



OPEN The power of 810 nm near-infrared photobiomodulation therapy for human asthenozoospermia

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Sperm motility is a crucial factor in male fertility. Photobiomodulation (PBM) has been reported to increase sperm motility, but a consistent approach suitable for identifying standardizable protocols is lacking. We collected asthenozoospermic ($n = 70$) and normozoospermic ($n = 20$) semen. The asthenozoospermic samples were irradiated with an 810 nm diode laser, in continuous wave mode, at 0.25 W, 0.5 W, 1 W and 2 W for 60 s on a circular area of 1 cm² through a novel handpiece with an innovative flat-top profile. Sperm motility was assessed immediately, after 30 and 60 min. A sample size calculator, unpaired t-test and one-way ANOVA with post-hoc Tukey HSD tests were used for statistics. One and 2 W were the most effective outputs in increasing progressive motility compared to control ($p < 0.001$). The maximum effect was immediately after 1 W-PBM ($p < 0.001$) and decreased after 60 min ($p < 0.001$). Time physiologically decreased vitality ($p < 0.001$), but less in the 1 W-PBM samples ($p < 0.05$). 1 W-PBM did not affect chromatin condensation. Asthenozoospermic samples displayed an impairment of 80% in oxygen consumption and ATP production and a slight inefficiency of oxidative phosphorylation compared to normozoospermic samples ($p < 0.001$). 1 W-PBM partially restored the functionality of aerobic metabolism ($p < 0.001$) by recovery of oxidative phosphorylation efficiency. PBM did not affect lactate dehydrogenase (glycolysis pathway). No irradiated samples increased accumulated malondialdehyde, a marker of lipidic peroxidation. In conclusion, PBM improves progressive motility in asthenozoospermia through increased mitochondrial energetic metabolism without harmful oxidative stress.

Keywords Low level laser therapy, Asthenozoospermia, Sperm motility, Mitochondrial metabolism, DNA fragmentation, Sperm vitality, Male fertility

Infertility is a global public health problem that affects about 10% of couples. Approximately 50% of cases involve a male factor alone (30%) or combined with a female factor (20%)¹. The prevalence of male infertility is steadily increasing worldwide as semen quality and other markers of male reproductive health have declined considerably over the past decade². Sperm motility is a crucial parameter in male fertility, and it is affected by energy consumption³.

Mature spermatozoa consist of a head with a nucleus, a neck, and a flagellum that allows motility. It comprises three parts; mitochondria are present only in the midpiece⁴. They are tightly interconnected and helically wound around the axoneme, forming the mitochondrial sheath. In humans, 10–12 mitochondrial cores are formed by 72–80 oblong mitochondria⁵. The primary function of mitochondria is to produce energy. Mammalian sperm uses ATP for motility, capacitation, hyperactivation, acrosomal reaction, and oocyte penetration. However, there is still debate about the metabolic pathways responsible for energy production in spermatozoa^{4,6}. Indeed, oxidative phosphorylation (OxPhos) and anaerobic glycolysis are not mutually exclusive, and sperms can use both pathways. Makai and Travis⁷ have suggested that ATP production in the mitochondrial membrane may not be sufficient for diffusion throughout the flagellum. It has been postulated that mitochondria produce ATP through OxPhos in the midpiece and that anaerobic glycolysis provides ATP in the midpiece. Piomboni et al.⁸

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reviewed the literature on mitochondrial energy production and its role in human sperm motility and found a link between structural abnormalities and impaired sperm mitochondrial function in asthenozoospermia.

The possibility of energizing mitochondria through the irradiation of visible or near-infrared light has attracted increasing interest over the last 40 years, starting with the first evidence suggested by Karu et al.^{9,10} and later by Passarella et al.¹¹ with a wavelength of about 630 nm. Recently, the possibility of stimulating photoacceptors in mitochondrial complexes with different wavelengths in the near-infrared has been characterized. All the research showed increased ATP production through the photo-energization of cytochromes. In particular, a diode laser light of 810–980 nm mainly modulated complex IV and, to a lesser extent, III, while 1064 nm stimulated complexes I, III, and IV^{12–16}. Medical and veterinary use of this phototherapy is known as photobiomodulation (PBM), formerly referred to as low-level laser therapy. It is used to support recovery from various diseases often associated with mitochondrial dysfunction^{17–21}.

Reports on animals suggest that PBM may improve spermatogenesis^{22–24} or increase sperm motility^{25–31} in fish, mice, dogs, boar, ram, and cattle models.

Several studies conducted *in vitro* on human spermatozoa highlighted the potential benefits of PBM on sperm function^{20,32–35}. Almost all studies have examined the positive impact of PBM therapy on the increase in the motility of human sperm from healthy subjects^{36–42}. Some studies included sperm from oligozoospermic and asthenozoospermic patients and showed that irradiation significantly increased sperm motility also in abnormal sperm^{37,39,42–44}. In two studies, PBM therapy before cryopreservation, even in normozoospermic semen samples, played a protective role against the detrimental effects of cryopreservation by preserving the functional parameters of spermatozoa^{45,46}.

Overall, as often happens in the field of PBM therapy, standardization of application protocols remains a challenge due to the heterogeneity of population characteristics and sample size, the type of intervention, and the different LED/laser light parameters (in terms of wavelength, output power, distance, energy density, and other characteristics of the radiation probes), duration of exposure and manifestation of effects. In addition, the biological mechanisms of PBM on sperm are not fully known, and limited safety assessments are available, sometimes with conflicting data. For instance, Lenzi et al.³⁶ have shown that an increase in progressive sperm motility after laser irradiation (647 nm) was associated with a faster rate of sperm ATP consumption, suggesting that laser irradiation may have an “energy modulation effect” on normal sperm. However, later, it was observed that near-infrared radiation resulted in a loss of cell viability, increased lipid peroxidation leading to diminished membrane function, and higher susceptibility of sperm DNA to fragmentation, resulting in induction of apoptosis⁴⁷.

Our work investigated how PBM with 810 nm wavelength irradiated at 0.25 W, 0.5 W, 1 W and 2 W for 60 s on a circular area of 1 cm² impacts the sperm of men with asthenozoospermia. The selected parameters were delivered using a new handpiece with a flat irradiation profile (FT-HP). In a previous work, we have described and characterised this handpiece as having improved performance compared to conventional fibres or standard Gaussian profiled handpieces^{48,49}. To validate an optimised protocol, we first identified which of the chosen irradiation parameters could maximise the performance of asthenospermic spermatozoa. Then, we analyzed the impact of PBM on sperm motility and its duration after irradiation. The effectiveness of photobiomodulation on sperm energy metabolism was tested by monitoring OxPhos and lactate dehydrogenase (LDH), which is involved in the glycolytic energy pathway. Photobiomodulation safety was assessed by analyzing sperm vitality, DNA fragmentation, and lipidic peroxidation.

Materials and methods

Technical characteristics of the equipment used and the irradiation setup

The irradiations were done with the ENEA GaAl-As diode laser model (Garda Laser S.A.S., Verona, Italy). The device allowed irradiating at a wavelength of 810 nm ± 2 for 60 s in continuous wave mode. The irradiation power was set at 0.25, 0.5, 1.0, or 2.0 W to generate an energy of 15.0, 30.0, 60.0, or 120.0 J (power density of 0.25, 0.5, 1.0, or 2.0 W/cm²; fluence of 15.0, 30.0, 60.0–120.0 J/cm²). The control samples' power was set to 0.0 W. Irradiations were delivered using a novel FT-HP, which can improve irradiation performance compared to conventional fibres and standard Gaussian profiled handpieces. Indeed, our previous characterization of the FT-HP showed that it can provide a uniform and consistent energy distribution over a spot area of 1 cm², which is independent by distance^{48,49}. A 635 nm red light pointer (negligible power, <0.5 mW) was used in both treatments to visualize the exposed area and maintain experimental blinding. A Pronto-250 power meter (Gentec Electro-Optics, Inc. G2E Quebec City, Canada) was used to ensure the accuracy of the irradiated laser parameters. The irradiations were carried out with the handpiece fixed to a stand and in contact mode with the surface of the multiwells plate (diameter of a single well approximately 1.1 cm). All irradiations were performed with the multiwells plate placed on a Metal Velvet light-absorbing plate (Acktar Ltd., 8643 Kiryat-Gat, Israel). The extremely low reflectance of the plates, which is guaranteed to be equal to or less than 1% in the near-infrared band, significantly reduces reflections from the top of the bench. Any undesirable thermal effects were avoided by monitoring the exposure with a FLIR ONE Pro-iOS thermal camera (FLIR Systems, Inc., Portland, OR, USA) (dynamic range: -20 °C/+400 °C; resolution 0.1 °C).

Study population and study design

We collected semen samples from donors undergoing semen analysis for fertility diagnosis at the SS Physiopathology of Human Reproduction, IRCCS Ospedale Policlinico San Martino, Genova, Italy, from December 2023 to May 2024. The study complied with the Declaration of Helsinki⁵⁰ and was approved by the Ethics Committee of Regione Liguria (219/2024 - DB id 13852). Written informed consent was obtained from all donors, and participation was voluntary. For the basic examination of semen samples, the following parameters were checked based on WHO criteria⁵¹: semen volume, sperm count per milliliter, sperm motility,

and percentage of sperm with normal morphology. Inclusion criteria: normozoospermia for control samples with $\geq 30\%$ progressive motility, according to the WHO criteria. Based on the same criteria, asthenozoospermia for irradiated samples was defined as $< 42\%$ total motility, $< 30\%$ progressive motility. Exclusion criteria: samples with < 1.5 ml semen volume, semen with sperm agglutinations, round cells, bacterial contamination, severe oligospermia (< 10 million sperms per ml, < 35 million sperms per ejaculate), genetic syndrome. Sperm morphology was not included among the inclusion/exclusion criteria since we focused on the effects of laser irradiation on sperm motility.

PBM therapy for sperm was carried out in native ejaculates. Each asthenozoospermic sample was divided into 5 equal aliquots (200 μ l each). Four out of 5 were irradiated at different powers (0.25 W, 0.5 W, 1 W, 2 W), respectively. One aliquot served as a control and received laser treatment with 0.0 W. A second operator trained in laser therapy then treated the samples. Sperm motility was assessed in a blinded manner immediately after irradiation (T0), after 30 (T30) and 60 min (T60) by an experienced operator. Sperm chromatin dispersion and membrane integrity were assessed blindly in untreated and 1 W irradiated asthenozoospermic samples after 60 min. Energetic metabolism, oxidative stress, and oxidative damage were analyzed in normozoospermic samples as a reference for analyzing metabolism and control and 1 W irradiated asthenozoospermic samples. Figure 1 summarizes the experimental setup.

Semen collection

All individuals collected semen by masturbation into a sterile container after 2 to 5 days of abstinence. Sperm samples were liquefied at room temperature for 30–60 min.

Sperm count and sperm motility

Sperm count and motility were assessed using a disposable counting chamber (AB Cell-VU, AB Analytica, Padova, Italy). It consists of a standard specially-designed glass slide and a coverslip with the counting grid laser-etched into its surface. The 1×1 mm grid is divided into 100 squares, each one of 0.1×0.1 mm, and the chamber has a depth of 20 μ m. This depth is optimal for sperm cells to form in a monolayer, so motility can be assessed and counts are made easily. Briefly, after mixing the undiluted sample thoroughly, one drop (approximately 5 μ l) of semen was placed at the sampling area of the slide and the coverslip was gently lower over the specimen, avoiding air bubbles on the counting area. Sperm concentration was determined by counting all motile and non-motile sperm within a row of 10 squares, that is one millionth of ml. Therefore, the number of sperm heads in 10 squares indicates their concentration in millions/ml.

The number of motile sperms within 10 squares of the grid was divided by the total sperm (motile + non-motile). The multiplication of this number by 100 established the percentage of motile sperm.

The sperm score for motility evaluation was expressed as follows: (a) rapid progressive motility refers to sperm that are swimming fast in a mostly straight line; (b) slow progressive motility refers to sperm that are swimming slowly in large circles; (c) non-progressive motility refers to sperm that swim in situ; (d) immobility refers to non-motile sperm. The progressive motility rate was calculated as the percentage of a + b.

Sperm vitality

To check for the safety of the sperm-PBM treatment, sperm vitality was estimated 60 min after irradiation by assessing the membrane integrity of the cells by dye exclusion (dead cells have damaged plasma membranes that allow entry of membrane-impermeant stains). Briefly, a 50- μ l aliquot of semen was mixed with an equal volume of eosin (Life test, AB Analytica, Italy); after 30 s, it was smeared on a glass slide and immediately examined under an Eclipse Si microscope (Nikon Europe B.V., Amstelveen, The Netherlands) at 20X magnification. Spermatozoa with red or dark pink heads were considered dead, whereas spermatozoa with white heads were considered alive. A minimum of 200 sperms per sample was counted, and the percentage of live sperms was calculated.

Sperm DNA fragmentation

For further confirmation of the safety of PBM treatment, measurement of sperm DNA fragmentation was performed 60 min after irradiation. To provide results as repeatable and consistent as possible we used a commercial kit (Halosperm G2 kit, Halotech DNA SL, Madrid, Spain) that is based on the Sperm Chromatin Dispersion (SCD) technique. Fresh sperms are immersed in an inert agarose microgel on a pretreated slide. An initial acid treatment denatures DNA in those sperms with fragmented DNA. Following this, a controlled DNA denaturation process facilitates the subsequent removal of most nuclear proteins in each spermatozoon. In this way, normal spermatozoa create halos formed by loops of DNA at the head of the sperm, which are not present in those with damaged DNA. When no massive DNA breakage is present, nucleoids from sperm with fragmented DNA either do not show a dispersion halo or the halo is minimal. Spermatozoa without DNA fragmentation show a dispersion halo and spermatozoa with fragmented DNA do not show a dispersion halo, or the halo is minimal. A minimum of 300 sperms per sample were counted using conventional microscopy (Eclipse Si, Nikon) at 20X magnification, and the percentage of sperms with fragmented DNA was calculated. Values of DNA fragmentation above 30% were considered pathological.

OxPhos function evaluation

The OxPhos function, in terms of ATP synthesis and oxygen consumption rate (OCR), was assessed in 50 μ g of total protein of normozoospermic samples, asthenozoospermic samples, PBM-treated asthenozoospermic samples immediately or after 30 min from the laser administration permeabilized with 0.01% digitonin and resuspended in phosphate-buffered saline (PBS).

ATP synthesis was measured by a GloMax 20/20 Luminometer (Promega, Milan, Italy) at 30-second intervals over 2 min using the luciferin/luciferase methods. OCR was measured using an amperometric electrode

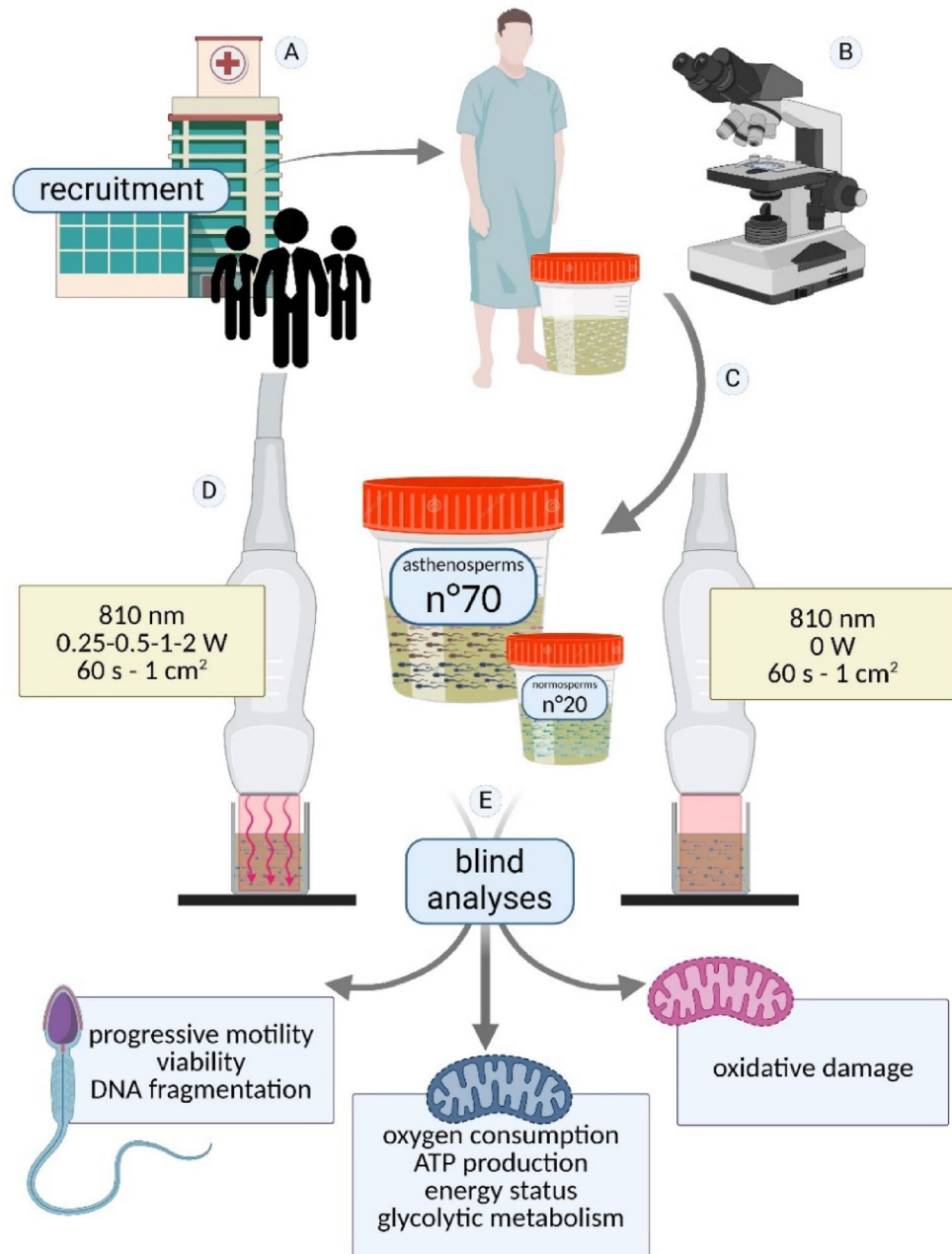


Fig. 1. Study design. The patients were recruited at the SS Physiopathology of Human Reproduction, IRCCS Ospedale Policlinico San Martino, Genova, Italy (A). Semen samples were analyzed (B), and according to the inclusion and exclusion criteria, 70 asthenozoospermic semen and 20 normozoospermic semen samples were included in our study (C). Asthenozoospermic samples were divided into aliquots and irradiated with 810 nm photobiomodulation therapies (D). Samples irradiated with laser switch-off were considered controls. To assess the beneficial or detrimental effects of the treatment, the asthenozoospermic samples were then analyzed in a blinded fashion (E). The normozoospermic samples' energy metabolism and oxidative stress were also examined and considered (E).

(Unisense Microrespiration, Unisense A/S, Aarhus, Denmark) in a sealed chamber. For both assays, samples were resuspended in PBS, and 10 mM pyruvate and 5 mM malate were used as respiring substrates and 0.1 mM ADP was added just before the start of the evaluations⁵².

To assess the OxPhos efficiency the P/O value was calculated as the ratio between the produced ATP and the consumed oxygen. Values around 2.5 indicate a whole coupling between energy synthesis and respiration; lower values indicate an uncoupling status^{53,54}.

Energy status evaluation

The energy status was evaluated as the ratio between ATP and AMP intracellular concentrations. ATP and AMP intracellular concentrations were quantified using the enzyme coupling method, employing 50 µg of total protein for each assay. ATP measurement involved monitoring NADP reduction at 340 nm. The assay medium contained 50 mM Tris-HCl pH 8.0, 1 mM NADP, 10 mM MgCl₂, and 5 mM glucose. Samples were analyzed spectrophotometrically before and after the addition of 4 µg of purified hexokinase plus glucose 6-phosphate dehydrogenase⁵⁵. AMP level was evaluated following the NADH oxidation at 340 nm, using an assay solution composed by 100 mM Tris-HCl pH 8.0, 75 mM KCl, 5 mM MgCl₂, 0.2 mM ATP, 0.5 mM phosphoenolpyruvate, 0.2 mM NADH, 10 IU adenylate kinase, 25 IU pyruvate kinase, and 15 IU of lactate dehydrogenase⁵⁵.

Lactate dehydrogenase activity assay

Lactate dehydrogenase (LDH; EC 1.1.1.27) activity was assessed using 20 µg of total protein by tracking NADH oxidation at 340 nm with a double-beam spectrophotometer (UNICAM UV2, Analytical S.n.c. Langhirano, Italy). The reaction mixture consisted of 100 mM Tris-HCl pH 7.4, 0.2 mM NADH, and 5 mM pyruvate⁵⁶. The assay started with the sample addition and was monitored for at least five minutes every 1 min.

Malondialdehyde intracellular concentration assay

Malondialdehyde (MDA) concentration was determined as a marker of lipid peroxidation to evaluate oxidative damage using the thiobarbituric acid reactive substances (TBARS) assay. The TBARS solution contains 15% trichloroacetic acid in 0.25 N HCl and 26 mM thiobarbituric acid. To evaluate the basal concentration of MDA, 600 µl of TBARS solution was added to 50 µg of total protein dissolved in 300 µl of milliQ water. The mix was incubated for 40 min at 95 °C. Then the sample was centrifuged at 14,000 rpm for 2 min and the supernatant was analyzed spectrophotometrically at 532 nm⁵⁶.

Statistics

According to Rosner⁵⁷, the sample size for the PBM therapy screening was calculated using the sample size calculator available at ClinCalc.com. Based on the preliminary data and considering a beta of 0.2, an alpha significance level of 0.05, and a power of 0.8 for the initial screening trials with the 0.25, 0.5, 1, and 2 W powers, a minimum of 18 patients was considered necessary to detect a clinically significant difference. One-way analysis of variance (ANOVA) was used to analyze the data. Tukey's multiple comparison test was then performed. The software used was Prism 8 (GraphPad software, Boston (MA), USA). The comparison between the means of two independent samples was performed using an unpaired t-test. The data are presented as the mean ± standard deviation (SD). The statistical significance was determined at a probability level of $p < 0.05$.

Results

Patient and baseline characteristics

Progressive motility screening (0.25-0.5-1-2 W) was performed in 20 asthenozoospermic patients according to the results of the sample size calculation. Afterwards, the study was conducted on a total of 70 asthenozoospermic patients using 810 nm PBM therapy at 1 W power. In the energy metabolism and oxidative stress studies, semen from 20 normozoospermic patients was used as a baseline for comparison. Overall, the cohort consisted of 100% Caucasian males, aged 34.1 ± 8.8 years for the asthenozoospermic patients and 35.1 ± 9.4 years for the normozoospermic patients, with no significant difference between groups. In May 2024, patient recruitment and sample analysis were completed.

Effect of different powers of the laser on sperm motility

All treated samples exhibited impaired sperm motility, with less than 30% initial progressive motility according to WHO criteria⁵¹. Impaired non-irradiated samples were used for comparison. Figure 2 reports the effect of an 810 nm laser on asthenozoospermic sperm progressive motility immediately after irradiation at different powers (0.25-0.5-1-2 W). The results show that only 1 W and 2 W significantly ($p < 0.01$) increased progressive motility relative to the control, inducing an increment of 50%.

PBM effects on sperm motility

Based on the above screening protocol results, 1 W and 2 W have been identified as the laser power capable of maximizing sperm motility parameters. Further experiments were performed at 1 W, which we previously demonstrated to be effective and safe in an urchin sea in vitro model⁵⁸. PBM improved progressive motility according to the post-exposure time: the maximum effect with respect to control was obtained immediately after irradiation (T0) (29% versus 36%, $p < 0.001$) and kept high within 30 min (T30) (29% versus 35%, $p < 0.01$) with a decrease after 60 min (T60) (27% versus 31%, $p > 0.05$) (Fig. 3A).

Compared to controls, the immotile fraction of sperms significantly reduced with an average percentage decrease of -11% at T0 and T30 ($p < 0.05$) and -9% at T60 ($p > 0.05$) (Fig. 3B).

PBM does not affect sperm membrane integrity

Figure 4A shows the presence of viable and nonviable sperm samples from 25 asthenozoospermic patients after eosin staining. Figure 4B shows the viability test results on the samples at the baseline and controls and the irradiated samples at 60 minutes after irradiation. Time physiologically decreased vitality with respect to the baseline (control T0) ($p < 0.001$). However, the process is less evident in the PBM samples (laser T60) than in the control T60 ($p < 0.05$).

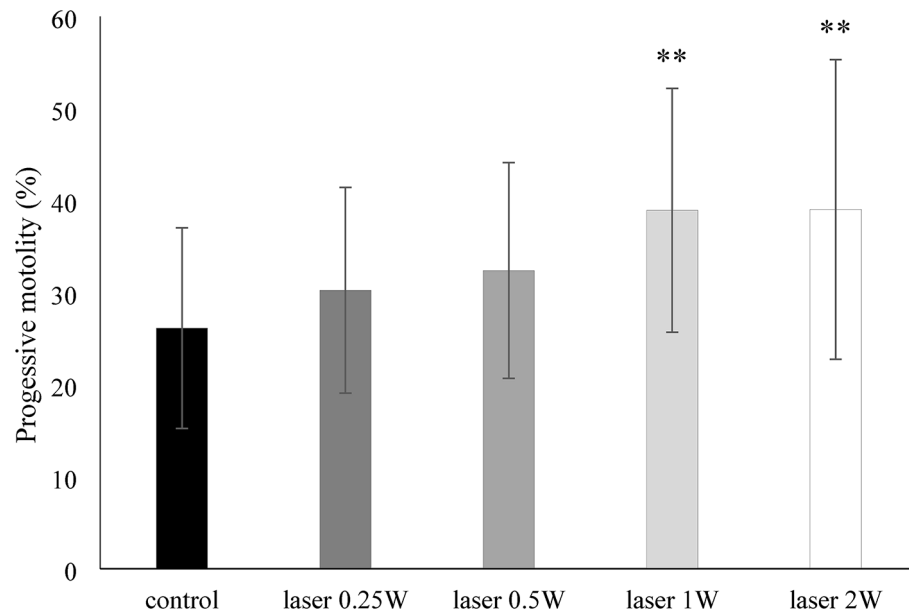


Fig. 2. Graph showing results of progressive motility in samples of 20 asthenozoospermic patients immediately after irradiation at different powers. The progressive motility rate was calculated as the percentage of a + b, where a corresponds to rapid progressive motility and b to slow progressive motility. Data are expressed as mean ± SD and compared with one-way ANOVA followed by Tukey’s multiple comparison test. ** indicates a significant difference ($p < 0.01$) compared to the control.

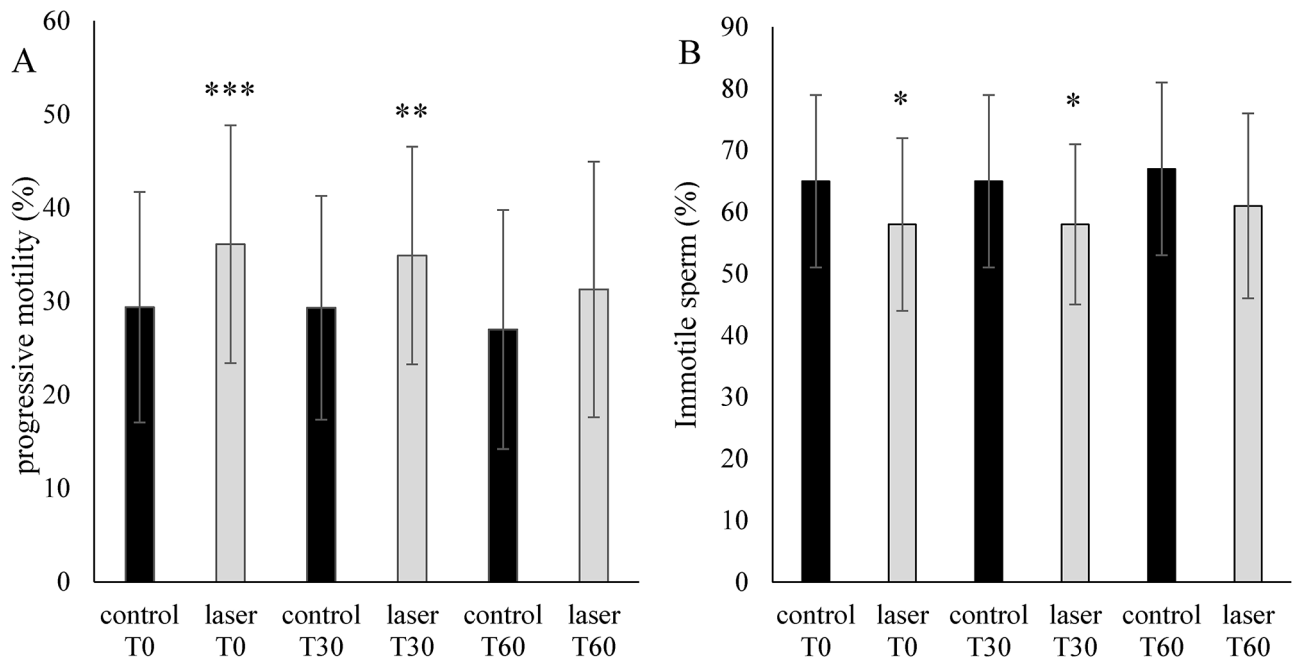


Fig. 3. Bar graphs showing PBM effects on sperm motility of 70 asthenozoospermic patients. (A) percentage of progressive motility, and (B) percentage of immotile sperm in control and treated samples over time (T0: immediately after irradiation; T30: 30 min after irradiation; T60: 60 min after irradiation). Data are expressed as mean ± SD. Comparisons were made by one-way ANOVA followed by Tukey’s multiple comparison test. *, **, and *** indicate a significant difference for $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively, between the control and the treated sample at the same time point.

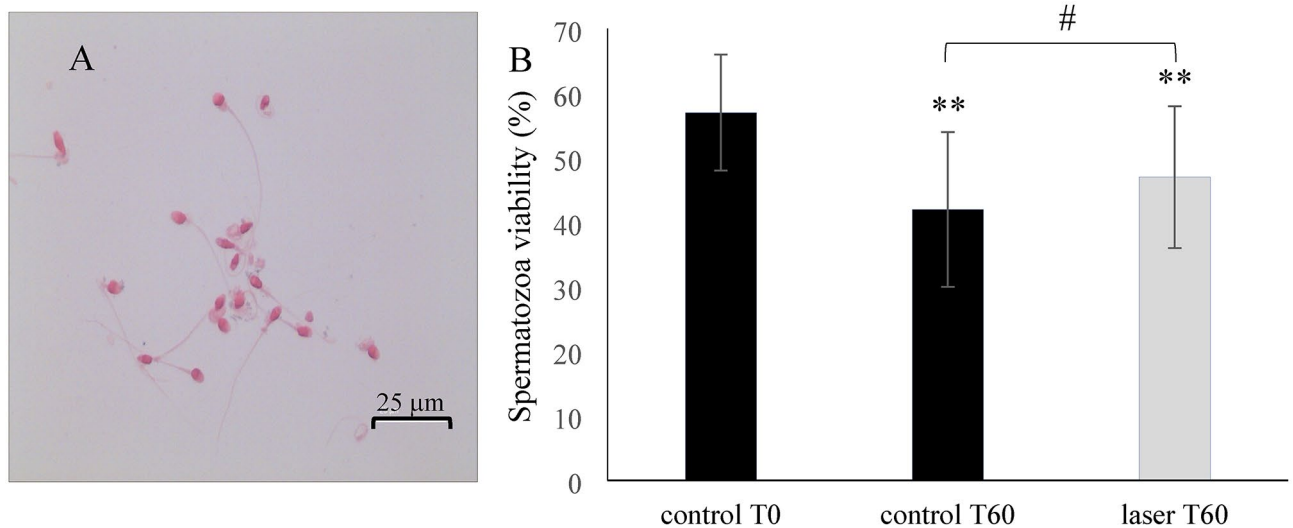


Fig. 4. Sperm viability test using eosin staining. **(A)** A representative image of sperm viability test. Live sperm heads were unstained, and dead sperm heads were stained red-pink (bar: 25 μm). **(B)** Graph showing the viability percentage at the baseline (control T0) and after 60 min from irradiation in controls (control T60) and treated samples (laser T60) of 25 asthenozoospermic patients. Data were expressed as mean \pm SD and compared with one-way ANOVA followed by Tukey's multiple comparison test. *** indicates a significant ($p < 0.001$) difference between control T0 vs. control T60 and laser T60, respectively. # indicates a significant ($p < 0.05$) difference between control T60 vs. laser T60.

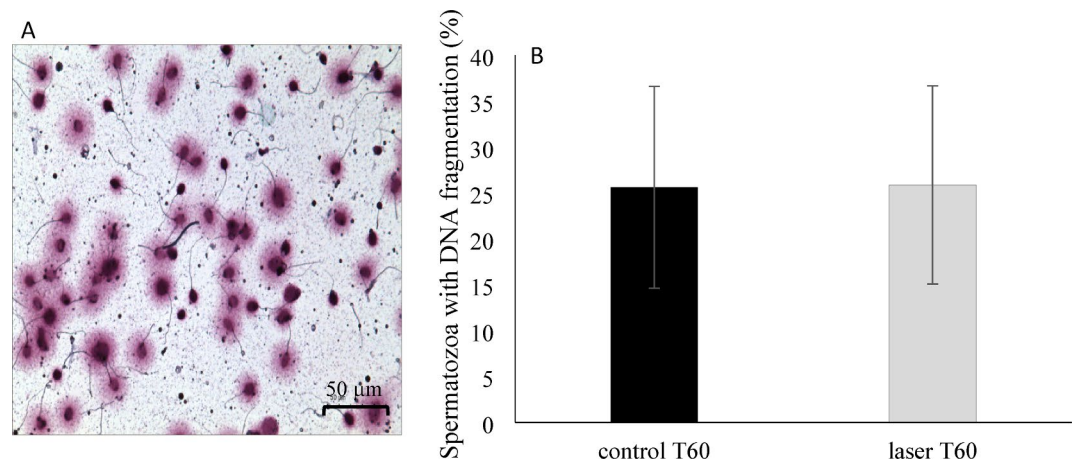


Fig. 5. Sperm DNA integrity test using Sperm Chromatin Dispersion (SCD) technique. **(A)** A representative image of sperm DNA integrity test. Sperm without DNA fragmentation shows a dispersion halo, sperm with fragmented DNA do not show a dispersion halo, or the halo is minimal (bar: 50 μm). **(B)** The percentage of sperm with fragmented DNA after 60 min from irradiation in controls (control T60) and treated samples (laser T60) of 25 asthenozoospermic patients. Data reported in Panel B were expressed as mean \pm SD and compared with the unpaired t-test.

PBM does not affect sperm DNA integrity

Our analysis showed that chromatin dispersion in sperm samples from 25 asthenozoospermic patients remained stable after 60 minutes from PBM ($p > 0.05$) (Fig. 5). We did not detect significant differences in the DNA fragmentation level after PBM compared to untreated sperm. Interestingly, even 5 samples with a positive SCD test ($> 30\%$) at the baseline ($41.2\% \pm 5.0\%$) did not display an increased value of chromatin dispersion after irradiation ($41.1\% \pm 5.1\%$).

PBM increases OxPhos activity in asthenozoospermic samples

To verify whether the increased motility observed in asthenozoospermic samples after PBM could be due to an enhancement in cellular energy metabolism, the OxPhos function was assessed by comparing the results obtained in samples from 20 asthenozoospermic and 20 normozoospermic patients. The data reported in Fig. 6 show that,

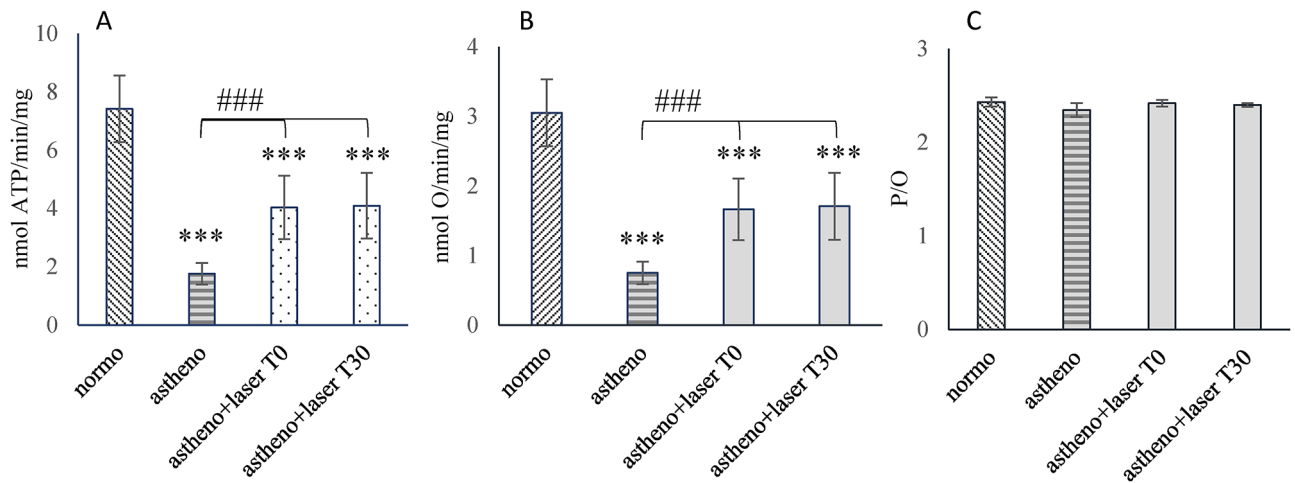


Fig. 6. PBM effect on OxPhos activity in samples from 20 asthenozoospermic and 20 normozoospermic patients. **(A)** ATP synthase activity; **(B)** oxygen consumption rate; **(C)** P/O ratio as a marker of OxPhos efficiency in normozoospermic samples (normo), asthenozoospermic samples (astheno) and asthenozoospermic samples lasered and immediately evaluated (astheno + laser T0) or evaluated after 30 min (astheno + laser T30). Data are expressed as mean \pm SD. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test. *** indicates a significant difference ($p < 0.001$) between normo and astheno, astheno + laser T0), or astheno + laser T30. ### indicates a significant difference ($p < 0.001$) between astheno versus astheno + laser T0 or astheno + laser T30.

under basal conditions, asthenozoospermic samples produce less ATP due to reduced oxygen consumption than normozoospermic samples, suggesting a deficit in OxPhos. This decrease only regards energy yield, as mitochondrial efficiency, indicated by the P/O ratio, appears similar to that observed in normozoospermic samples. The reduction in aerobic metabolism in asthenozoospermic samples is partially reversed after treatment with PBM, as both ATP synthesis and OCR increase by approximately 50% compared to the untreated sample immediately after irradiation and in the following 30 min. This increase does not alter the coupling between chemical energy synthesis and cellular respiration, as P/O values remain consistently around 2.5.

The PBM-induced OxPhos increment in asthenozoospermic samples leads to an enhanced cellular energy state

Following the increase in OxPhos, it was examined whether the cellular energy state also increased by evaluating the ATP and AMP intracellular concentrations and their ratio (Fig. 7). Samples from 20 asthenozoospermic and 20 normozoospermic patients were analyzed. The data show that asthenozoospermic samples are characterized by low ATP levels and high AMP concentration compared to normozoospermic samples. This results in a low cellular energy state that could explain the reduced motility. The increase in OxPhos in asthenozoospermic samples induced by PBM causes a 1.5-fold increase in intracellular ATP concentration and a reduction of about 50% in AMP levels, leading to a 3.5-fold increase in the ATP/AMP ratio. This effect is observed both immediately after irradiation and 30 min post-treatment. However, it is a reversible effect, as 60 min after laser administration, ATP, AMP levels, and the ATP/AMP ratio return to values similar to those of untreated asthenozoospermic samples. This finding is not surprising, as it has been demonstrated that the effect of PBM on isolated mitochondria is reversible⁵⁹ However, the increase in the energy state of asthenozoospermic samples could ensure improved motility for a sufficient time to allow fertilization.

The altered OxPhos activity in asthenozoospermic samples is not compensated by an enhancement in anaerobic glycolytic metabolism

Usually, if OxPhos is defective, cells attempt to compensate for the lack of ATP synthesis and the conversion of NADH to NAD⁺ by increasing anaerobic glycolysis. However, samples from 20 asthenozoospermic patients do not seem to employ this strategy, as the levels of lactate dehydrogenase (LDH), the enzyme that catalyzes the conversion of pyruvate to lactate, are not higher than those in samples from 20 normozoospermic patients, nor does PBM treatment alter its activity (Fig. 8).

PBM does not induce an accumulation of oxidative damage in asthenozoospermic samples

Considering that aerobic metabolism is always associated with the production of oxidative stress, even under conditions of complete coupling between ATP synthesis and respiration, the concentration of malondialdehyde (MDA), a marker of lipid peroxidation, was assessed. Samples from 20 asthenozoospermic and 20 normozoospermic patients were analyzed. The data shown in Fig. 9 indicate that asthenozoospermic samples under basal conditions display a higher concentration of MDA compared to normozoospermic samples. However, the MDA level does not increase following PBM treatment, suggesting that the increase in asthenozoospermic samples OxPhos does not induce further oxidative damage.

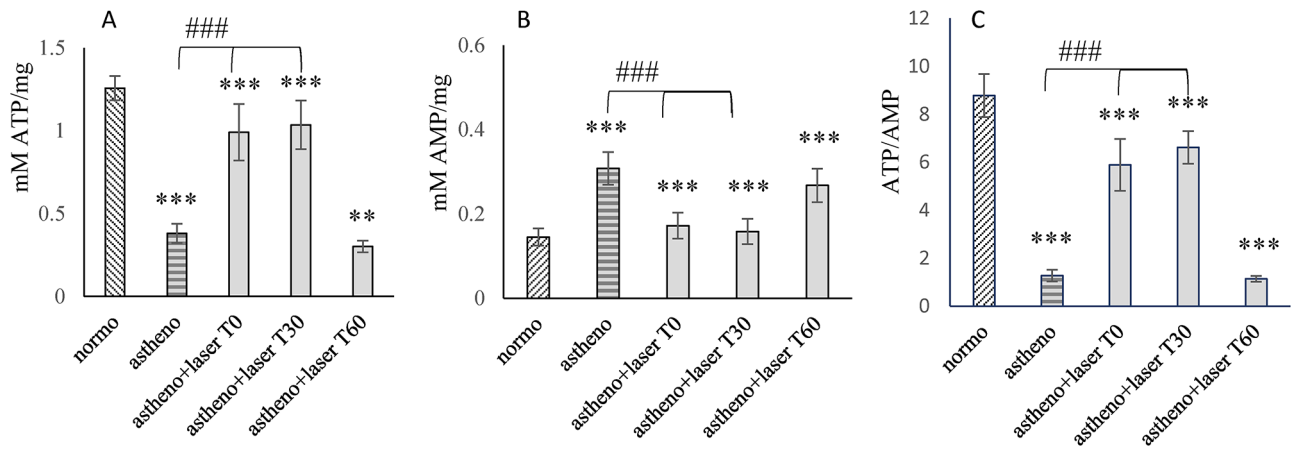


Fig. 7. PBM-induced changes of cellular energy status in samples from 20 asthenozoospermic and 20 normozoospermic patients. **(A)** ATP intracellular concentration; **(B)**; AMP intracellular concentration; **(C)** ATP/AMP ratio as a marker of cellular energy status in normozoospermic samples (normo), asthenozoospermic samples (astheno) and asthenozoospermic samples lasered and immediately evaluated (astheno+laser T0) or evaluated after 30 minutes (astheno+laser T30). Data are expressed as mean + SD. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test. *** indicates a significant difference ($p < 0.001$) between normo and astheno, astheno+laser T0), or astheno+laser T30. ### indicates a significant difference ($p < 0.001$) between astheno versus astheno+laser T0 or astheno+laser T30.

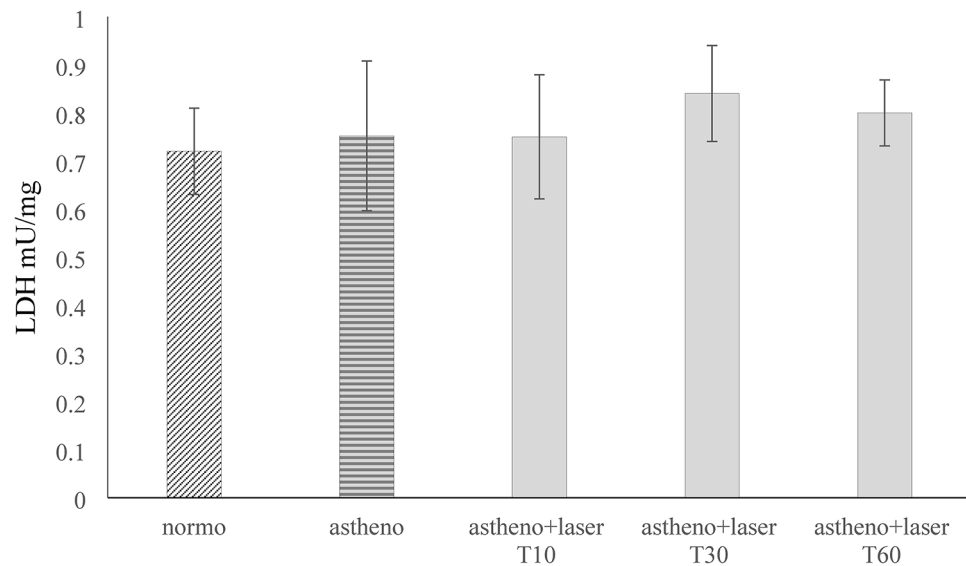


Fig. 8. PBM effect on LDH activity in samples from 20 asthenozoospermic and 20 normozoospermic patients. Graph reports the LDH activity in normozoospermic samples (normo), asthenozoospermic samples (astheno), and asthenozoospermic samples lasered and immediately evaluated (astheno+laser T0) or assessed after 30 minutes (astheno+laser T30). Data are expressed as mean + SD. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test, and no significant differences were observed.

Discussion

The positive contribution of PBM on sperm parameters has been reported in several studies^{20,32–34}. However, while PBM offers a translational potential to treat spermatozoa with poor motility, there is no common standard, making it difficult to directly compare the various data and draw a firm conclusion about the safety of PBM on semen samples in clinical practice. Our results showed that laser irradiation of human asthenozoospermic samples significantly improves progressive motility through increased mitochondrial energetic metabolism without harmful oxidative stress nor negative impact on sperm membrane integrity and chromatin condensation.

We used a diode laser light of 810 nm that we previously demonstrated to induce PBM on *Paramecium primaurelia* and mammalian mitochondrial activity without generating thermal damage^{13,60,61}. It also did

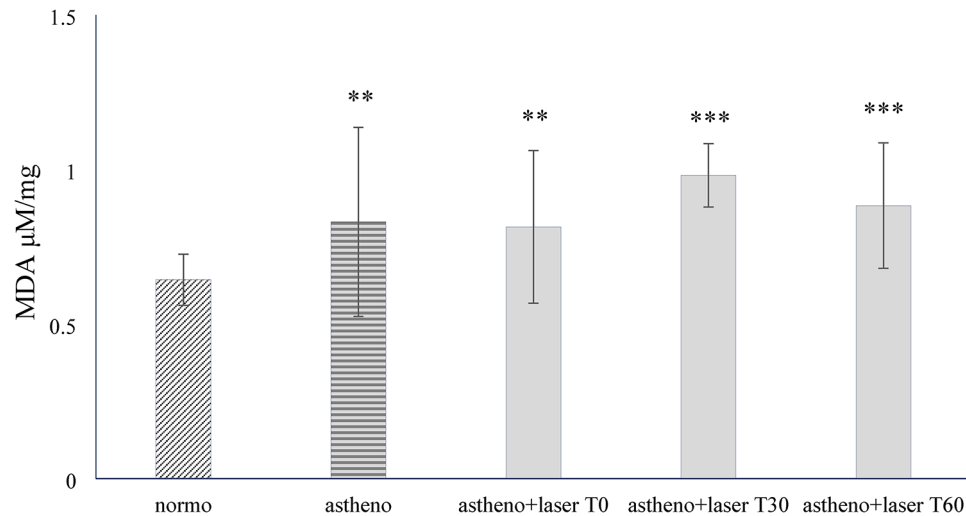


Fig. 9. PBM effects on lipid peroxidation accumulation were evaluated in samples from 20 asthenozoospermic and 20 normozoospermic patients. Graph reports the MDA intracellular concentration as a marker of oxidative damage in normozoospermic samples (normo), asthenozoospermic samples (astheno) and asthenozoospermic samples lasered and immediately evaluated (astheno + laser T0) or assessed after 30 min (astheno + laser T30). Data are expressed as mean \pm SD. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test. ** and *** indicate a significant difference ($p < 0.01$ and 0.001 , respectively) between normo and astheno, astheno + laser T0), or astheno + laser T30. No significant differences have been observed between astheno *versus* astheno + laser T0 or astheno + laser T30.

not negatively affect fertilization and early embryo development after irradiation of sea urchin eggs and spermatozoa⁵⁸. Moreover, other Authors reported promising results at 810 nm in human normozoospermic semen samples⁴¹. After identifying 1 W and 2 W as the most influential powers in increasing progressive motility and decreasing the percentage of immotile sperm, we chose 1 W for the following experiments. Indeed, the therapy showed preliminary aspects of efficacy and safety based on our evidence in vitro on protozoa and in vivo on sea urchins and humans. Indeed, 810 nm 1 W for 60 s over an area of 1 cm² increased the ATP production and the movement of the cilia in *Paramecium primaurelia*^{62,63}; the mechanisms underlying the movement of the cilia in protozoa have homologies with the flagellar movement of spermatozoa^{64,65}. It also improved fertilization rates in healthy sea urchin spermatozoa⁵⁸, and allowed complete recovery in patients with Bell's palsy who were unresponsive to drugs⁶⁶. No adverse effects were reported after follow-up.

We focused exclusively on a population of asthenozoospermic patients to avoid possible confounding factors. Specifically, we considered that normozoospermic samples could not benefit from laser therapy or do so in a reduced way. On the other hand, the low sperm number in oligozoospermic samples may make it challenging to count enough sperm to appreciate the laser effect on motility. To the best of our knowledge, this is the first study that included only fresh abnormal sperm and enrolled the largest cohort of semen samples from asthenozoospermic patients.

Semen parameters of asthenozoospermic patients were shifted above a threshold of 30% progressive motility, representing the standard criteria for normozoospermic, while in parallel, the fraction of immotile sperm decreased. Our results are in accordance with previous publications that reported enhanced sperm motility in semen samples treated with an infrared range of PBM^{36,39,41}.

The underlying pathways of PBM therapy are not well established and may vary among different sperm states (fresh *versus* frozen, normal *versus* abnormal). At present, at least four mechanisms are believed to be related to the response of sperm to PBM: (i) changes in mitochondrial function, (ii) generation and/or release of reactive oxygen species (ROS) from sperm cells; (iii) formation of nitric oxide, (iv) activation of opsins coupled to G-proteins³⁴. In this study, we investigated the involvement of mitochondria since they serve as the primary energy source for sperm movement, and in asthenozoospermia, sperm motility is impaired due to mitochondrial malfunction or structural defects of the tail⁸. In addition, the release of nitric oxide and the effects on the opsins are mainly the result of an interaction in the visible range of 450 to 650 nm^{19,67}. Our PBM protocol increased mitochondrial energy metabolism, providing biochemical evidence that mitochondrial activation is implicated in the increased sperm motility observed.

To broaden the knowledge of PBM treatment of human sperm, it is essential to analyze side effects. To date, it is challenging to draw a firm conclusion about the safety of PBM on semen samples in male subjects because there are some significant differences in the data available in the literature in terms of population characteristics, sample size, and type of methods used to conduct safety tests. In general, most of the human sperm DNA damage during manipulation is oxidatively induced⁶⁸ because spermatozoa are poor in enzymes that metabolize ROS, like catalase and glutathione peroxidase^{68,69} and have limited DNA repair capability⁷⁰. This may be why 810 nm PBM did not reduce oxidative stress damage in irradiated asthenozoospermic samples, as has been observed in other cell types⁷¹.

Oxidative stress causes lipid peroxidation of the plasma membrane, which is rich in polyunsaturated fatty acids, leading to the breakdown of membrane-dependent functions in these cells, including motility and sperm-oocyte fusion⁷⁰. Since we did not observe MDA accumulation, a marker of lipidic peroxidation, in irradiated asthenozoospermic samples, we argue that our PBM treatment does not increase oxidative stress in sperm. Noteworthy, to investigate this aspect without introducing potential biases into the system, we applied PBM only to native spermatozoa within the seminal plasma since manipulation (centrifugation, resuspension) of seminal fluids results in an artificially high level of ROS^{72,73}. Moreover, seminal plasma is one of the most potent antioxidant fluids⁷⁰.

Functionally, our laser therapy protocol did not adversely affect the integrity of the sperm membrane, preserving vitality and chromatin condensation, and did not introduce DNA fragmentation or worsen it when already present.

All these safety aspects are essential for applying PBM in clinical practice as an asthenozoospermia therapy. One of the putative applications would be a laser-induced improvement of sperm motility to increase pregnancy rates achieved by intrauterine insemination. The percentage of motile sperm that has the ability for forward progression is one of the most critical prognostic indicators of fertilization, which can increase the likelihood of pregnancy⁷⁴. Bypassing time, sperm cells decrease their ability to move⁷⁵. Boosting the availability of total motile sperm can improve the chance of pregnancy. This finding could be helpful in cases of moderate male infertility to encourage the use of intrauterine insemination as a cost-effective first-line treatment⁷⁶. On the one hand, it is noteworthy that in our experiments, the effect of irradiation on sperm motility was kept high within 30 min after irradiation, unlike other studies where the effect decreased significantly after 10 min of phototherapy⁷⁷. On the other hand, we have shown a significant increase in progressive sperm motility from 29 to 35%, but we cannot conclude that this increase predicts a better clinical outcome.

More benefits could be expected in selecting viable ejaculated or surgically retrieved testicular/epididymal spermatozoa in patients with immotile sperm before microinjection into the oocyte. Sperm motility is an essential selection criterion for embryologists during intracytoplasmic sperm injection (ICSI). A method of testing sperm viability is to induce sperm motility by increasing cyclic adenosine monophosphate (cAMP) levels by treating a semen sample with phosphodiesterase inhibitors, such as theophylline and pentoxifylline⁷⁸. Although pentoxifylline is a popular *in vitro* sperm motility enhancer in many embryology laboratories, its application is not commonly approved in clinical practice because the consequences of pentoxifylline treatments in terms of potential damage to sperm DNA, oocytes, embryos, and newborns are contradictory⁷⁹. In this context, stimulating sperm motility by PBM could advance sperm selection.

Another clinical application of PBM could be the treatment of frozen and thawed semen, especially in programs of fertility preservation of cancer patients before chemo- and radiotherapy. Despite its widespread use, the cryopreservation technique can cause damage to sperm through oxidative stress that induces lipid peroxidation, DNA fragmentation, and apoptosis⁸⁰. In addition, sperm motility and fertilizing potential are reduced due to membrane, cytoskeletal, and acrosome damage^{81,82}. The harmful side effects of the freezing and thawing process may be countered by PBM-preconditioning of fresh semen before the cryopreservation procedure, hopefully resulting in increased mitochondrial membrane potential and decreased levels of intracellular ROS and lipid peroxidation^{45,46}.

Of course, some ethical concerns must be resolved before the use of PBM of sperm in assisted reproductive technologies (ART), whose ultimate goal is a healthy baby. Indeed, laser technology is now being applied in ART to reduce procedure times and increase the consistency of traditional techniques such as assisted hatching, embryo biopsy in the preimplantation genetic diagnosis program, and sperm immobilization⁸³. It is also important to note that the energy used to enhance sperm motility in PBM is much lower than those used for, *i.e.*, assisted hatching, since it is only used to stimulate the photosensitive enzyme cytochrome c oxidase and below the threshold for ionization. However, there is undoubtedly little published information about the potential adverse effects that laser application - even for PBM of sperm - could have on the developing human embryo, making further research necessary.

Overall, our work encompasses both advantages and limitations. First of all, we explored very deeply the specific mechanism behind the positive effect of PBM on sperm motility. In fact, the analysis of energy metabolism in irradiated and non-irradiated asthenozoospermic spermatozoa and the comparison with healthy spermatozoa clearly showed that the macroscopic effect on cell motility is a consequence of the impact of our light therapy on mitochondrial oxidative phosphorylation. However, the glycolytic pathway, also known to play an energetic role in sperm motility, does not appear to be activated by light, as well as opsins and nitric oxide are predominantly stimulated in the visible range. Second, the strength of our work lies in our approach, which represents the first standardisable protocol easily adaptable to the clinical environment, shifting the focus from preliminary data and evidence towards a coherent and consistent demonstration of the photobiomodulation phenomenon in the context of male infertility. In fact, the short exposure time prevents samples from being exposed to sub-optimal conditions for too long, and the immediate effect, which lasts consistently up to 30 min (after 60 min, the effect returns to the level of untreated samples), ensures consistency of the procedure. Additionally, using an innovative FT-HP, which we have previously characterized, to deliver photon energy allowed irradiation to be independent of distance from the target and uniform across the entire 1 cm² surface area. These properties make the approach repeatable, operator-independent and more effective than standard delivery systems used in photobiomodulatory therapy^{48,49}.

We are aware that there are some limitations. First, in this study, we checked the number of spermatozoa and sperm motility by microscopic investigation. However, the method followed the practical guidelines in the WHO manual. We know semen examination is complicated and procedurally challenging to standardize, mainly because it is based on operator-dependent procedures. Anyway, the accuracy of the data shown in this study was ensured because in our laboratory we monitor the fundamental parameters of sperm count, morphology, and

motility through internal and external quality control procedures. Our operators are qualified, and performance is periodically monitored to detect systematic and random differences between individuals performing analyses. Moreover, our laboratory, as the Regional referral center for semen diagnostic analysis, adheres to an external quality verification program to detect systematic variations and evaluate accuracy through comparisons between different laboratories. In addition, to limit bias, semen parameters were assessed blindly and through commercial disposables and kits for clinical use to provide repeatable and consistent results.

A second limitation is that we did not check the straightness of sperm movement and the flagellar waveform, which helps evaluate sperm hyperactivation⁸⁴. However, the complex parameters of the flagellar movement of hyperactivated spermatozoa are challenging to identify reliably and unambiguously through manual analysis. Motility stereotypes can be assessed by computer-aided sperm analysis (CASA). However, the comparability of measurements among different instruments is not yet known, and their biological value is not fully understood⁵¹. Lastly, we did not evaluate the effectiveness of our PBM protocol in improving fertilization potential and the impact on embryo quality. This was due to the Italian law prohibiting the insemination of human oocytes outside the standard procedures recognized as non-experimental. However, in the sea urchin, a well-known model for studying the molecular mechanisms underlying fertilization and human embryonic development⁸⁵, we have obtained an encouraging increase in sperm fertilization rates, without the occurrence of embryonic and larval anomalies⁵⁸.

Conclusion

Laser irradiation at 810 nm through the FT-HP of human sperm significantly improves progressive motility. The effect is due to increased metabolic energy (ATP) resulting from photons interacting with the OxPhos system instead of the glycolytic pathway represented by lactate dehydrogenase (LDH). What makes these data even more interesting is that the treatment did not cause harmful oxidative stress or negative effects on membrane integrity and chromatin condensation.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Author contributions

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Declarations

Competing interests

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Institutional review board statement

Ethics Committee of Regione Liguria (219/2024 - DB id 13852).

Informed consent statement

Written informed consent was obtained from all donors, and participation was voluntary.

Additional information

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