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Effect of near-infrared light on *in vitro* cellular ATP production of osteoblasts and fibroblasts and on fracture healing with intramedullary fixation

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ABSTRACT

Objective: Evaluate the effect of near-infrared light (NIR) on immediate production of ATP by osteoblasts and fibroblasts *in vitro*, and the healing process of rat femur fractures with intramedullary fixation.

Background: NIR is one potential treatment option for complications of fracture healing, which has shown to stimulate cellular proliferation and to enhance the healing process.

Methods: Cell culture – MC3T3-E1 and 3T3-A31 cells were subjected to NIR at 660 nm, 830 nm, or both combined. ATP was assayed at 5, 10, 20, and 45 min after exposure. Animal study – 18 rats had surgery with retrograde intramedullary pins inserted into their femurs, which then underwent closed, transverse femur fracture. Rats were randomly divided into 3 study groups of 6 each: nonirradiated controls, 660 nm, and 830 nm NIR. Healing process was assessed by a blinded radiologist, assigning a healing score of 1–6 for radiographs taken on days 0, 7, 14, and 21.

Results: Cell culture – All groups gave significant increase in ATP within 5–10 min, with decay to baseline by 45 min. 660 nm NIR was significantly more effective than 830 nm with fibroblasts or either wavelength with osteoblasts. Animal study – A significant increase in the fracture healing grade in the 660 nm group at day 14, but with no differences at day 21.

Conclusion: The study demonstrated an immediate increase in ATP production *in vitro* and an initial acceleration of callus formation in the fracture healing process, in the presence of NIR.

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1. Introduction

Musculoskeletal injuries comprise a major proportion of unintentional injuries incurred in the United States. Fractures

account for approximately 25% of the reported 61.2 million musculoskeletal injury episodes each year.¹ Known complications of fractures in orthopedic patients include nonunion, delayed union, malunion, and refracture at the previous fracture site during early callus formation. Unfortunately,

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approximately 5–10% of fractures will show delayed healing.² Therefore, treatment modalities that potentially augment and accelerate the healing process may help decrease the rate of these complications seen in the orthopedic population. In particular, accelerated formation of callus may result in decreased complications due to a reduced time period for movement-induced disunions.

Many supplemental techniques in addition to the standard immobilization and fixation of fractures have been investigated to accelerate the healing process, including various pharmacologic investigations and mechanical adjuncts. Recently, laser therapy in the red to near-infrared (NIR) range, roughly 630–1000 nm, has been studied for improving bone healing in several conditions, such as in dental implants,^{3,4} autologous bone graft,^{5,6} and several types of bone defects.^{7–9} A wide variety of wavelengths have been used, with positive results reported for 633, 660, 685, 735, 780, 790, 802, 820, 830, 850, and 904 nm.^{10–14} Some studies have indicated that irradiated bone showed increased osteoblast proliferation, collagen deposition, and new bone formation when compared with nonirradiated bone, and the effect was dependent on total dose and mode of irradiation.⁷ There is *in vivo* evidence demonstrating increased bone healing in laser-irradiated animals after surgery when compared to controls.^{4,7,15}

The potential of NIR therapy for application in fracture healing is evidenced by data showing increased mitochondrial ATP production, increased local lymphatic circulation, increased osteoblast activity and proliferation,¹⁶ upregulation of pro-osteoblast gene expression, and increased nitric oxide (NO) in presence of NIR.^{17–19} One of the proposed mechanisms for NIR-induced healing and cellular protection is by a NO-dependent mechanism based on data that in the presence of NO scavengers, the previously shown protective effect of NIR on hypoxia/reperfusion is neutralized.¹⁹ NIR has been shown to increase intracellular NO, reverse NO-induced inhibition of oxygen consumption by cytochrome oxidase in mitochondria, and increase energy synthesis; it has been postulated that heme-containing proteins, such as cytochrome oxidase, myoglobin, and hemoglobin, may be NO donors in the presence of NIR treatment.¹⁹

In fractures, the healing process involves multiple events, including angiogenesis and intramembranous and endochondral ossification.²⁰ Following a fracture, osteoprogenitor cells are activated to proliferate in the periosteum and cartilage formation, calcification, removal, and subsequent bone formation by osteoblasts take place.²⁰ These fracture healing events require rapidly dividing cells and production of large quantities of proteins and induction of genes promoting osteoblast activation. Consequently, this process requires efficient energy consumption and mitochondrial function in the chondrocytes and osteoblasts during the ossification process. Considering the positive cellular effects of NIR demonstrated in previous *in vitro* and *in vivo* models, we propose that NIR application after fracture may accelerate the healing process.

The study was conducted in two parts – the effect of NIR on cellular ATP production, and on fracture healing. As the effect of NIR on osteoblastic cell proliferation seems to be equivocal,⁷ we set as our goal in the first part a more direct effect on cellular energetics, the generation of ATP by individual cells,

independent of cellular proliferation. The second part was a study of callus formation in a rat-fracture model using the same wavelengths and power density. The wavelengths of 660 and 830 nm were chosen to be representative of prior art in our group and of the field at large.

Thus, the aims of this study were to evaluate the effects of NIR light at 660 nm or 830 nm on the initial ATP production of fibroblast (3T3) and osteoblast (MC3T3) cells, and on the formation of callus in the healing process of femoral fractures in rats treated by intramedullary pin fixation, using the rat-fracture model of Bonnarens²¹ with radiographic analysis at weekly time points. This model affords us closed (at the fracture site), controlled, reproducible fractures, with minimal soft tissue trauma. The use of the intramedullary pin in simple transverse fractures allows for more reliable stability at the fracture site during healing, and is analogous to a commonly used clinical method. Weekly radiographic analysis allows us to minimize animal usage by eliminating intermediate time points that require sacrifice, while unfortunately also eliminating the possibility of histological and mechanical measures relevant to these intermediate stages of healing. In addition, future human clinical trials will likely be limited to imaging techniques and clinical outcomes as endpoint measures. Till date, there seems to have been few attempts to move this therapeutic modality into the clinical realm. An early review²² reports on a trial where 632 nm NIR was said to have resulted in better fracture consolidation, as assessed radiographically, and with fewer complications. A case report is also given where 890 nm NIR was said to have resulted in callus formation after 4 weeks in a case of delayed union dating eight months from fracture. A clinical trial in India²³ on tibial stress fractures using 830 nm NIR gave earlier resolution of symptoms, while a trial in Bangladesh²⁴ using 830 nm on long bone fractures gave better early bone regeneration and callus formation. Clearly, there is potential here for a clinically useful modality, but the work of human trials has hardly yet begun. To our knowledge, this type of study on rat femur fractures has not been done before and will provide further information on NIR and its applications in fracture healing.

2. Methods

All experimental procedures and protocols used in this investigation were reviewed and approved by the Animal Care and Use Committee of the Medical College of Wisconsin, Milwaukee, Wisconsin. All conformed to the Guiding Principles in the Care and Use of Animals of the American Physiologic Society and were in accordance with the Guide for the Care and Use of Laboratory Animals (8th ed, ILAR).

2.1. Cell culture

Mouse calvarial osteoblastic MC3T3-E1 and mouse fibroblast BALB/3T3-A31 cells were used for this study. Cells were grown in Medium Essential Medium Alpha (MEM Alpha, osteoblast) or Dulbecco's Modified Eagle's Medium (DMEM, fibroblast) (Gibco Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 1:100 diluted penicillin/streptomycin 10,000 u/ml, and 1:100 dilution L-Glutamine 200 mM in a 37 °C humidified

incubator with 5% CO₂ (NuAire IR Autoflow, NuAire Inc., Plymouth, MN). Cells were plated at 25,000 cells/well in 500 µl in two 24-well plates, one well in one plate and four wells in the other plate. Cells were grown at 37 °C for 72 h, whereupon they generally reached 80–100% confluency. The plates were removed to the bench top and allowed to equilibrate to room temperature for 30 min. Just before NIR application, the culture media was removed and refilled with 300 µl of fresh media. NIR application was done to the plate with four seeded wells using a custom-made prototype light source (Hanger Orthopedic Group, Inc., Bethesda, MD), with a 3.8 × 3.8 cm array, and featuring the ability to select and combine the wavelengths of 660 nm and 830 nm. Application was at 50 mW/cm² for a time period of 80 s, resulting in a total exposure of 4 J/cm². The plate with the single seeded well was reserved for the nonirradiated control. At the appropriate time points postexposure, the seeded wells were tested for ATP content using CellTiter-Glo Luminescent Cell Viability Assay (Promega Corp., Madison, WI). Culture media was not removed prior to addition of the cell lysing reagents. The control plate was tested concurrently with the 45 min postexposure well. These procedures were repeated in toto three times for each wavelength and cell type. Temperatures in the wells were monitored by placing a thermocouple junction within a dummy well containing media, and tracking the temperature throughout the experiment using an Omega HH802W (Omega Engineering, Stamford, CT) digital thermometer with wireless USB recording capabilities. Statistical analysis was by one-way ANOVA with the addition of Tukey's HSD *post hoc* analysis. Values for significance were set at $p < 0.05$.

2.2. Animal study

We conducted a pilot study where 18 rats each underwent right closed, transverse, bicortical, middle-third diaphysis femur fractures with a fracture apparatus technique previously described.²¹ The rats (male, Sprague Dawley, 315–400 g) were anesthetized with an intraperitoneal injection of ketamine (75 mg/kg) and xylazine (10 mg/kg), and received buprenorphine (0.5 mg/kg subcutaneous, Buprenorphine SR, ZooPharm, Windsor, CO) for 72 h postoperatively for analgesia. As the surgery was performed using aseptic technique, no antibiotic therapy was administered. Rats were prepared for surgery by shaving and cleansing of the surgical site with betadine followed by 70% isopropyl alcohol. Prior to the closed fracture of the femur, a retrograde intramedullary pin was placed into the femur for rigid fixation by making an incision medial to the patella, and then dislocating the patella laterally to expose the

femoral condyles. A 20-gauge needle was used to make a pilot hole between the femoral condyles, and then a 0.45-mm Steinmann pin was introduced into the femoral canal in a retrograde fashion and driven out the greater trochanter. The pin was cut flush at the distal entry point to allow for full range of motion, and then an incision was made over the greater trochanter to expose the proximal end of the pin. The pin was bent to 90° and cut to leave a small handle that was then buried under muscle. The distal and proximal wounds were then closed in layers with suture.

The femoral diaphysis was fractured by means of a blunt guillotine driven by a dropped weight. The anesthetized rats were placed on the fracture apparatus in a supine position with the prepinned femur in abduction and external rotation. A 1620-g weight was dropped from a height of 35 cm onto the guillotine for executing the fracture. After the procedure, the fracture and pin placement were verified by radiography at day 0 with the rat in the prone position with fully abducted femurs. Rats that had a fracture in the wrong configuration or in the wrong anatomical location (i.e. fracture extended through the femur into the tibia, etc.) were excluded from the study. To be included in the study, the fracture needed to be transverse, bicortical, and middle-third diaphysis of the femur (see Fig. 1). A total of 21 rats were used in the study, with a mortality rate of 14.2%. Three rats were excluded because of development of an underlying hematoma at the fracture site.

Rats were randomly divided into 3 study groups: 6 controls (the control is fracture with intramedullary pin, no irradiation), 6 treated with 660 nm NIR daily, and 6 treated with 830 nm NIR daily. All of the study rats were allowed to ambulate on the fractured leg and were monitored daily for signs of pain, including failure to groom, decreased activity level, increased respiratory rate, and anorexia. No abnormalities in activity or signs of systemic illness were observed in any of the study rats. The surgical site in all rats was monitored for signs of infection/inflammation (as indicated by yellow or greenish discharge [pus] or redness, warmth, or swelling of the incision areas) by both the investigative staff and the veterinary staff. All rats had *ad libitum* access to the vivarium's standard rodent chow and reverse osmosis, hyperchlorinated water.

The treatment groups received NIR from a LED-array with a power density of 50 mW/cm² at the skin surface each day for 15 min, for a total daily dose of 45 J/cm². Cumulative dose for the three-week period was 945 J/cm² to the surface of the leg. The light source was a custom-made prototype as described above. The light was directed at the fracture site from a distance of 1 cm through the walls of a Plexiglas restrainer to prevent movement during treatment. Radiographs were taken

Fig. 1 – Day 0 lateral radiographs of femur fracture with intramedullary pin in place; fracture healing score 1.

Fracture Healing Score

Grade 1 - no calcification
 Grade 2 - patchy-calcification
 Grade 3 - calcification takes on the appearance of a callus
 Grade 4 - callus bridging across the fracture gap
 Grade 5 - indicates continuity of bone trabeculae
 Grade 6 - remodeling to normal bone

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Fig. 2 – Radiographic fracture healing score.

at 0, 7, 14, and 21 days postoperatively. Prior to the radiograph, the rats were placed in a transparent induction chamber where they received isoflurane (3–5% induction, 1–3% maintenance) via a precision vaporizer and compressed oxygen. Once the rat was unconscious, it was removed from the chamber and an X-ray was taken of the right femur. On day 21, the rats were euthanized with CO₂ from a compressed gas source. All radiographs were obtained with the same settings on the same X-ray device. A single radiologist read all radiographs. The radiologist was blinded to both the exposure to NIR and to the age of the fracture. Fracture healing was scored on a radiographic healing scale previously described for mouse fractures.²⁵ Fig. 2 describes each score, as the radiologist would classify the radiograph. These scores were assigned to each film taken on days 0, 7, 14, and 21. Statistical analysis of the radiograph scores was performed by two-tailed t tests with significance level set at $p < 0.05$, with application of the Bonferroni correction for multiple comparisons at each time point.

3. Results

3.1. Cell culture

ATP assays performed on cell cultures shortly after irradiation with NIR show increased ATP content, expressed as percent of baseline, within 10 min after irradiation, with decay back to baseline within 45 min (Table 1 and Fig. 3). The conditions of

Fig. 3 – ATP content of cell culture wells for osteoblasts and fibroblasts at various wavelengths and time points. Values given are mean $\pm$ standard error. * Significant difference of value at the indicated time point from control values of same group. + Significant difference of indicated cell type wavelength results from each other at that particular time point. Values for significance were set at $p < 0.05$.

the assay were such that it is not possible to distinguish intra from extracellular ATP. All combinations of cell type and wavelength gave significant increases from baseline at 5 or 10 min, while none did so at 45 min. The results do not support a rise and fall of ATP content within this time span; it is just as likely that there was an immediate increase during irradiation, with a fall off as ATP was consumed or hydrolyzed.

The osteoblasts showed equivalent results whether 660 nm, 830 nm, or a combination of both was used. This was not the case for the fibroblasts. The 830 nm fibroblast results were similar to the osteoblast results, while the 660 nm fibroblast results gave a much larger ATP increase, while the combined wavelengths fell in between. At both the 5 and 10 min time points, the 660 nm and 830 nm fibroblast results showed significant differences from each other, but not from the mixed wavelength results.

As there was some concern that the ATP increases seen might be attributable to heat, rather than the NIR, temperatures within the culture plate wells were monitored during the experiments. The culture medium was verified to be at room

Table 1 – ATP content of cell culture wells at various wavelengths and time points.

	Osteoblast 100% 660 nm	Osteoblast 50% 660 nm 50% 830 nm	Osteoblast 100% 830 nm	Fibroblast 100% 660 nm	Fibroblast 50% 660 nm 50% 830 nm	Fibroblast 100% 830 nm
Control	100 $\pm$ 5.5	100 $\pm$ 5.5	100 $\pm$ 1.3	100 $\pm$ 16	100 $\pm$ 3.3	100 $\pm$ 7.5
5 min	128 $\pm$ 6.6 ^a	111 $\pm$ 1.9	117 $\pm$ 3.7	240 $\pm$ 14 ^{a,b}	187 $\pm$ 8.3 ^a	147 $\pm$ 8.9 ^{a,b}
10 min	129 $\pm$ 4.7 ^a	132 $\pm$ 1.6 ^a	121 $\pm$ 5.5 ^a	254 $\pm$ 12 ^{a,b}	177 $\pm$ 7.1 ^a	140 $\pm$ 6.6 ^b
20 min	116 $\pm$ 4.5	130 $\pm$ 1.3 ^a	119 $\pm$ 3.2	163 $\pm$ 9.9	125 $\pm$ 7.1	116 $\pm$ 10
45 min	100 $\pm$ 2.4	103 $\pm$ 6.4	94 $\pm$ 5.7	95 $\pm$ 4.3	93 $\pm$ 5.5	66 $\pm$ 5.8

Values are expressed as percent of control from same group, and are given as mean $\pm$ standard error. Each value represents an $n = 3$ of independent experiments.

^a Significant difference of value at the indicated time point from control value of same group.

^b Significant difference of indicated cell type wavelength results from each other at that particular time point.

Values for significance were set at $p < 0.05$.

Fig. 4 – Fracture grading results for various healing periods, with or without NIR treatment at 660 nm or 830 nm. Values at each time point for control, 660 nm, and 830 nm groups are 1.5 ± 0.2 , 1.7 ± 0.3 , and 1.7 ± 0.3 at 7 days, 3.0 ± 0.2 , 3.7 ± 0.2 , and 3.0 ± 0.2 at 14 days, respectively. Values at day 0 were uniformly 1. $N = 6$ for each group. Values given are mean $\pm$ standard error. * Indicates significant difference ($p < 0.05$) between NIR group and respective control value.

temperature before irradiation and before ATP assays, and the total temperature rise during irradiation was no more than 1°C (data not shown).

3.2. Animal study

All animals entered in the study completed their full three-week experimental protocol. None of the rats displayed any clinical signs of infection or inflammation around the incision areas. Results of the radiographic scoring are displayed in Fig. 4. At day 0, all 3 groups were scored as 1, indicating the successful execution of the transverse femur fracture. At day 7, there was no significant difference in radiologic fracture scoring between the control, 660 nm, and 830 nm groups. However, at day 14, there was a significant difference seen in the 660 nm group. The 660 nm group average radiologic score was 3.7, significantly greater than the control group average of 3.0 and the 830 nm group average of 3.0. Representative radiographs of a control rat at day 14 and a rat in the 660 nm treatment group at day 14 postoperatively are seen in Figs. 5 and 6, respectively. At 21 days postfracture, there was no

Fig. 6 – Lateral radiograph of 660 nm NIR-treated rat at day 14 postoperatively; fracture healing score 4.

significant difference in radiographic scoring between all 3 groups.

4. Discussion

4.1. Cell culture

Experiments on osteoblast-related cell cultures with NIR have yielded a mixed bag of results till date. With the commonly used wavelength of 830 nm, cell proliferation has been reported for osteoblasts²⁶ and osteoblast-like cells,^{27,28} with a decrease seen for osteoblasts using glass-ceramic scaffolds.²⁹ Osteosarcoma cells showed no change with 830 nm.^{26,30} Osteoblasts and bone marrow stem cells showed proliferation at two other wavelengths as well, 630^{31,32} and 635 nm.³³ Osteosarcoma cells showed increased proliferation at 670 nm and 780 nm.²⁶

Our study was designed to show, rather than cell proliferation, immediate ATP production. While the exact mechanism for NIR-induced therapeutic benefits remains unclear, a common assumption is that an improvement in cellular energy mechanisms may lie at the heart of the question.³⁴ From the experiments reported in this study, however, it appears that any effect, as measured by ATP production, may be transient not persistent. This has always been a perceived flaw in the energy metabolism mechanism for NIR effects. Another possibility, not normally considered in the field of photobiomodulation, is the role of ATP as a

Fig. 5 – Lateral radiographs of a control rat at day 14 postoperatively; fracture healing score 3.

signaling molecule.³⁵ ATP has been seen to promote cellular proliferation in embryonic stem cells,³⁶ osteosarcoma cells,³⁷ smooth muscle cells,^{38,39} and retinal cells.⁴⁰ ATP had effects on cell growth through modulation of the cell cycle in smooth muscle cells⁴¹ and retinal cells.⁴⁰

We propose for consideration a hypothesis that ATP generated by the application of NIR has stimulatory effects on cell proliferation through signaling pathways, possibly affecting the progression through the cell cycle. Numerous studies (*vide supra*) have shown such effects for ATP in cell culture, with two showing effects on the cell cycle itself. Our study reveals an immediate, but transient, increase in ATP content that could account for cellular proliferation through this mechanism. At this point, it remains untested whether this is intracellular ATP or ATP released into the medium, but either, or both, could be operative.⁴²

Although an immediate enhancement of production of ATP by NIR is not usually studied, there is one such report. Oron et al.⁴³ showed an immediate increase (10 min after irradiation) in ATP content using 808 nm in conjunction with human neuronal cells. Again, as in our study, it is not known if this is intracellular or released ATP. A possibly related phenomenon is the pulsed-ultrasound-induced release of ATP into the medium, accompanied by proliferation, by osteoblasts.⁴⁴

4.2. Animal study

Our data showed a significant increase in the fracture healing score of rats treated with 660 nm NIR light at day 14, lending evidence that NIR light may accelerate the fracture healing process as demonstrated by callus formation. By day 21, there was no significant difference in scoring between the control group and treatment groups. This implies that NIR light may initially increase the rate of fracture healing and affect early stages of healing, such as hematoma absorption and bone remodeling. Fractures of this type seem to heal rapidly in rats. In a study⁴⁵ using the same fracture model with radiographic detection of bridging callus, at day 14 in the control group, no bridging was seen, while at day 21, 33% of the animals showed callus bridging the fracture gap. These results are the same as our control group at day 14, and similar at day 21, where we had 50% of the animals with bridging. In this model, early detection of treatment effects is necessary, as differences tend to be obscured at later time points.

These clinical findings are supported by data from Ozawa et al.,²⁷ who showed increased osteoblast proliferation, differentiation, and nodule formation in tissue culture after irradiation with low-level laser therapy when applied at earlier stages. There is benefit to an initial increase in healing rate, such as a theoretically decreased incidence of delayed union, nonunion, and refracture at the injured site. Gerbi et al.⁹ used low-level laser therapy on rat femur defects also and demonstrated an increase in collagen fibers seen on histologic examination at the early stage of bone healing at 15 days *versus* nonirradiated rats. This is consistent with our radiographic data showing increased healing at day 14. A number of other preclinical studies have also shown improvements in bone healing due to NIR within the day 15 window.^{46–51} Combined, these data may indicate that NIR light has a positive effect on the early stages of bone healing.

These results are not only seen in the rat femur with intramedullary pinning as done in our study. Liu et al.⁵² demonstrated increases in fracture callus volume, bone mineral density, and histologic grading in rat tibia fractures with external fixation treated with NIR light at 830 nm *versus* controls. In that study at 4 weeks, postoperative radiographs did not show statistically significant differences in fracture callus thickness between the control and treatment groups. Since most fractures will eventually heal (given favorable circumstances), differences in the rate of healing that are apparent only in the early stages are still beneficial to the clinical progress of any patient.

In this study, treatment with 660 nm NIR seems to have been beneficial in the rat-fracture model, while that with 830 nm was not. Few studies have been reported so far using the 660 nm wavelength. One study⁴⁶ reports an effect of NIR on osteoblast and osteoclast populations and enhanced bone repair in a rabbit tibia model. Another⁴⁷ shows histologically apparent improvements in bone healing at day 15 in a rat femur model. Although we did not see a benefit from use of 830 nm NIR in the animal portion of this study, it has been shown to be beneficial in a number of other studies.^{5,8–10,48–51} It should be noted that none of these studies employed the same animal model, and more importantly, none of these used the same endpoint. As our endpoint was very specifically given as callus formation as measured by radiographic scoring, it is not unfair to suppose that other measures, as employed by other groups, may have shown differences unnoted by our study. It is acknowledged that this is a shortcoming of our rather focused study design.

In our *in vitro* portion of this work, we saw significant differences between these two wavelengths in immediate ATP production using fibroblast cell cultures, but not osteoblast cultures. In addition, the improvement in ATP production using 660 nm with fibroblasts was roughly 80% greater than that using 830 nm, and roughly twice as large as any of the values seen for the osteoblasts. The initial stages of bone healing involve the production of callus, which is made up of fibrous tissue, cartilage, and young immature fibrous bone. If fibroblasts are much more sensitive to 660 nm NIR than 830 nm, it is not unreasonable to predict an improvement in initial callus formation using this wavelength, in a time period where fracture healing is in the fibrous stage, as opposed to 830 nm or nonirradiated controls. Gerbi et al.⁹ did show an increase in collagen fibers, detected histologically, within 15 days from injury. Although the treatment was at 830 nm, we have shown some effect on fibroblast cultures at that wavelength, and histological detection of collagen fibers is likely to be more sensitive than radiographic scoring. Both wavelengths showed some effect on osteoblast cultures, but on a much smaller scale. As our endpoint was not designed to detect differences in later, osteoblast-related, endpoints, any improvements in these measures would necessarily elude us. The *in vivo* differences seen here compared to other studies may also be related to the exposure parameters chosen or differences in light application techniques between our study and others, or may simply be a factor of the limited power of such a small pilot study. It would be unwise at this point to eliminate any particular wavelength from all future consideration.

5. Conclusion

This pilot study lends further evidence of the positive effect of NIR light therapy for fracture healing, as shown with the significant increase in radiographic scoring seen at day 14 postoperatively. In addition, we have demonstrated an immediate effect on *in vitro* cellular ATP content that cannot be attributed to cellular proliferation. A plausible hypothesis suggests that this immediate increase in ATP production may be related to long-term cellular proliferation via an ATP signaling pathway that mediates cell growth through effects on the cell cycle. The effect of NIR on fibroblast cultures was larger with 660 nm than fibroblasts with 830 nm, or osteoblasts at either wavelength, and may explain the improved callus formation at 14 days for 660 nm but not 830 nm. This pilot study is also unique in the fact that, to our knowledge, this is the first study to evaluate NIR light therapy on fracture healing using a femur fracture model in rats with internal fixation via an intramedullary pinning technique. Human orthopedic patients undergo this type of surgical procedure for mid-diaphysis femur fractures, if surgically indicated. An animal model for this type of surgery is valuable for research purposes and for further evaluating the application of NIR therapy clinically. Additional studies with this model with increased sample sizes, increased healing time, evaluation of the healing bone with additional methods such as histology, mechanical testing, bone mineral density, and fracture callus volume would be helpful in further delineating the effects of NIR on fracture healing, but this initial study shows there is a potential clinical application of NIR in fracture treatment.

Conflicts of interest

The authors have none to declare.

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