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The effect of red light irradiation on spermatozoa DNA

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ABSTRACT

A key goal in the conservation of endangered species is to increase successful reproduction. In cases where traditional methods of in vitro fertilization are unsuccessful, new methods of assisted reproduction are needed. One option is selective fertilization via optically trapped sperm. A more passive option is red light irradiation. Red light irradiation has been shown to increase sperm motility, thus increasing fertilizing potential. However, there is some concern that exposure to laser irradiation induces the production of oxidative species in cells, which can be damaging to DNA. In order to test the safety of irradiating sperm, sperm samples were exposed to 633 nm laser light and their DNA were tested for oxidative damage. Using fluorescence microscopy, antibody staining, and ELISA to detect oxidative DNA damage, it was concluded that red light irradiation does not pose a safety risk to sperm DNA. The use of red light on sperm has potential in both animal conservation and human reproduction techniques. This method can also be used in conjunction with optical trapping for viable sperm selection.

Keywords: sperm, oxidative DNA damage, red light irradiation, sperm motility, fertility

1. INTRODUCTION

The extinction of animal species poses a threat to biodiversity. The conservation of endangered species has proved to be a difficult challenge due to the limited availability of resources as well as the complexity of wild animal reproductive systems. Artificial insemination, wherein collected sperm is inserted directly into the female's uterine tract, has limited success in many species due to the lack of understanding of female reproductive cycles[1]. The use of intracytoplasmic sperm injection has shown greater success, but requires direct injection of sperm into the oocyte. In vitro fertilization (IVF) requires incubation of sperm and egg in a dish for fertilization to occur. These artificial reproductive techniques can benefit from increased sperm motility. One method for determining the quality of sperm is to measure curvilinear velocity (VCL), or the sperm's velocity in a curved path. The ability of sperm to escape an optical trap can provide insight to the swimming force of sperm.

1.1 Red light as a therapeutic agent

Red and near-infrared light has long been studied as an aid to wound healing. Low-level laser therapy (LLLT) has been shown to increase cell proliferation and stimulate angiogenesis[2–5]. The mechanism for the therapeutic effects of LLLT is believed to be the absorption of photons by mitochondrial cytochrome c oxidase, increasing ATP production and overall energy supply[6]. In sperm, it is believed that this absorption leads to increased swimming speeds, and thus increased fertilization potential. Recent studies have indicated that red light irradiation of sperm increases motility. Cohen et al found that exposure to 630 nm light improved fertilization rates in mouse sperm[7]. Firestone et al increased the swimming speed of human sperm using 905 nm light[8]. Yeste et al observed increased VCL of boar sperm using red LED light[9]. The effect of red light irradiation on sperm swimming force can also be evaluated using optical tweezer systems. In these experiments, computer-assisted sperm-analysis (CASA) systems were used to evaluate sperm motility, which introduces a delay between stimulation and tracking. This approach assumes that the stimulatory effects continue post-irradiation. In this study, a novel method of sperm tracking in real-time is used to overcome this delay.

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1.2 Possible consequences of red light exposure

Although many studies have indicated the positive effects of red light irradiation on sperm, its effect on sperm DNA has not been well established. Laser light stimulation has been shown to increase reactive oxygen species (ROS) production. Although low levels of ROS act as an important messenger during fertilization[10], higher levels may react with and damage DNA. As a reproductive aid, it is important that red light irradiation does not induce DNA damage. Cohen et al found that red light stimulation increased mitochondrial production of hydrogen peroxide (H_2O_2), a form of ROS, in mouse sperm[7]. Therefore, irradiated sperm may be susceptible to oxidative DNA damage. It has been shown that sperm can form γH2AX, a double strand break marker, in response to H_2O_2[11]. γH2AX can be visualized through antibody staining 1-2 megabases beyond the damage site[12]. This allows for the determination of the source of DNA damage by indicating the site of breaks. Additionally, the DNA base guanine has been known to form 8-oxo-dG in response to ROS. Therefore, detection of 8-oxo-dG in irradiated samples is important for quantifying DNA damage. In this study, these forms of oxidative damage are analyzed to validate the safety of red light irradiation.

2. METHODS

2.1 Sample preparation

Cryogenically frozen human sperm samples were thawed at 37°C in a water bath. Samples were centrifuged for 10 minutes at 208 g. Samples were washed in 1 mL modified human tubal fluid (HTF) (Irvine Scientific) with 5% serum substitute supplement (SSS) (Irvine Scientific). Samples were resuspended in 1 mL HTF with 5% SSS. Samples were kept at 37°C until use.

2.1.2 Red light irradiation

Sperm samples were irradiated in 3.5 cm clear glass-bottom dishes. Sperm were irradiated from above using a 633 nm monochromatic, coherent laser light source (Intense 7404) coupled to a multimode, homogenizing fiber (Medilight FD) at a power density of 5.66 mW/cm^2 for 30 minutes. For oxidative damage assessment, sperm were irradiated at a power density of 31 mW/cm^2 for 30 minutes. The experimental set up is shown in Figure 1. To minimize the effect of temperature variation on sperm motility, all experiments were conducted at a temperature of 37°C. To minimize the effect of wavelengths outside of those studied, experiments were conducted without external lighting.

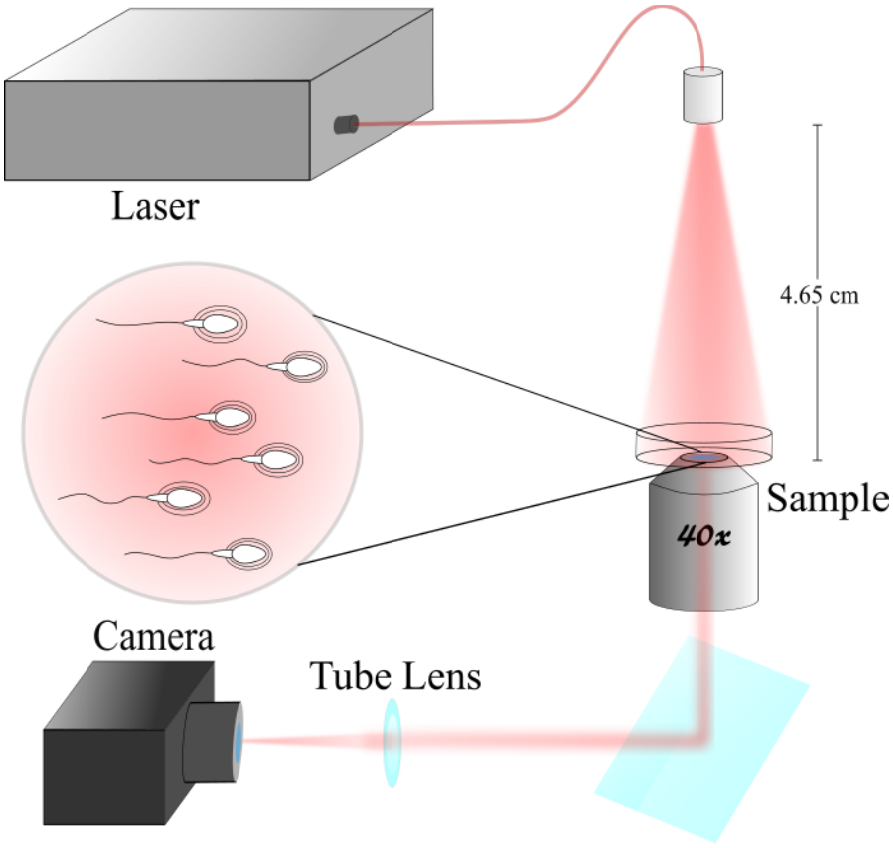

Figure 1. Setup of Microscope System with Red Light as the Illumination Source. 633 nm red light from a coherent laser light source illuminates the sample. The image is focused through an infinity-corrected tube lens. Coherent light is scattered in the presence of particles – both debris and cells – making it difficult to track sperm by shape. The light source can be switched out for imaging.

2.1.2 Exposure to H_2O_2

Double strand breaks were induced in sperm for subsequent antibody staining. 500 µL sperm samples were prepared in Eppendorf tubes. Samples were exposed to 10 mM H_2O_2 (Sigma) for 2 hours.

2.2 Analysis of motility

A wavelet-based tracking algorithm was developed to track sperm swimming speeds under all light sources, including coherent red laser light. Coherent light causes diffraction rings to appear around the tracked particle due to the scattering of light, which may complicate the detection of single particles. The algorithm is implemented in LabVIEW and allows individual sperm to be identified based on their movement, rather than their shape. A video of swimming sperm is inputted into the algorithm and sperm are tracked using their centers of mass. Wavelet analysis is used to sort the frames based on the frequency of sperm movement to separate particles that are likely to be motile sperm from artefacts in the background. The VCL of each sperm is determined using Kalman filtering, where the trajectory of each sperm is predicted and compared with its current position.

The measured VCLs from the algorithm were comparable with those from the IVOS CASA system (with the CASA measurements being 1.59 ± 0.18 times the algorithm measurements). The speeds obtained from the two methods were compared by transferring a 6 µL sample of sperm between the two systems and alternatingly taking measurements for 3-5 runs per system (Table S1). The speeds from the runs were averaged to account for discrepancies in time. These averages were compared over multiple sets to determine the validity of the algorithm.

Samples were imaged using a Zeiss Axiovert S100 2TV microscope with a 40x, NA 1.3 phase III, oil immersion objective and a Cohu 7800 CMOS camera. Speed measurements were taken by splitting thawed sperm samples into two aliquots and treating one with red light, but not the other. The speeds of each sample were measured using the algorithm, alternatingly taking measurements for each sample and averaging the results to account for time delays.

2.3 Assessment of DNA damage

2.3.1 Assessment of γH2AX formation (antibody staining)

Three 50 μL sperm samples – one exposed to red light, one exposed to H_2O_2, and one untreated sample – were dried on poly-L-lysine dishes. The samples were fixed with 4% paraformaldehyde for 1 hour. The samples were then permeabilized with 200 μL blocking buffer for 1 hour. The samples were stained with 1:1000 anti-γH2AX antibody (Millipore) in phosphate-buffered saline (PBS) for 1 hour. The samples were incubated with 1:2000 secondary antibody in PBS for 1 hour. The samples were stained with 1:600 DAPI (Invitrogen) in PBS for 10 minutes for visualization of DNA.

Fluorescent images were taken using a Zeiss microscope with a 63x, NA 1.4 phase III, oil immersion objective and a Hamamatsu ORCA-flash 4.0 CMOS camera. γH2AX formation was assessed by determining mean pixel intensity of the sperm head using ImageJ software. An ANOVA and Student's t-test were used to determine if the means were statistically significant. The number of γH2AX foci in the sperm head was assessed by determining the points of maximum brightness with ImageJ. This indicates the approximate locations of double strand breaks. A Student's t-test was used to determine whether the numbers of foci between the irradiated and untreated samples were significantly different.

2.3.2 Assessment of 8-oxo-dG formation (ELISA)

Oxidative DNA damage was quantified by measuring the levels of 8-oxo-dG using an 8-OHdG ELISA Kit (Cayman Chemical). One mL of untreated and irradiated human sperm samples were prepared. DNA was extracted from the samples with DNAzol reagent (Invitrogen). The ELISA was performed as specified by the kit protocol. Samples were assayed in triplicate and read at a wavelength of 412 nm. Concentrations of oxidatively damaged DNA were determined using a standard curve. The concentrations were compared using an unpaired t-test with Welch's correction to determine whether the means between the samples were statistically significant.

3. RESULTS

3.1 Sperm swimming speed

The swimming speeds of irradiated sperm were compared to those of untreated sperm, as shown in Figure 2. Each irradiated sample showed a 17%-47% increase in speed when compared to their untreated counterpart. The speed increase was observed throughout irradiation.

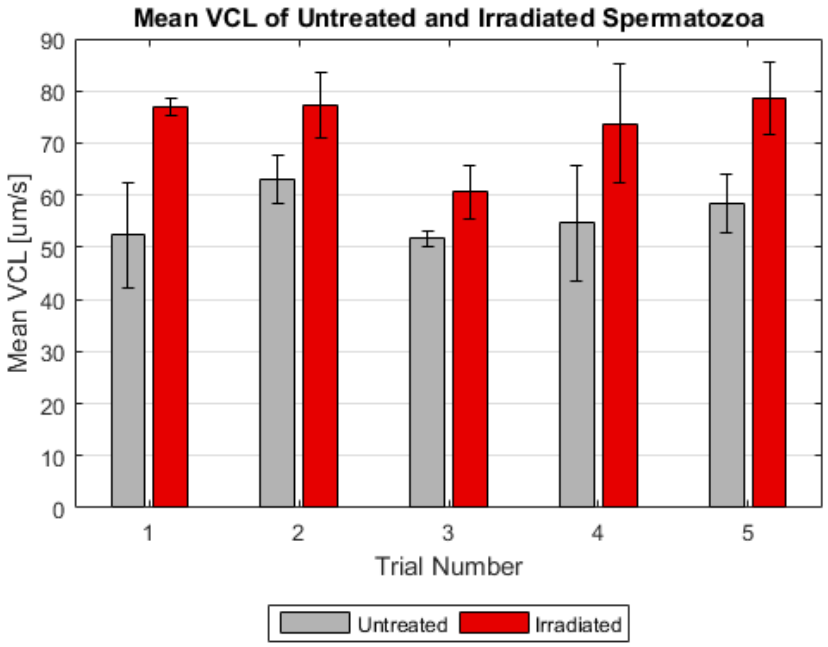

Figure 2. Comparison of Swimming Speeds of Irradiated and Untreated Sperm. Gray bars represent untreated samples while red bars represent sperm irradiated with 633 nm light at 5.66 mW/cm2. Error bars show standard deviations. VCL was determining using the wavelet-based tracking algorithm.

3.2 Oxidative DNA damage

3.2.1 Assessment of γH2AX formation

24 sperm exposed to H_2O_2, 34 sperm exposed to laser light, and 32 untreated sperm were imaged. Images of fluorescently dyed sperm from each sample type are shown in Figure 3. The presence of γH2AX as well as the number of γH2AX foci in the sperm head were quantified. The levels of γH2AX in each sample are shown in Figure 4A. An ANOVA test determined that the H_2O_2 treated sperm showed significantly higher levels of γH2AX than the untreated and irradiated samples ($p < 0.05$). A Student's t-test showed that there was no significant difference in γH2AX formation between the untreated and irradiated samples. The average number of γH2AX foci in the untreated and irradiated samples are shown in Figure 4B. A Student's t-test determined that there was no significant difference between the number of foci in the untreated and irradiated samples ($p < 0.05$).

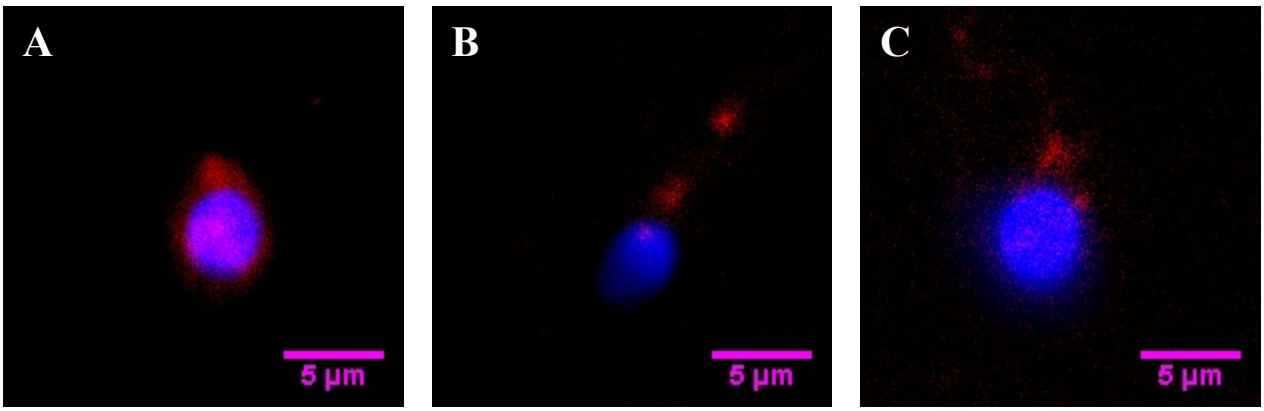

Figure 3. Visualization of γH2AX Localization. DAPI staining is shown in blue, indicating nuclear DNA in the sperm head, while anti-γH2AX staining is shown in red. **(A) H_2O_2 Treated Sperm.** The overlay of red with blue at the sperm head indicates a high level of double strand breaks in response to H_2O_2. **(B) Untreated Sperm.** γH2AX is concentrated in the sperm midpiece and tail. **(C) Irradiated Sperm.** γH2AX is shown in the sperm midpiece and tail rather than in the sperm head.

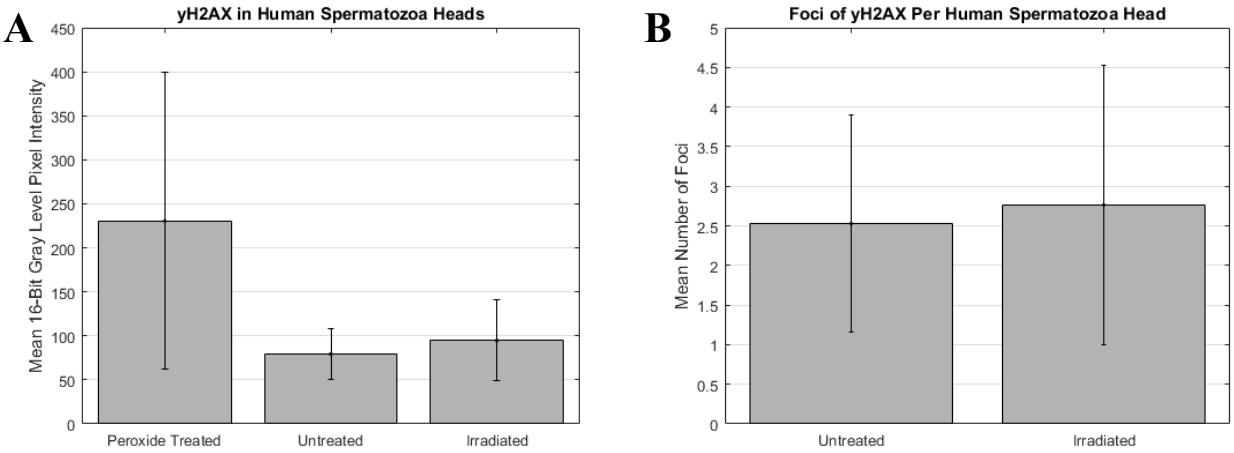

Figure 4. γH2AX Formation in Spermatozoa. (A) γH2AX in Human Spermatozoa Heads. The formation of γH2AX was determined using anti-γH2AX antibody and measuring the fluorescence in the sperm head. Higher pixel intensities represent higher levels of γH2AX and thus DNA damage. Error bars represent standard deviation. **(B) γH2AX Foci in Human Spermatozoa Heads.** The number of γH2AX foci was determined by counting the number of bright points in fluorescent images of anti-γH2AX stained sperm heads. Error bars represent standard deviation.

3.2.2 Assessment of 8-oxo-dG formation

Untreated and irradiated samples showed insignificant differences in 8-oxo-dG concentration, as shown in Figure 5. Samples were assayed at concentrations of $1.31*10^7$ pg/mL. The untreated sample contained 425.4 pg/mL of 8-oxo-dG while the irradiated sample contained 470.9 pg/mL of 8-oxo-dG.

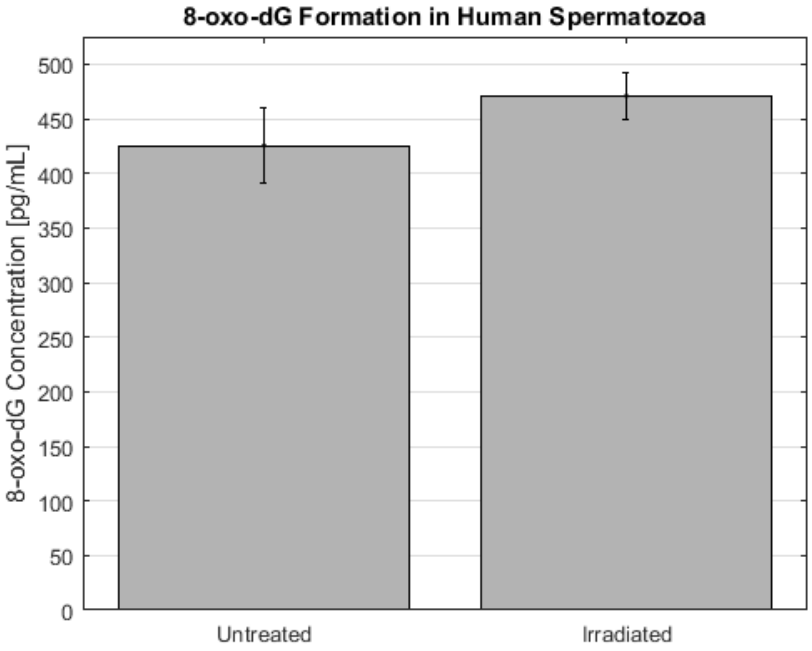

Figure 5. Concentrations of 8-oxo-dG in Untreated and Irradiated Samples. Concentrations were determined by comparing against a standard curve prepared using samples of known 8-oxo-dG concentration. The error bars represent standard error of the mean.

4. DISCUSSION

This study indicated that 633 nm irradiation of spermatozoa does not induce oxidative DNA damage and increases sperm motility. This validates the efficacy and safety of red light irradiation as a potential treatment for infertility. Although sperm were irradiated at different power densities for motility tests and DNA tests, the higher power (31 mW/cm^2) showed insignificant levels of DNA damage while the lower power (5.33 mW/cm^2) indicated increased motility. It can be inferred that DNA damage would not occur at powers $\leq$ 31 mW/cm^2. It should be noted that sperm of different species have differently shaped and sized heads, which may alter the effectiveness of red light towards increasing motility.

The developed wavelet-based tracking algorithm proved to be a viable alternative to conventional CASA systems, especially when tracking under a particular light source is necessary. Algorithms that track particles based on shape are prone to failure in cases where particle shape varies greatly or in cases where the true shape of the object is obscured by diffraction rings. The wavelet-based algorithm was used to determine the immediate effect of red light irradiation on sperm, whereas with a CASA system, there is necessarily some delay between irradiation of the sample and motility tracking.

Further studies are currently being conducted to assess the effect of red light on sperm swimming forces. Though sperm velocity has been shown to change with red light stimulation, it is unclear whether swimming force is affected in the same way. Individual sperm were trapped using a 1064 continuous-wave IR laser at a power of 500 mW before the objective. Using the microscope set up illustrated above (Figure 1), the trapped sperm were alternatingly illuminated under phase contrast and red light in 30 second intervals. The x and y positions as the sperm moved in the trap were recorded using LabVIEW and their motion characteristics were then analyzed in MATLAB. From the position data, the power spectral densities for the trapped sperm were determined (Figure 6).

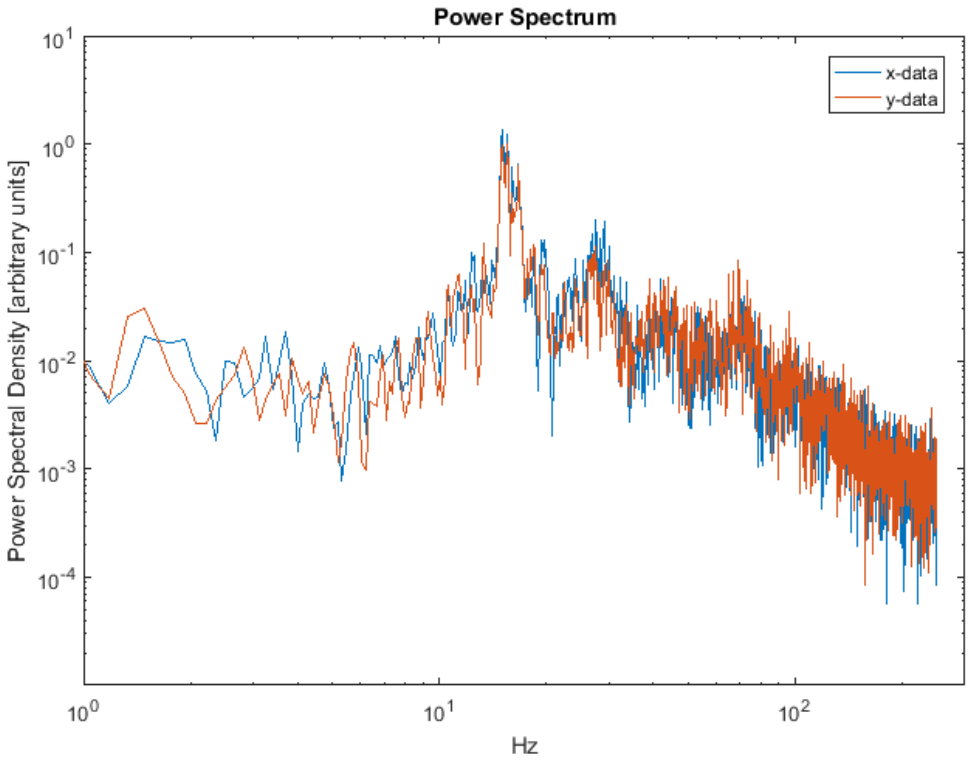

Figure 6. Power Spectra for Trapped Sperm. This data shows that it is possible to determine the power spectra for trapped sperm using x and y position data. Peaks in the power spectra represent the frequency of sperm tail movement.

Power spectra show the distribution of frequencies in a moving particle. The low frequency information is representative of Brownian motion while the higher frequency data at the low end of the power spectrum represents noise. For trapped sperm, the peaks in the spectrum occur at the frequency of sperm tail movement (beat cross frequency), since the trap eliminates all other sources of motion. Understanding the effect of red light on the frequency of sperm tail movement can provide insight to the mechanisms of sperm motility. Though clear differences can be seen between the phase contrast and red light data, it is currently not possible determine a relationship between red light illumination and force; future studies will be conducted to more definitively determine the effect of red light on sperm swimming frequency and force.

Red light irradiation does not cause oxidative DNA damage in spermatozoa, as measured by staining for γH2AX as well as by testing for 8-oxo-dG. DNA damage in sperm has been linked to miscarriage and problems with fertility[13]. Previous studies have tested for DNA damage, but the focus on oxidative damage is important when considering the possibility of excess mitochondrial ROS production as a result of laser stimulation. Firestone et al tested for DNA fragmentation using acridine orange and was also unable to find significant levels of DNA damage[8]. While this method is widely used to determine sperm chromatin quality, it is unable to determine the cause of DNA damage. Assessing DNA damage by testing for γH2AX can indicate the source of damage by producing a visualization of double strand break locations in intact spermatozoa. In H_2O_2 treated sperm, it is shown that there are double strand breaks in nuclear DNA (Figure 3A). In contrast, untreated and irradiated sperm show γH2AX localization mostly on the sperm midpiece and very little in the sperm head (Figures 3B and 3C). It may be inferred that the presence of γH2AX at the sperm midpiece is a result of damage to mitochondrial DNA, but mitochondrial DNA lacks the H2AX histone needed for γH2AX formation. Thus, it is likely that γH2AX detection outside of the sperm head is a result of nonspecific binding.

Both antibody staining for double strand breaks and quantification of oxidatively damaged guanine showed the presence of some DNA damage in the untreated and irradiated samples (Figures 4 and 5). Because the difference in the levels of DNA damage between the two samples is statistically insignificant in all cases, it can be assumed that the cause of this damage is artefactual in these samples. DNA damage may be the result of cryopreservation, centrifugation, or regular

mitochondrial ROS production. Because the irradiated sample did not have significantly higher levels of oxidative DNA damage than the untreated sample, it can be concluded that 633 nm laser light irradiation at a power density of 31 mW/cm^2 does not induce significant levels of DNA damage. However, further studies should be conducted to determine the effect of red light stimulation on the oocyte.

5. CONCLUSION

Red light irradiation has been used to increase sperm swimming speed, but it is not fully understood whether it damages DNA. 633 nm laser irradiation of spermatozoa shows promising results as a fertility treatment. Red light irradiation is able to increase sperm swimming speeds without compromising DNA integrity, as measured by antibody staining for γH2AX and by 8-OHdG ELISA. A wavelet-based tracking algorithm can be used to measure sperm swimming speeds during irradiation, as opposed to after irradiation when using a CASA system. This method provides an alternative to artificial insemination in animals where knowledge of reproductive biology is lacking. This technique can also be combined with traditional IVF to increase the probability that the sperm will fuse with the egg. This method can be applied to both conservation of animal species as well as in human fertility clinics.

6. SUPPLEMENTAL FIGURES

Trial	CASA [μm/s]	Algorithm [μm/s]
1-1	80.18	48.33
1-2	87.33	48.705
1-3	89.28	39.09
1-4	83.44	43.53
1-5	74.12000	57.4878
2-1	100.75710	59.92406
2-2	76.40000	52.04287
2-3	80.73300	70.80056
3-1	90.93330	63.68310
3-2	75.08000	51.72906
3-3	77.60000	43.07923
3-4	77.00000	52.65801
4-1	108.78000	50.88964
4-2	80.17500	47.11488
4-3	75.56000	54.64050
4-4	77.11250	43.61597

Table S1. Comparison of Speed Measurements from CASA and Wavelet-Based Algorithm. Each "Trial" is numbered "set-run" where each "set" contained the same sample of sperm and each "run" represents the number of times that set had been measured using each system.

7. ACKNOWLEDGEMENTS

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