Photoacoustic laser effects in live mouse blastocysts: pilot safety studies of DNA damage from photoacoustic imaging doses

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Abstract

Objectives: To investigate the laser safety of photoacoustic imaging. In photoacoustic imaging, a pulsed laser of several nanoseconds is used to illuminate biological tissue, and photoacoustic waves generated by optical absorption are used to form images of the tissue. Photoacoustic imaging is emerging in clinical applications; however, its potential use in reproductive medicine has yet to be reported.

Design: Assessment of photoacoustic laser safety before its adoption by clinical reproductive medicine.

Setting: Academic medical center.

Animals: Blastocyst-stage mouse embryos.

Interventions: Potential DNA damage of photoacoustic laser exposure on preimplantation mouse blastocyst stage embryos was examined. Different embryos groups were exposed to either
5- or 10-minute 15-Hz laser doses (typical clinical doses) and 1-minute 1-kHz laser dose (significantly higher dose), respectively.

**Main Outcome Measures:** A terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was used to identify the rate of DNA damage in the laser-exposed blastocysts.

**Results:** The negative control blastocyst group (no laser exposure) had a mean of 10.7 TUNEL-positive nuclei. The 5- and 10-minute 15-Hz laser—exposed groups had a mean of 11.25 and 12.89 TUNEL-positive nuclei, respectively. The embryos exposed to the 1-kHz laser for 1 minute had an average mean of 12.0 TUNEL-positive nuclei.

**Conclusion:** We demonstrated that typical lasers and exposure times used for photoacoustic imaging do not induce increased cell death in mouse blastocysts.

**Keywords**

Photoacoustic imaging; embryo; reproductive organ; safety; cell death; development

The photoacoustic effect, that is, the conversion of light to sound, was first described by Bell in the late 1800s (1). With the advances of lasers, ultrasound transducers, and tomographic reconstruction techniques, photoacoustic imaging (PAI) has become one of the fastest growing fields in biomedical imaging (2, 3). In PAI, nanosecond laser pulses are used as probing energy. The laser induces photon absorption and thermoelastic expansion in the examined tissue, which results in ultrasound waves. Thus, PAI reveals biological tissue optical contrast with ultrasound resolution. Of significance, physiological parameters, such as relative total hemoglobin concentration and oxygen saturation, could be defined with PAI.

The potential use of PAI on reproductive organs such as the ovary and uterus raises a question about the effect of its exposure on embryos. The effects of a photoacoustic laser on preimplantation embryos have not, to our knowledge, been reported. Previous studies have reported that intense lasers can induce alterations in DNA, mitochondria, spindle apparatus, as well as cell metabolism and survival (4–8). Yet, the laser parameters used in those studies differ significantly from the laser parameters used in photoacoustic imaging systems. In addition, responses of cell lines to the laser used in these studies are expected to differ from the responses of embryos. To establish a stage-specific safety threshold of PAI on early embryos, studies need to be done with photoacoustic laser parameters during specific stages of preimplantation embryo development such as the blastocyst stage. To the best of our knowledge, this study is the first to examine laser-induced DNA damage on preimplantation mouse embryos using photoacoustic imaging lasers.

**MATERIALS AND METHODS**

**Mouse Embryo Sources**

Frozen two-cell mouse embryos were purchased from Embryotech Laboratories. Embryos were thawed according to the manufacturer’s protocol and subsequently cultured for 72 hours in vitro in 50-µL drops of Continuous Single Culture-NX complete medium (Fujifilm Irvine Scientific) under light mineral oil (Fujifilm Irvine Scientific). Embryos were group cultured at a maximum density of 10 embryos per drop of medium in ASTEC IVF Cube low
oxygen incubators (ASTEC Co. Ltd.) at 37°C and 6% CO₂, 5% O₂, and 89% N₂. Our average mouse blastocyst formation rate is approximately 89%. The study was exempted by the Institutional Animal Care and Use Committee.

**Laser Exposure**

The embryos were suspended in 30 µL of medium in Eppendorf polymerase reaction (PCR) tubes for laser exposure. The 5-minute 15-Hz and the 10-minute 15-Hz laser doses represent the expected doses in typical human reproductive tissue imaging studies. The 1-minute 1-kHz dose represents a level of exposure that is significantly above that which may be used clinically. None of the control blastocysts were exposed to the laser. Test samples were exposed to the laser at 780 nm for 5 minutes or 10 minutes by delivering pulsed laser light through one 1.5-mm core multimode optical fiber (FP1500ERT, 0.5 NA, Thorlabs) as shown in Figure 1. The light photons penetrate through the Intralipid fat emulsion (Baxter) solution layer inside the three-dimensionally printed water tank and the culture medium in the PCR tubes, and finally reach the embryos on the bottom of the tubes as shown in Figure 1. The 10-mm-thick calibrated Intralipid solution layer with an absorption coefficient (μ_a) of 0.02 cm⁻¹ and reduced scattering coefficient (μ'_s) of 4 cm⁻¹ is used to mimic a soft tissue layer between the probe and the embryos. One plastic film without optical attenuation on the bottom of water tank is sandwiched by the Intralipid solution layer and the culture medium layer with good contact on both sides. The laser used for illumination consists of a Ti:Sapphire (LS-2134, Symptotics TII Corp.) optically pumped with a Q-switched Nd:YAG laser (LS-2122, Symptotics TII Corp.; 10-ns pulse duration, 15-Hz pulse repetition rate) (9, 10). The fiber tip output power is 0.55 mJ, which corresponds to the maximum permissible exposure (MPE) (~28 mJ/cm² at 780 nm) (11) based on simulations and measurements. The fluence level delivered to the Intralipid solution layer surface was experimentally measured as follows: The beam output from the fiber tip was Gaussian shaped, and its diameter at the point where its peak intensity drops to 1/e was measured using a variable iris diaphragm and energy meter that corresponds to approximately 63.2% of the energy from the fiber tip. The beam area used for calculating the fluence was measured to be the iris aperture that allowed 63.2% of the light energy to pass through it when the beam was centered on the iris (12). Fluence equals the total input energy divided by the circular area calculated by the detector radius. In experiments, the required laser input energy reaching surface MPE (~28 mJ/cm² at 780 nm) is 0.55 mJ. We also used another Nd:YAG laser with a much faster repetition rate (DPS-1064-Q, CNI Laser; com; 1064 nm, 1-kHz repetition rate, 7-ns pulse duration). The fiber tip output power is 2 mJ, which corresponds to the MPE (~ 100 mJ/cm² at 1064 nm) (11) based on simulations and measurements.

**Staining Procedure**

After the test groups were exposed to the laser, all groups of embryos were stained for imaging. Embryos were suspended on negatively charged microscope slides within a hydrophobic PAP pen circle. Embryo presence was verified using an Olympus SZX12 microscope (Olympus).
Medium was drawn off the slides, and the embryos were subsequently fixed in a 4% paraformaldehyde (PFA) solution and incubated at room temperature in a humidified chamber for 25 minutes. The slides were stored in a −20°C freezer overnight after fixation.

Slides were warmed and embryos were resuspended in 1x PBS. Embryos were incubated in a 1% Triton solution for 25 minutes at room temperature in a humidified chamber to allow permeabilization of the cellular membranes. One slide was then treated with DNase and incubated for 10 minutes at 37°C in a humidified chamber to generate the positive control slide. The negative control slide was not exposed to the laser, but underwent the same terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining protocol as the other groups.

A TUNEL assay kit was used to identify locations of DNA damage within the blastocysts. The assay solution was prepared according to the manufacturer’s specifications. Slides were incubated with TUNEL reagent in the dark for 1 hour in a 37°C incubator in a humidified chamber. The embryos were also incubated in a 4 μM To-Pro-3-Iodide nuclear staining solution at room temperature for 25 minutes in a humidified chamber.

After all staining procedures, excess liquid was removed, and slides were sealed with a coverslip. Slides were stored at −4°C until imaging.

**Blastocyst Microscopic Imaging**

The mounted blastocysts were imaged by a confocal laserscanning microscope (FV 1000, Olympus). The embryos were first located using the 10X objective, and then the 60X objective was used for imaging. The upper and lower bounds were found by adjusting the focus, and then five slices were taken to ensure that all cells contained within the embryo could be counted. The excitation wavelengths for the TUNEL stain (TMR red) and To-Pro-3-Iodide are 589 nm and 642 nm, respectively, and the lasers available for use had wavelengths of 543 nm and 633 nm. The emission wavelengths of TMR red and To-Pro-3-Iodide are 615 nm and 661 nm, and the emissions were read at 568 nm and 633 nm, respectively.

**Laser Damage Calculation**

The cells with damaged DNA were then counted using the manual cell counter extension of ImageJ software (National Institutes of Health), which allows for moving between slices while maintaining one constant count and leaving tick marks to track previously counted cells. An example of how the cells were counted in the positive control can be seen in Figure 2. The To-Pro-3-iodide stain produced overexposed images, so the total number of cells could not be counted from the nuclear stain.

**Statistical Analysis**

The mean and standard deviation of each of the five treatment groups were calculated based on the number of damaged cells counted using ImageJ. Since some cells had weak nuclear stains, it was difficult to obtain the actual total cells to calculate the percentage of cell death; therefore, we counted total number of damaged cells from each group. A two-tailed,
independent *t*-test was conducted to compare the total number of TUNEL-positive nuclei in embryos that were or were not exposed to the laser. A value of $P < .05$ was considered statistically significant.

**RESULTS**

**Embryo Microscopic Imaging**

The images shown in Figure 3 are examples of all three groups examined using the confocal microscope. Since the positive control embryos were treated with DNase, the cells within each embryo were damaged and were visualized as strong red fluorescence under the microscope. The gain on the TMR red color of the TUNEL stain was left the same for all embryos in all trials, and was used to determine whether embryos incurred any DNA damage. The nuclear stain gain was also left the same among different embryos.

**Quantitative Analysis of Effects of Different Laser Doses**

Once the embryo images were acquired, they were imported into and processed using ImageJ. The ImageJ manual cell counter was used to count the number of cells with DNA damage stained by TUNEL, and those counts were used for the statistical analysis.

The negative control blastocyst group (no laser exposure) ($n = 10$) had $10.7 \pm 6.56$ (mean ± standard deviation) TUNEL-positive nuclei (Figure 4). The 5-minute ($n = 16$) and 10-minute ($n = 9$) 15-Hz laser—exposed groups had $11.25 \pm 5.66$ and $12.89 \pm 4.26$ TUNEL-positive nuclei. The embryos exposed to the 1-kHz laser for 1 minute ($n = 14$) had an average of $12.0 \pm 6.51$ TUNEL-positive nuclei ($P = $ not significant for all comparisons).

**DISCUSSION**

The results provide a preliminary safety assessment of PAI on mouse embryonic cells. We demonstrated that typical laser exposure times used for photoacoustic imaging do not induce increased cell death in mouse blastocysts. Our study targeted blastocysts with a defined set of laser parameters typically used in photoacoustic imaging clinical examinations. For example, in our transvaginal photoacoustic imaging study of ovarian cancer (9), the 15-Hz Q-switched Nd:YAG laser delivered laser energy transvaginally through optical fibers to illuminate ovarian tissue. An ultrasound transducer detected the photoacoustic signals and mapped out lesion relative total hemoglobin concentration and oxygen saturation. These measurements were successfully used to differentiate normal/benign ovarian masses from invasive epithelial cancers. Using this method, the laser fluence was controlled under MPE and the total scanning time to obtain the data was typically about 5 to 10 minutes. The results reported in this article suggest that the laser energy used and the scanning duration of 5 to 10 minutes are safe for a pregnancy during transvaginal PAI evaluation. In a rectal cancer study, the 1-kHz Nd:YAG laser delivered the laser energy endoscopically via an optical fiber to illuminate the rectum. The imaging head, consisting of the fiber and an ultrasound transducer, was used to screen for residual rectal cancer after neoadjuvant treatment. Within a 1-minute period of time, 60 imaging frames was acquired safely.
PAI’s noninvasiveness, ease of integration with an ultrasound imaging system, and the potential of functional imaging have resulted in increasing interest from physicians to incorporate it into clinical practice. So far, human in vivo pilot studies using PAI have been mainly in the areas of oncology (9, 13–21) and inflammatory diseases (22, 23). The largest clinical trial thus far was a 3-year prospective, multicenter study focused on breast cancer diagnosis. The study concluded that combined ultrasound and photoacoustic imaging helps radiologists to assess breast masses with increased specificity compared to gray scale ultrasound imaging alone (19).

To date, the use of PAI in the field of reproductive medicine has not been reported. The ability of PAI to be coupled with an ultrasound system will provide complementary information, such as vascular distribution and blood oxygen saturation, to the detailed imaging of the reproductive organ structures. This additional information may prove beneficial during in vitro fertilization (IVF) cycles. For example, during controlled ovarian stimulation, ultrasound is used to monitor follicular development. Also, during the oocyte retrieval procedure, physicians use ultrasound guidance to locate and to subsequently aspirate ovarian follicles. PAI, if incorporated, offers information beyond the location, size, and number of follicles, such as the degree of follicular vascularization. Studies have shown that follicles that appear to be similar in size and location can be surrounded by a drastically different vascular environment (24). The additional information can be recorded and correlated with the developmental potential of eggs and thus can serve as a potential biomarker of egg quality. Furthermore, PAI could be used to measure the uterine endometrium during IVF cycles, which might better define the optimal window of implantation of both fresh and frozen embryo transfer cycles.

Thus, PAI is potentially a powerful tool that can be useful in clinical reproductive medicine. The instruments could measure oxygen levels, which would provide additional information for clinicians to use to monitor follicular development during ovulation induction cycles, to measure the suitability of the endometrium for implantation during the luteal phase, to measure precancerous or cancerous changes in the endometrium or ovary, and to measure the progress of an early pregnancy.

The use of PAI in clinical settings will result in exposure of reproductive organs to photoacoustic laser rays. Current laser safety guidelines, however, are established based on ocular and skin damage (11, 25, 26). Photoacoustic lasers affect sensitive organs beyond the eyes and skin, as in recent studies involving ovarian tissue (9). To establish robust safety thresholds for PAI, studies using photoacoustic laser parameters on specific biological targets are necessary. A typical photoacoustic laser system consists of pulsed tunable near-infrared wavelength lasers with pulse width (<10 nanoseconds), fluence (20–100 mJ/cm² in the range of 700–1024 nm), and pulse repetition frequency (several Hz to a few kHz) to achieve high-quality real-time images of tissue (27, 28). In safety studies, tissues should be exposed to these laser parameters at time intervals that reflect the duration of laser exposure in clinical settings. Post-laser exposure tissue measurements such as cell death can help researchers and clinicians to establish more informative safety thresholds for PAI. These thresholds are critical for successful translation of PAI into clinical use.
Although the pilot study gives a preliminary safety data for PAI on preimplantation embryos, experiments need to be performed to establish a dose-response curve of laser exposure on the rate of cell death. For example, duration of exposure beyond 10 minutes for the 15-Hz laser needs to be explored with a set of laser parameters typically used in clinical settings. Further experiments are warranted to improve the experimental setup. For example, the embryos were centrifuged onto the tube bottom before laser exposure. The distribution of embryos on the tube bottom, however, are not well defined. Slightly non-uniform embryo distribution will induce slightly unequal laser fluence onto each individual embryo. A safety threshold for duration of exposure could be established for this given set of laser parameters. Long-term embryo development, in addition to cell death following the time of exposure, should be assessed.

In summary, our study reports the first evidence that laser-induced DNA damage to mouse embryos is negligible when the typical lasers used for photoacoustic imaging within a short exposure time (15-Hz laser of 5 and 10 minutes, with 1-kHz laser of 1 minute representing a high dose) are utilized. Further studies are warranted to establish a fuller safety profile for embryonic tissues and reproductive organs for this novel imaging technology.

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FIGURE 1.
Light path from the fiber tip to the embryos. Embryos inside the polymerase chain reaction tube were exposed to the 15-Hz laser at 780 nm for 5 minutes and 10 minutes, and 1-kHz laser at 1064 nm for 1 minute by delivering pulsed laser light through one 1.5-mm optical fiber.
FIGURE 2.
A positive control embryo showing the counting tick marks provided by ImageJ software.
FIGURE 3.
Stained images of (A) positive control (DNase-treated), terminal deoxynucleotidyld transferase dUTP nick end labeling (TUNEL) stain only; (B) positive control, nuclear stain only; (C) positive control, both stains; (D) negative control (no laser exposure), TUNEL stain only; (E) negative control, nuclear stain only; (F) negative control, both stains; (G) 5-minute 15-Hz laser-exposed, TUNEL stain only; (H) 5-minute 15-Hz laser exposed, nuclear stain only; (I) 5-minute 15-Hz laser-exposed, both stains; (J) 10-minute 15-Hz laser-exposed, TUNEL stain only; (K) 10-minute 15-Hz laser-exposed, nuclear stain only; (L) 10-
minute 15-Hz laser-exposed, both stains; (M) 1-kHz laser-exposed, TUNEL stain only; (N) 1-kHz laser-exposed, nuclear stain only; (O) 1-kHz laser-exposed, both stains.
FIGURE 4.
Number of cells per embryo with damaged DNA in all embryo groups. N is the number of embryos in each group.