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Effects of 670-nm Phototherapy on Development

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ABSTRACT

Objective: **The objective of the present study was to assess the survival and hatching success of chickens (*Gallus gallus*) exposed *in ovo* to far-red (670-nm) LED therapy.** ***Background Data:*** **Photobiomodulation by light in the red to near-infrared range (630–1000 nm) using low-energy lasers or light-emitting diode (LED) arrays has been shown to accelerate wound healing and improve recovery from ischemic injury. The mechanism of photobiomodulation at the cellular level has been ascribed to the activation of mitochondrial respiratory chain components resulting in initiation of a signaling cascade that promotes cellular proliferation and cytoprotecton.** ***Materials and Methods:*** **Fertile chicken eggs were treated once per day from embryonic days 0–20 with 670-nm LED light at a fluence of 4 J/cm^2.** ***In ovo*** **survival and death were monitored by daily candling (after Day 4).** ***Results:*** **We observed a substantial decrease in overall and third-week mortality rates in the light-treated chickens. Overall, there was approximately a 41.5% decrease in *mortality rate* in the light-treated chickens (NL: 20%; L: 11.8%). During the third week of development, there was a 68.8% decrease in the mortality rate in light-treated chickens (NL: 20%; L: 6.25%). In addition, body weight, crown–rump length, and liver weight increased as a result of the 670-nm phototherapy. Light-treated chickens pipped (broke shell) earlier and had a shorter duration between pip and hatch.** ***Conclusion:*** **These results indicate that 670-nm phototherapy by itself does not adversely affect developing embryos and may improve the hatching survival rate.**

INTRODUCTION

LOW-ENERGY PHOTON IRRADIATION by light in the far-red to near-infrared spectral range (630–1000 nm) using low-energy lasers or light-emitting diode arrays has been found to modulate various biological processes *in vitro* and *in vivo*.[1–5] Photobiomodulation has been applied clinically in the treatment of soft tissue injuries and to accelerate wound healing for more than 30 years.[1,2,5–7] Phototherapy using low-energy red laser lights and red LED arrays (640–690 nm; 670-nm peak) is now being used in a variety of clinical and experimental settings to promote wound healing and tissue regeneration.[3,8,9] At the cellular level, photoirradiation at low fluences can generate significant biological effects, including cellular proliferation and the release of growth factors from cells.[10–12] Investigations into low-energy stimulation of tissues by lasers have shown increased cellular activity during wound healing, including increased collagen production and angiogenesis.[10–12] Photobiomodulation by light-emitting diode arrays (LED) has also been shown to increase retinal cytochrome oxidase activity in rats and cellular proliferation in diabetic mice.[8,13] Based on recent studies, these low-energy light therapy effects appear to be at least partially due to potent effects on gene expression that have not been fully characterized.

The present study was undertaken to investigate the actions of low-energy light therapy on embryonic development, using the chicken embryo as a model system. Domestic chickens (*Gallus gallus*) have been widely investigated as an animal model for vertebrate embryonic development for over a century.[14] Chicken embryonic development is well characterized anatomically, physiologically, biochemically, and in terms of the molecular cues that control development.[15] Moreover, chicken embryos are sensitive to many developmental toxins and are therefore an ideal laboratory model for this study.[14] As a corollary to the current tests and applications of the LED therapy, we set out to assess the developmental impacts of 670-nm LED photobiomodulation using the chick embryo model.

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MATERIALS AND METHODS

Fertile domestic chicken (*Gallus gallus*) eggs were collected from Purdue University Poultry Farm (W. Lafayette, IN) and hand delivered to the laboratory. Upon arrival, all eggs were cleaned, candled, and inspected for cracks. Eggs were equally distributed based upon weight into LED-treated and control groups. In all, four separate batches of eggs were incubated throughout the course of the study. Eggs were incubated for the 21-d incubation period in a Petersime Model 4 incubator (99.5°F dry bulb; 87°F wet bulb) (Petersime Incubator Co., Gettysburg, OH). All eggs subjected to 670-nm phototherapy were treated with a far-red light-emitting diode (LED) array (640–690 nm; 670-nm peak; 4 J/cm^2) (Quantum Warp 10, Quantum Devices, Barneveld, WI) *in ovo* once every 24 h for 80 s. LED-treated eggs and control eggs were handled identically during delivery and upon arrival at the laboratory. Both experimental groups were incubated in the same incubator for the same amount of time and were removed from the incubator in LED-treated and control pairs for the daily treatment.

Embryonic development, movement, and growth and proliferation of the vasculature were monitored by candling each egg daily. Pipping and hatching activity were monitored approximately eight times per day during Days 20–22 of development. Hatched chicks were provided with food (Purina Mills Start and Grow Poultry Feed) and water *ad libitum* upon hatching. All chickens were euthanized by decapitation within 30 h of hatching. Brains and livers were harvested and all archived tissues were post-fixed for at least two weeks in 10% neutral buffered formalin (Fisher Scientific International, Inc.) at 4°C. Animals were handled in accordance with the Guide for the Care and Use of Laboratory Animals, as adopted and promulgated by the National Institutes of Health. Animal care and handling protocols were approved by the Indiana University Bloomington Animal Care and Use Committee (BIACUC).

As is true for all mammalian development, avian *in ovo* development falls grossly into three stages. Each stage is approximately one week in duration. Organ development is initiated within the first five days of incubation, but complete organogenesis is considered to take approximately the first full week of incubation.[16]

During the second week of chicken *in ovo* development, organs and tissues continue to grow, refine connections, and differentiate. As in the last trimester of mammalian development, chicken embryos mature and undergo a substantial growth spurt during the last week of incubation. It is during this period that most of the yolk is metabolized *in ovo*. Residual yolk, which is internalized into the abdominal cavity prior to hatching, is used to support the energy demands of the newly hatched chick during the first few days of life.[15,16]

Pipping (i.e., the chick breaking through the shell with intense pecks of the beak) usually occurs around Day 20 of incubation, approximately 12–24 h before hatching. Pipping requires consistent utilization of neck muscles, and the embryo has a cache of brown fat in its neck providing energy reserves.[16] Stressed, energy-depleted, and chemically intoxicated birds have a tendency not to survive this energy-demanding process.[17]

Therefore, to investigate the effect of low-level light treatment on development, based upon prior studies of avian embryonic survival, we evaluated the following endpoints: body weight, crown–rump length, yolk weight, liver weight, heart weight, pip time, pip-to-hatch duration, and residual yolk weight as a function of post-hatch survival time.

All endpoints were evaluated both graphically (Microsoft Excel) and statistically (SAS System, SAS Institute, Cary, NC). Statistical analysis included ANOVA (PROC GLM) to compare the endpoints for statistically significant differences. Regression (PROC REG) analysis was conducted to illustrate the strength of the correlate between residual yolk weight and post-hatch survival time (hatch–sacrifice duration). This regression was conducted to assess the strength of the relationship and to account for differences between hatch–sacrifice duration as a result of early pipping and hatching of the light-treated chicks. Statistical significance was determined using an alpha of 0.05.

RESULTS

Mortality

Under control conditions embryo mortality was approximately 20%. Treatment with 670-nm LED decreased the mortality rate by 41.5%. Mortality was then divided into 3-wk periods, based upon the 21-d gestation period of domestic chickens. For our purposes, "early death" is defined as death during the first week of embryonic development and "late death" is defined as death during the third week of embryonic development. Early death determinations did not include eggs that appeared to be infertile based upon the lack of evidence of any changes in the yolk, including mottling, membrane clearing, and vasculature development. No embryos died during the first week of incubation, and one LED-treated embryo died during the second week of incubation. Typically, chickens that die during the third week have survived to full term and either do not pip, or start to pip (one small hole was present in the shell) but never hatch. There was a substantial decrease (68.8%) in the third-week mortality rates in the LED- treated chickens (Fig. 1).

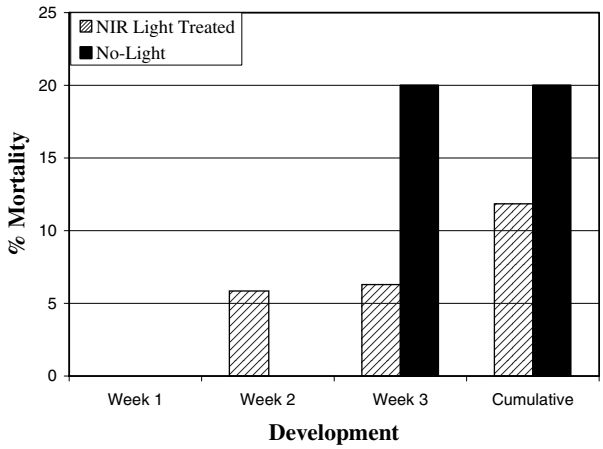

FIG. 1. Comparison of weekly and cumulative mortality during embryonic development. Notice the clear trend in decreased mortality throughout development in the near-infrared (NIR) light-treated group.

TABLE 1. TRENDS IN SIZE, WEIGHT, AND SOMATIC INDICES CORRELATED WITH NEAR-INFRARED-THERAPY

	Near-infrared light-treated (mean ± SD) n = 14	*No-light (mean ± SD) n = 12*	*Avg. difference (%) ([near-infrared-no-light]/ no-light)*
Body weight (g)	38.798 ± 3.378	38.022 ± 2.688	2.04
Yolk weight (g)	3.993 ± 1.011	4.118 ± 0.865	−3.13
Body weight–yolk weight (g)	34.805 ± 3.019	33.905 ± 2.444	2.59
Liver weight (g)	0.885 ± 0.062	0.834 ± 0.150	6.12
Corrected liver weight (liver wt/[body wt-yolk wt])	0.0256 ± 0.0031	0.0247 ± 0.0046	3.64
Heart weight (g)	0.3369 ± 0.02713	0.3337 ± 0.0302	0.96
Corrected heart weight (heart wt/[body wt-yolk wt])	0.0097 ± 0.00103	0.0098 ± 0.00094	−1.02
Crown–rump length (mm)	94.714 ± 6.232	92.5 ± 5.248	2.34

Growth and somatic parameters

In chickens that survived to hatch, 670-nm light treatment produced an increase in mean body weight, crown–rump length, liver weight, and corrected liver weight (Table 1). The crown–rump length/body-weight ratio was approximately 2.5 for both treatment groups, and was independent of light. Within batch one, liver weight ($p = 0.0140$) and somatic liver weight ($p = 0.0457$) were statistically higher in the light-treated chickens.

In the LED-treated chickens, the decrease in hatchling residual yolk weight (as a function of post-hatch survival time) was statistically significant ($p = 0.0209$, $R^2 = 0.3704$) (Fig. 2). In contrast, a statistically significant decrease in hatchling residual yolk weight (as a function of post-hatch survival time) ($p > 0.15$, $R^2 = 0.1767$) was not observed in control animals. We postulate that these findings are indicative of increased nutrient utilization during development as a result of 670-nm phototherapy.

On average, the light-treated (L) chickens pipped 2.92 h *earlier* (Day 20 to pip) than did the no-light (NL) chickens (NL: 26.007 h +/− 6.91, range: 20–43.5; L: 23.084 h +/− 6.50, range: 19.50–43.75) (Fig. 3). The increased pip time variability (reflected in the standard deviation) is due to one LED-treated and one control bird that pipped on Day 22. Removing these outliers reduces the standard deviation and improves the statistical difference (NL: 24.417 h +/− 4.37, range: 20–32.5; L: 21.494 h +/− 2.73, range: 19.5–27.67) ($p = 0.0581$).

The mean duration between pip and hatch time was 2.91 h *shorter* in the LED-treated chickens (NL: 16.20 h ± 7.08,

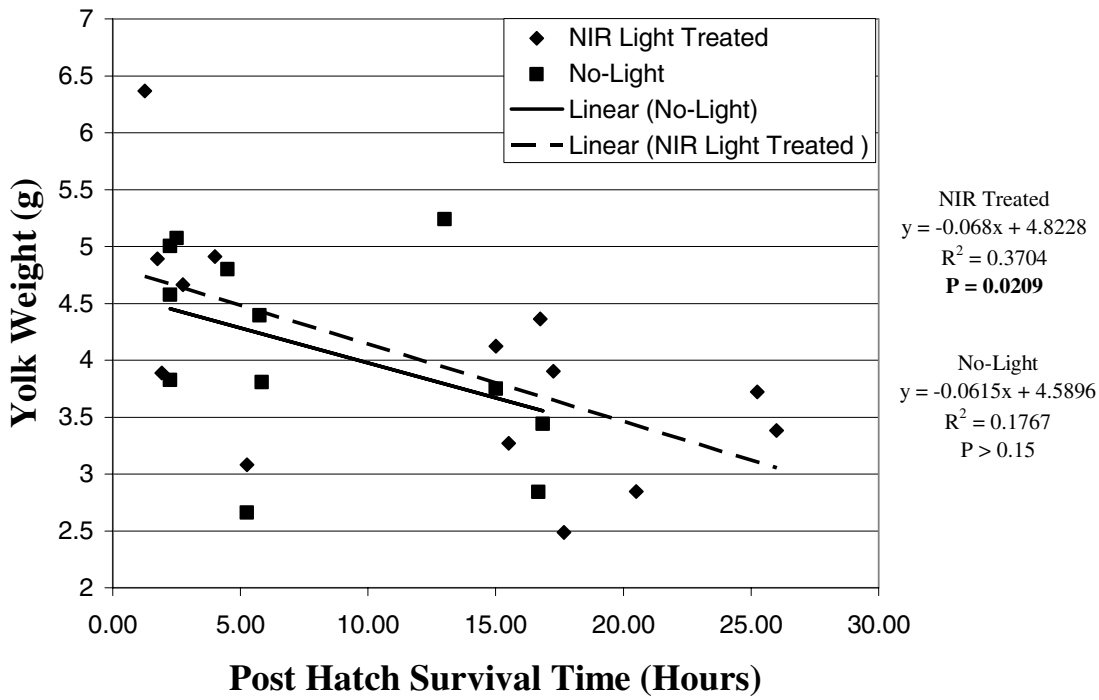

FIG. 2. Residual yolk weight vs. post-hatch survival time regressions for near-infrared (NIR) light-treated and no-light hatchlings.

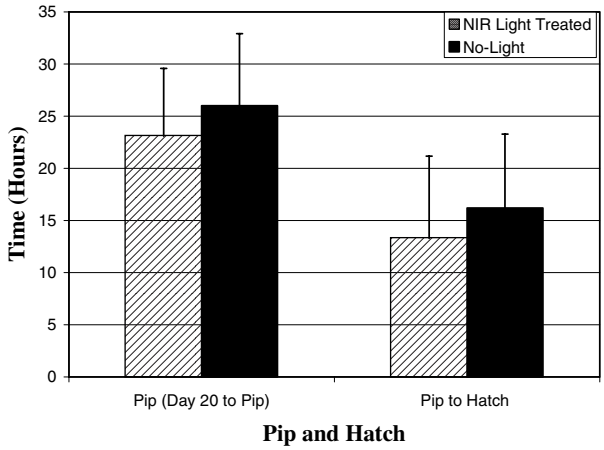

FIG. 3. Near-infrared-LED-induced decrease in pip and hatch times (avg + stdev).

range: 1–24.5; L: 13.29 h ± 7.88, range: 1.75–26.25) (Fig. 3). The two outliers mentioned above hatched within the normal (12–24 h) time period following pipping.

DISCUSSION

Studies from our laboratory[8,9,13,18,19] and others[1,2,12,20,21] have shown that exposure to far-red to near-infrared light from low-energy lasers or light-emitting diode arrays delivered at energy densities between 2 and 10 J/cm^2 promotes mitochondrial energy metabolism, cell division, and wound healing. With respect to LED wavelength, the majority of our studies have been conducted using 670-nm LED light, and we have accumulated substantial evidence that near-infrared-LED treatment at 670 nm is beneficial both *in vitro* and *in vivo*.[9,13,18] Based on these observations, we examined the effect of *in ovo* 670-nm LED treatment on the development, hatching efficiency, and survival of chickens. This study demonstrates that 670-nm LED treatment did not adversely effect the survival of chicken hatchlings exposed to one daily light treatment throughout the incubation process. Moreover, we observed an overall improvement in the embryo/hatchling survival rate in the LED-treated group.

The mechanism of photobiomodulation by red to near-infrared light at the cellular level has been ascribed to the activation of mitochondrial respiratory chain components resulting in initiation of a signaling cascade that promotes cellular proliferation and cytoprotection.[1,2,8,13,18] A growing body of evidence suggests that cytochrome oxidase is a key photoacceptor of light in the far-red to near-infrared spectral range.[1,2,8,13,18] A comparison of the action spectrum for cellular proliferation following photoirradiation with the absorption spectrum of potential photoacceptors first led Karu and colleagues to postulate that cytochrome oxidase is a primary photoreceptor of light in the red to near-infrared region of the spectrum.[1,2] More recent studies in our laboratory have confirmed this postulation and have demonstrated that the action spectrum for stimulation of cytochrome oxidase activity and cellular ATP content parallels the near-infrared absorption spectrum of cytochrome oxidase.[18,22] Cytochrome oxidase is an integral membrane protein that contains four redox active metal centers and has a strong absorbance in the far-red to near-infrared spectral range detectable *in vivo* by near-infrared spectroscopy.[23–25] Moreover, 660–680 nm irradiation has been shown to increase electron transfer in purified cytochrome oxidase[1,2] to increase mitochondrial respiration and ATP synthesis in isolated mitochondria[1,2] and to upregulate cytochrome oxidase synthesis and activity in cultured neuronal cells.[19]

Hatching is an energetically demanding process. Many chicks that do not survive the process (especially those that die after pipping) are thought to have exhausted their ability to generate cellular energy in the form of ATP and other high-energy phosphates. Our observations that photobiomodulation decreased third-week mortality, combined with the findings that near-infrared LED irradiation enhances cellular energy metabolism, lead us to suggest that *in ovo* photobiomodulation increased the energy available to the chicks during the hatching process.[15,16] The 670-nm phototherapy clearly decreased third-week mortality, presumably by increasing the energy available to the chicks during the hatching process. Moreover, we observed clear differences in the behavior of LED-treated versus non-LED–treated chickens that were also indicative of improved energy metabolism. LED-treated chickens were more social and energetic than the untreated control group. These behavioral differences were observed in all four batches of hatchlings.

In addition to the decreased mortality in the LED-treated group, LED treatment throughout incubation also improved the fitness of the embryo/hatchlings. Shorter periods before starting to pip and between pipping and hatching indicate that the LED-treated embryos were prepared to hatch earlier and were more physically prepared for the hatching process. The increased body weight is another indication of overall improved fitness in the LED-treated groups. The increased liver-weight and body-weight normalized liver somatic indices may also be attributable to increased cellular and enzymatic activity induced by the near-infrared-LED treatment.

Two additional indications of the increased fitness of the light-treated hatchlings were the difference in time to pip and the amount of residual yolk in each chick. Despite a slightly larger average egg size, the light-treated chickens contained less yolk on average than the control hatchlings (Fig. 2 and Table 1). This was partially attributable to an increase in yolk mobilization during the 24-h post-hatch period. This correlation was only statistically significant ($p \leq 0.05$) for the LED-treated hatchlings. We suspect this may be related to the difference in behavior (24 h post-hatch) in the hatchlings. Control chickens that did not receive *in ovo* LED treatment tended to sleep and were therefore much less likely to be energy demanding than the very active light-treated hatchlings. The earlier pip time, in addition to the shorter pip–hatch time, corresponds with the larger size and increased activity in the near-infrared-light-treated chickens. Future studies will focus on further evaluating these trends and improving statistical power.

CONCLUSIONS

The results of this study provide evidence that developmental exposure to 670-nm light treatment does not adversely affect the

development of the chicken embryo as assessed by hatching, survival, and morphological criteria. Furthermore, our data suggest that developmental 670-nm light exposure may exert beneficial effects on growth and development, including improved survival. Future studies will be directed at elucidating the molecular mechanisms of these observed changes.

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