen in each age treatment group of chick embryos in this study. (BM) Beak malformations, (MGR) Major growth retardation, (FD) Feather distribution, (B) Bleeding, (AH) Abdominal hernia.

Embryonic age		Congenital malformations seen				
	Treatment	BM	MGR	FD	B	AH
7 Days	Control	0%	-	-	6.7%	-
	Sham	-	-	-	6.7%	-
	treated	20%	6.7%	-	20%	-
10 Days	Control	-	-	-	-	-
	Sham	-	6.7%	-	-	-
	treated	6.7%	6.7%	-	-	-
14 Days	Control	-	-	-	-	-
	Sham	-	-	13.3%	6.7%	6.7%
	treated	-	-	26.7%	6.7%	6.7%

Hatchability

Hatching rate in control was 100%, sham 25% and treated 87%. Chicken embryos in all groups started hatching on day 21 until day 24. In the sham group, 75% were formed in the egg; however, they did not hatch and were dead. In the treated group, one egg hatched on day 28 with leg malformations and then died after a week. Another one hatched on day 24 with bulges in its head and upper dorsal body, it died immediately (see figure 4).

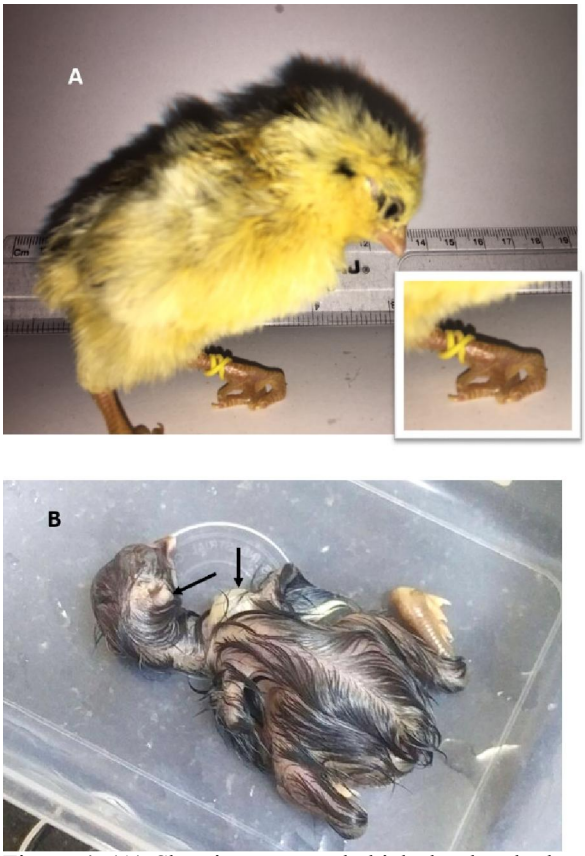

Figure 4. (A) Showing a treated chick that hatched on day 28 of incubation with a clear malformation in both legs. The insert magnifies the malformation. (B) showing a dorsal view of a treated chick that hatched on day 24 of incubation with bulges in his head and upper back, it died immediately after hatching.

Morphometric studies

Effect of diode laser on chick embryo whole body length

The mean of normal whole body length of chick embryo in this study was 3.6 cm for 7 days, 5.7 cm for 10 days, and 8.5 cm for 14 days of incubation.

There was a non-significant difference in the whole body length between the treated group, sham group, and control group in 7-day embryos. While the whole body length of 10 day treated embryos was significantly decreased compared to the sham group (P=0.023) and compared to the controls (P=0.001). Also a non-significant decrease was seen between treated 14 days embryos and the sham group, while there was a significant decrease in the length of sham group (P=0.045) compared to controls, and the treated group (P=0.002) compared to the controls (see figure 5).

Effect of diode laser on chick embryo whole body weight

The mean of normal whole body weight of chick embryo in this study was 0.7g for 7 days, 2.9987g for 10 days, and 8.5247 g for 14 days of incubation.

There was no significant difference in the whole body weight between the treated groups, sham and the control in 7-day embryos. While the whole body weight of 10 day treated embryos was significantly decreased compared to the sham group (P=0.037) and compared to the controls (P=0.001). No significant difference was seen between treated 14 days embryos and the sham group, while there was a significant decrease in the weight of the sham group (P= 0.009) compared to the controls, and the treated group (P=0.001) compared to the controls, (see figure 6).

Effect of diode laser on chick embryo beak length

The mean of normal beak length of chick embryo in this study was 0.577 cm for 10 days and 0.894 cm for 14 days of incubation. There was no significant difference in the beak length between the treated group, sham group, and control group in 10-day embryos. No significant difference was seen between treated 14 day embryos and the sham group, while there was a significant decrease in the length of treated group (P=0.026) compared to the controls (see figure 6).

4. Discussions

In this study, we used the chick embryo as a model to investigate the potential teratogenic hazards of diode laser emitted from a barcode scanner device. Using the chick embryo model allowed us to expose embryos to equal amounts of laser radiation. The eggshell, egg membranes and albumin surrounding the embryo might have worked as partial barriers absorbing part of the laser radiation. These barriers might have been similar to the different body layers surrounding mammalian embryos.

The control group In the present study had growth parameters such as whole body length and whole body weight similar to the growth parameters seen in other studies (Al-Qudsi and Azzouz 2012; Rahman *et al.,* 2014). In this study the combined effects of diode laser and slight changes in temperature and humidity (due to experimental set-up) caused growth retardation to chick embryos such as reduced whole body length and weight, these were clearly seen on day 10 of incubation, as the parameters of the treated group were significantly decreased compared to the sham group. The sham group was also affected as it significantly showed signs of growth retardation due to changes in temperature and humidity. However as there were significant difference between the growth of the treated and sham groups, it can be concluded that diode laser affected chick embryonic growth on 10 days of incubation.

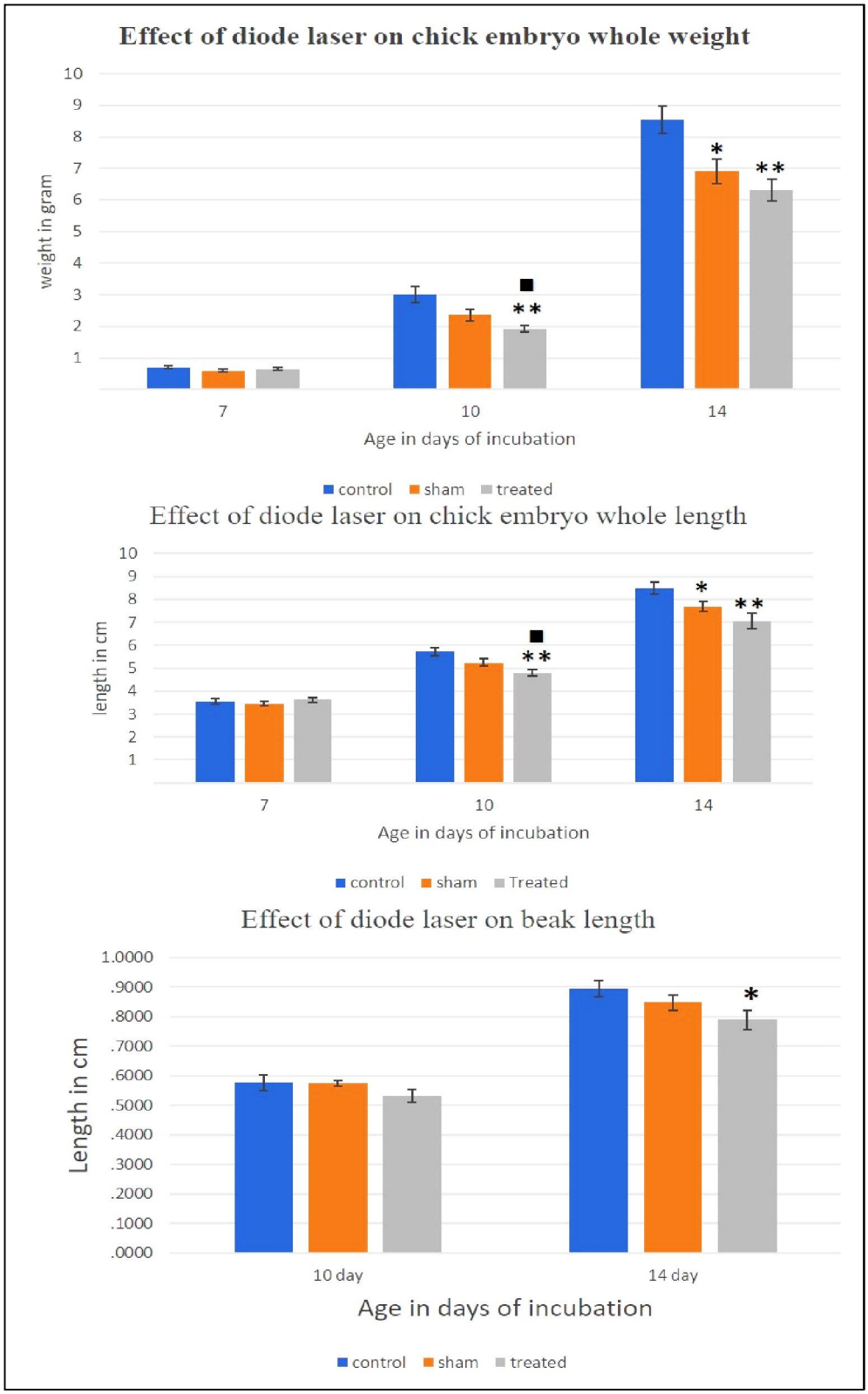

Figure 5. Graph showing the effect of diode laser on chick embryo whole body length, whole body weight and beak length. Values are mean ± SE taken from 15 samples for each group age treatment. (*) p< 0.05 with control, (**) p=0.001 with control, (■) p< 0.05 with sham.

In a study where glioblastoma cell cultures were irradiated with a Low-power 808-nm laser irradiation (LLI), the laser irradiation inhibited cell proliferation without cell death, it also inhibited cell viability without morphological changes. It was concluded that LLI might have regulated the expression of cyclin-dependent kinase inhibitors, therefore, arresting the cell cycle and keeping the cells in the non-dividing G0 phase (Murayama *et al.,* 2012), also other possibilities could not be excluded, such as stimulating/inhibiting the innate metabolism of a cell (Karu, et al., 1993; Karu, 2008). Murayama *et al.,*, 2012 concluded that LLI had stimulating and inhibitory effects on cell proliferation and summarized the actions of LLI in three points which were, that LLI influenced cell viability via the mitochondrial respiratory chain, not affecting the DNA synthesis, and that LLI did not cause necrosis (Murayama *et al.,* 2012). All these factors might have been involved in the growth retardation seen in the present study.

Many studies have shown that LLI at wavelengths in the visible red to near-infrared range enhances proliferation of satellite cells of skeletal muscle (Ben-Dov *et al.,* 1999), peripheral blood lymphocytes (Stadler, Istvan, *et al.,*2000), articular chondrocytes (Jia and Zhou-Yi 2004) and Schwann cells (Van et al., 1993). While other studies showed that LLI. caused inhibitory proliferation on fibroblast (Moore *et al.,* 2005).

Several studies proved that there is a similarity between embryonic cells and cancerous cells regarding cell multiplication (Smith and Sturmey 2013), therefore it might be logic to compare the effect of diode laser studies on cancer cells with the effect of diode laser on embryonic cells. The growth retardation seen in the treated embryos of this study might be due to the inhibitory effect of diode laser on cell proliferation.

In the present study diode laser caused severe craniofacial congenital malformations in one of the 10 day embryos, that included a third eye, two beaks, a non-closed neural tube exposing upper spinal cord and brain. Similar congenital malformations were caused by dioxin in chick embryo (Yeager *et al.,* 2006). Other minor congenital malformations were seen in the treated group more than the sham or control group such as growth retardation, beak absence, bleeding and abnormal feather distribution. Some of these malformations were seen in chick embryos treated with endosulfan (Mobarak *and* Al-Asmari 2011).

Studies have shown that stem cell migration was affected by low level laser treatment as it increased (Gasparyan and Brill 2004; Mvula *et al.* 2008). In this study it seems that chick embryonic stem cells migration such as neural crest cells migration might have been affected, resulting in the several congenital malformations seen, such as feather formation and distribution that resulted in this study.

In this study, the sham group had the lowest hatchability. Studies proved that fluctuations of temperature and humidity decreased hatchability (Nakage *et al.,* 2003). However the laser treated group had higher hatchability than the sham group, which might indicate that laser radiation had a role in protecting embryos from temperature and humidity fluctuation.

In a study to investigate the effect of short term laser application on broiler chickens hatchability. Experment eggs were irradiated with red light laser irratiation with 6 and 10 mw power, 633 nm wavelength for 90s, 12hrs before incubation. No significant difference was seen in hatchability of irradiated eggs compared to the control (Ghalehkandi *et al.,* 2015). On the other hand previous studies had shown that low power 0.1 mw\cm irradiation had significantly impoved hachability in broiler eggs (Yakimenko *et al.,* 2002) and duck eggs (Melnikova *et al.,* 1985).

Most of the previous studies on the effect of laser on hatchability irradiated the fertilized eggs before the incubation, while in the present study, the eggs were irradiated daily for fourteen days during incubation, and this might be the cause of the differences seen in the results with other studies.

As diode laser is used daily in check-out scanners, library scanners, and other devices, by several females that might be pregnant. Also with regard to the congenital malformations seen in this study during the embryonic development and after hatching. It is important to do more studies and investigations on the potential teratogenic hazards of diode laser devices.

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