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ORIGINAL ARTICLE

Effect of 830-nm diode laser irradiation on human sperm motility

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Abstract Sperm motility is known as an effective parameter in male fertility, and it depends on energy consumption. Low-level laser irradiation could increase energy supply to the cell by producing adenosine triphosphate. The purpose of this study is to evaluate how the low-level laser irradiation affects the human sperm motility. Fresh human semen specimens of asthenospermic patients were divided into four equal portions and irradiated by 830-nm GaAlAs laser irradiation with varying doses as: 0 (control), 4, 6 and 10 J/cm^2. At the times of 0, 30, 45 and 60 min following irradiation, sperm motilities are assessed by means of computer-aided sperm analysis in all samples. Two additional tests [HOS and sperm chromatin dispersion (SCD) tests] were also performed on the control and high irradiated groups as well. Sperm motility of the control groups significantly decreased after 30, 45 and 60 min of irradiation, while those of irradiated groups remained constant or slightly increased by passing of time. Significant increases have been observed in doses of 4 and 6 J/cm^2 at the times of 60 and 45 min, respectively. SCD test also revealed a non-significant difference. Our results showed that irradiating human sperms with low-level 830-nm diode laser can improve their progressive motility depending on both laser density and post-exposure time.

Keywords Sperm motility · Low-level laser · HOS test · SCD test

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Introduction

Motility is one of the most important characteristics associated with the fertilizing ability of spermatozoa and is an expression of their viability and structural integrity. It is evident that sperm functionality is strongly related to the intracellular energy management mechanisms. Hence, mitochondria merit specific consideration. Not only does it provide the adenosine triphosphate (ATP) needed for the tail to maintain its contractibility [1] but also regulates the calcium flux which plays a key role in the initiation of sperm motility [2]. The close spatial relationship between the middle piece of sperm which contains mitochondria and the flagellum (tail) indicates a concomitant close relationship between the functionality of both organelles. It has already been shown that laser irradiation could improve sperm fertilizing potential. Although the exact mechanism of action of the laser beam with living cells is not yet understood, some explanations based on the light absorption by primary endogenous chromophores such as mitochondrial enzymes have been proposed to describe biological effects of laser light [3].

Considerable literature exist on photo-stimulative effect of low-level laser irradiation on somatic cells such as: increasing fibroblast proliferation and reducing cell death [4],

enhancement of osteoblasts activity and bone formation [5] and accelerating the phagocytic activity of human monocytes [6]. Another area that has received much attention is the use of low-power laser in the gametic and embryonic cells in vitro [7, 8]. Furthermore, numerous studies have discussed the effect of laser on spermatozoa especially in mammalians. Cohen et al. [9] stated that laser irradiation stimulates fertilizing ability of mouse sperm. Ocana-Quero et al. [10] studied biological effects of He–Ne laser on acrosome reaction of bull sperm cells. Another study by Iaffaldano et al. [11] showed that irradiation of rabbit sperm cells with He–Ne laser prevents in vitro liquid storage dependent damage.

Although several studies have used laser beam to immobilize spermatozoa, especially for intracytoplasmic sperm injection [12], only little information exists regarding its photo-stimulative effect on human sperm. The main purpose of this study is to evaluate how exposure to an 830-nm continuous wave laser beam would affect human sperm motility. In addition, we assayed the Hypo-Osmotic Swelling (HOS) test and the sperm chromatin dispersion (SCD) test to study the effect of irradiation on functional capacity of spermatozoa.

Materials and methods

Semen samples

Twenty-two human semen samples with impaired sperm motility (Asthenzoospermia) and volume ≥4 ml were selected and used in this study. Semen samples were provided by masturbation of infertile men (25–40 years old) who were referred to infertility clinic of Royan Institute. They were seeking for treatment and had received no medication before. All samples were collected in special containers and underwent the routine semen analysis by computer-aided sperm analysis (CASA). Each selected sample was divided into four groups, one control group and three groups to be irradiated.

Laser specifications and irradiation procedure

A continuous wave GaAlAs laser hand-held probe of KL06 (Mustang 2000, Russia), with 830 nm wavelength, 200 mW output power and 0.67 cm^2 aperture size, was used in this study. The output power was brought to 100 mW during the experiment.

Each selected semen sample was divided into four aliquots: one control group (non-irradiated) and other three groups exposed to laser beam at energy densities of 4, 6 and 10 J/cm^2. The irradiation time for each group was calculated using the following equation (offered by Swedish Laser Society), and it is shown in Table 1:

$$\text{Irradiation time} = \frac{\text{energy density}}{\text{output power}} \times \text{aperture size} \quad (1)$$

Each aliquot of samples was put in a 2-ml spectrophotometry cuvette in dark condition. The laser probe was placed just above the cuvette opening so that the beam spot was as big as the cuvette surface with 3 cm distance. Irradiation was done from above so that the whole sample was homogeneously irradiated (usual way to irradiate liquid samples). The chosen irradiation doses were exactly those used by Corral-Baques et al. [13] on dog sperm.

Sperm motility analysis

A CASA automatic system was used to evaluate the sperm motility because it provides greater objectivity, reliability, sensitivity and security than the optical method. The CASA system commonly consists of a bright-field microscope connected to a high-resolution video camera and a computer for data organization and statistical analysis. In this study, the software used was the Videotest Image Analysis-Sperm 2.1 (Videotest, Moscow, Russia).

At the first step, 5 μl of each sample was transferred on a Makler counting chamber (Sefi Medical Instrument Ltd.) to evaluate the primary sperm motility status by CASA. After irradiation, similar measurements at 30, 45 and 60 min were performed on all groups (control, 4, 6 and 10 J/cm^2). Hence, for each sample, 13 measurements and for all 22 samples 286 measurements were performed.

During all steps of analysis, the samples were placed in a 37 °C incubator to fix the temperature effect on sperm motility. For each specimen, the tracks of at least 200 motile sperms were analysed, and all samples were assessed by the same technician to achieve acceptable precision and accuracy. Analysis of sperm motility was performed according to the WHO recommendation [14], and motility grading is shown in Table 2.

Sperm chromatin dispersion test

Sperm DNA fragmentation was assessed by SCD test according to Fernandez et al. [15] with some modifications.

Table 1 Time of irradiation (*T*) of each sample, depending on the energy density (ED), the power (*P*) and the spot of the laser beam

ED (J/cm^2)	*P* (mV)	Spot (cm^2)	*T* (s)
4	100	0.67	26.8
6	100	0.67	40.2
10	100	0.67	67

Table 2 WHO (1999) grading of sperm motility

Motility grade	Motility specification
Grade A (rapid progressive)	≥25 μm/s at 37 °C (or a 5 sperm head length) and ≥20μm/s at 20 °C
Grade B (slow progressive)	Sluggish progressive or 5–24 μm/s
Grade C (non-progressive)	Local motility only or <5 μm/s
Grade D (immotile)	Sperms do not move at all

The test was performed on control and 10 J/cm^2 (the highest dose) irradiated groups, both at 3 h after irradiation. Briefly, semen samples were washed two times with PBS and diluted in PBS to obtain sperm concentrations of about 5–10 million/ml. Thirty microliters of sperm suspension was added to 70 μl of 1 % low-melting aqueous agarose at 37 °C to obtain a 0.7 % agarose concentration. This mixture was pipetted onto a glass slide which was pre-coated with 0.65 % agarose, then covered with a coverslip (24× 60 mm) and left to solidify at 4-8 °C for 5 min. Afterwards, the coverslip was removed carefully and slide immediately immersed horizontally in a tray with freshly prepared acid denaturizing solution (0.08 N HCl) for 7 min at room temperature in the dark, to generate restricted single-stranded DNA motifs from DNA breaks. Then, slides were transferred to a tray containing neutralizing and lysing solution (0.4 M Tris, 0.8 M 2-mercaptoethanol, 1 % SDS and 50 mM EDTA, pH 7.5) for 25 min at room temperature. Slides were thoroughly washed two times in distilled water (2 min each), dehydrated in sequential 70, 90 and 100 % ethanol baths (2 min each) and dried at room temperature. Finally, the slides were stained with Wright-Giemsa stain (1:1 ratio with PBS). In this method, stained sperm cells were categorized in five groups as: large, medium, small, and without halo and degraded forms. In each smear, 200 sperm cells were counted as replicate under bright field microscope by magnification ×1,000, and replicate values were compared to see if they are acceptably close. If so, DNA Fragmentation Index (DFI) is reported as the percentage of sum of small, without halo and degraded sperm cells; if not, the smears are re-read.

Table 4 Results of Grade B motility (mean±SEM%) over time (minute) according to the irradiated dose

Groups	Time (min)			
	0 (n=22)	30 (n=22)	45 (n=22)	60 (n=22)
Control	28.16± 2.16a	26.27± 2.14b	26.52± 2.55ab	23.82±2.14c
Dose 4 J/cm^2	28.16± 2.16b	28.95± 2.30ab	30.56± 2.27ab	31.61±2.01a
Dose 6 J/cm^2	28.16± 2.16a	28.35± 2.07a	31.51± 2.16b	30.43±2.39ab
Dose 10 J/cm^2	28.16± 2.16a	30.32± 2.25a	29.28± 1.82a	28.50±1.70a

Times that do not share a letter are significantly different

Hypo-osmotic swelling test

The hypo-osmotic swelling test (HOST) evaluates the functional capacity of the spermatozoids. When spermatozoids are placed in a hypoosmotic environment, they react, when functional, by swelling and bending their tails. The percentage of swollen tails is, therefore, directly proportional to sperm quality [16–19]. HOS test was performed on the same two semen sample groups as they used for SCD test. In a test tube, 0.1 mL of liquefied semen was added to 1 mL of hypoosmotic solution prepared by dissolving 3.75 g of sodium citrate and 13.5 g of fructose in 1 L of distilled water in accordance with a previously described technique [20] (Jeyendran et al.). Test tubes were incubated for 45 min at 37 °C; then, for each sample, two smears were prepared and stained by Papanicolaou staining method. In each slide, 200 sperm cells were counted as replicate, and replicate

Table 3 Results of Grade A motility (mean±SEM%) over time (minute) according to the irradiated dose

Groups	Time (min)			
	0 (n=22)	30 (n=22)	45 (n=22)	60 (n=22)
Control	7.85± 1.19a	6.34± 1.05b	5.69± 0.99b	5.01± 1.12b
Dose 4 J/cm^2	7.85± 1.19ab	7.71± 1.36b	9.05± 1.37a	8.80± 1.36ab
Dose 6 J/cm^2	7.85± 1.19a	8.18± 1.18a	9.26± 1.54a	9.24± 1.57a
Dose 10 J/cm^2	7.85± 1.19a	8.21± 1.38a	8.50± 1.44a	7.72± 0.67a

Times that do not share a letter are significantly different

Table 5 Results of Grade A+B motility (mean±SEM%) over time (minute) according to the irradiated dose

Groups	Time (min)			
	0 (n=22)	30 (n=22)	45 (n=22)	60 (n=22)
Control	36.01± 2.94a	32.61± 2.76b	32.18± 3.18b	28.82± 2.83c
Dose 4 J/cm^2	36.01± 2.94a	36.66± 2.96a	39.61± 3.08b	40.86± 2.92b
Dose 6 J/cm^2	36.01± 2.94a	36.53± 2.78a	40.72± 3.22b	39.66± 3.60ab
Dose 10 J/cm^2	36.01± 2.94a	38.98± 3.47a	37.93± 2.80a	36.32± 2.48a

Times that do not share a letter are significantly different

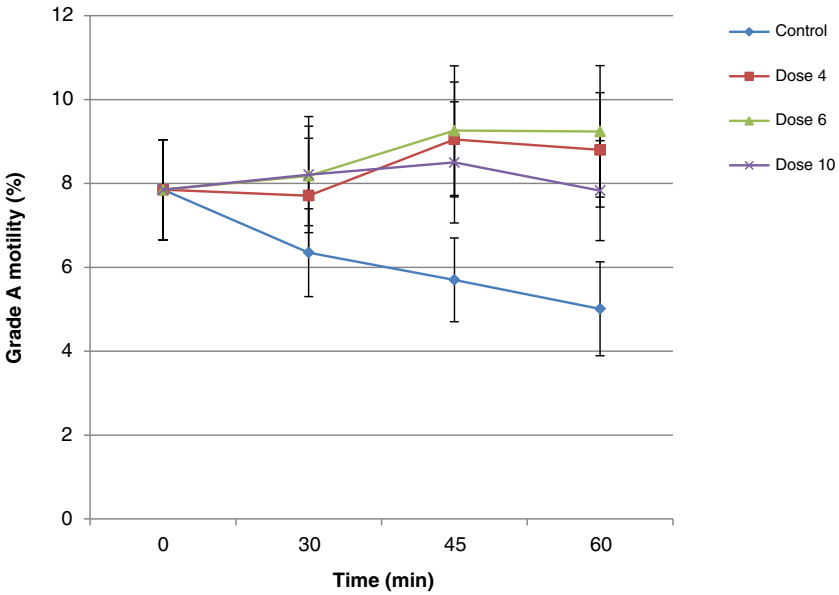

Fig. 1 Evolution of the Grade A motility (percentage) over time (minutes) according to the irradiated dose (D0=control, D4=4 J/cm^2, D6=6 J/cm^2, D10=10 J/cm^2). Results are expressed as means±SEM for 22 samples

values were compared to see if they are acceptably close. If so, the results are reported as the percentage of swelling cells and non-swelling cells; if not, the slides are re-read.

Statistical analysis

The repeated measure ANOVA analysis was applied for comparing mean results obtained from different groups and times by CASA. Results of motility percentage are expressed as mean±SEM. Wilcoxon signed rank test was applied to compare the results of HOS test and DFI analysis. SPSS version 16 was used for statistical analysis. Statistical significance was set at $P<0.05$.

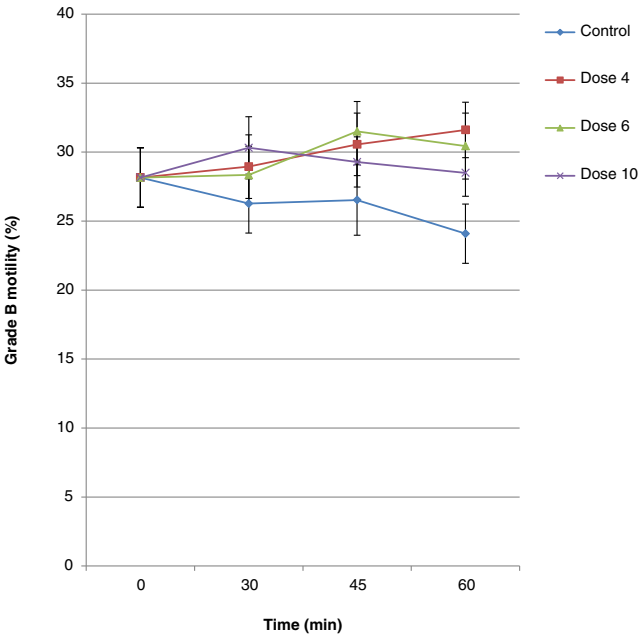

Fig. 2 Evolution of the Grade B motility (percentage) over time (minutes) according to the irradiated dose (D0=control, D4=4 J/cm^2, D6=6 J/cm^2, D10=10 J/cm^2). Results are expressed as means±SEM for 22 samples

Results

Sperm motility grades

According to the motility grading shown in Table 2, the CASA results for all samples were clustered into three groups: Grade A, Grade B and Grade A+B, which is the union of two previous grades indicating total progressive motility. The results of Grade A, Grade B and Grade A+B motility percentage over time for control and irradiated groups are shown in Tables 3, 4 and 5, respectively. Figures 1, 2 and 3 represent the evolution of each motility grade over time for control and irradiated groups. All of these figures show that the sperm motility of the control groups significantly decreased ($P<0.05$) after 30, 45 and 60 min of irradiation, while those of irradiated groups remained constant or slightly increased by passing of time. In particular, significant increases have been observed in doses of 4 and 6 J/cm^2 at the times of 60 and 45 min, respectively.

Figures 4, 5 and 6 represent the effect of irradiation on sperm motility as a function of different fluences after 30, 45 and 60 min of exposure to the laser beam. Although the progressive motility significantly increased in all three doses with respect to the non-irradiated groups at each of these times, the pattern of enhancement was different in each case. As shown in Fig. 4, the progressive motility monotonically increased with applying higher laser doses which means that highly irradiated group indicated better motility than the other groups after 30 min of irradiation. By passing of time,

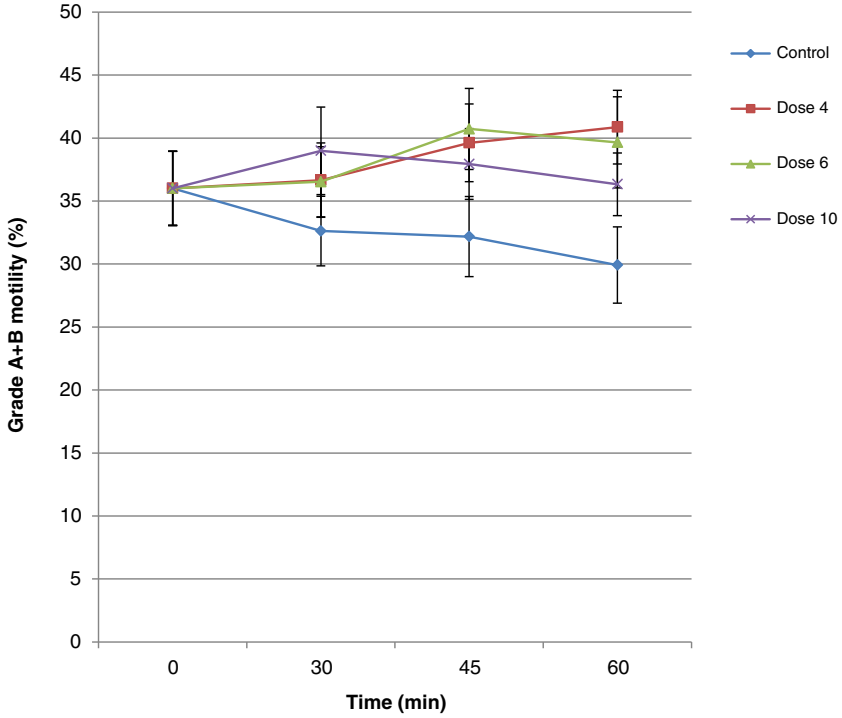

Fig. 3 Evolution of the Grade A+B motility (percentage) over time (minutes) according to the irradiated dose (D0=control, D4=4 J/cm^2, D6=6 J/cm^2, D10=10 J/cm^2). Results are expressed as means±SEM for 22 samples

the maximum point on the motility curves moved to left representing 6 and 4 J/cm^2 as the most effective fluences at 45 and 60 min, respectively (Figs. 5 and 6).

HOS test

Figure 7 shows HOS test results from control and highly irradiated groups according to the percentage of swelling and non-swelling cells after 3 h of irradiation. The mean percentages of swelling cells were 48.63±2.06 and 46.58±2.42 % in control and 10 J/cm^2 irradiated group, respectively.

SCD test

Figure 8 displays the percentages of DFI which resulted from performing SCD test on both control and highly irradiated groups, 3 h following irradiation. Although DFI in irradiated group (69.63±3.16 %) slightly increased in comparison with the control group (66.47±3.46 %), this change is not considered significant ($P>0.05$).

Discussion

Motility is the most single important feature in mammalian sperm because sperms have to deliver the male's nuclear package to the oocytes during fertilization. So, the spermatozoa must be motile to perform their journey to the egg, and the sperm tail is the main organelle linked to motility of

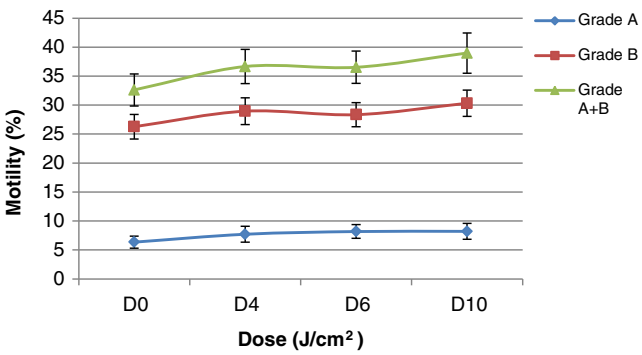

Fig. 4 Evolution of motility (percentage) after 30 min of irradiation according to the applied dose (*D0* control, *D4* 4 J/cm^2, *D6* 6 J/cm^2, *D10* 10 J/cm^2). Results are expressed as means±SEM for 22 samples

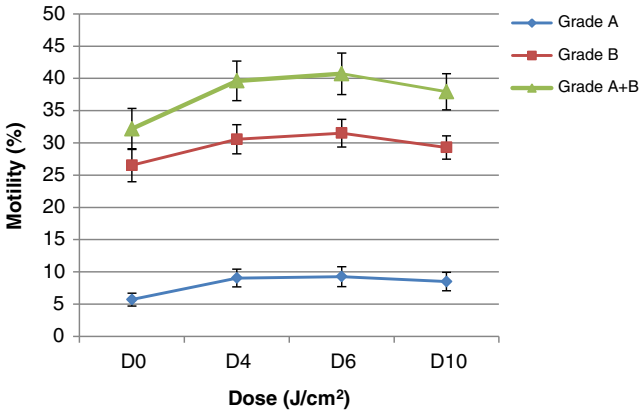

Fig. 5 Evolution of motility (percentage) after 45 min of irradiation according to the applied dose (*D0* control, *D4* 4 J/cm^2, *D6* 6 J/cm^2, *D10* 10 J/cm^2). Results are expressed as means±SEM for 22 samples

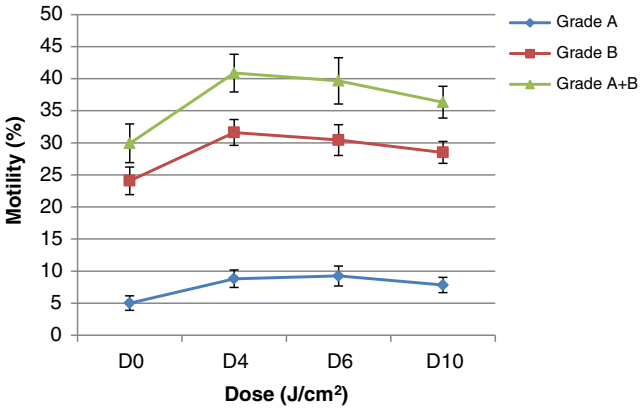

Fig. 6 Evolution of motility (percentage) after 60 min of irradiation according to the applied dose (*D0* control, *D4* 4 J/cm^2, *D6* 6 J/cm^2, *D10* 10 J/cm^2). Results are expressed as means±SEM for 22 samples

sperm [13]. Increase in sperm motility as one of the most important prognostic indicators of fertilization would result in higher chances of pregnancy. In this paper, we have studied the effects of laser irradiation on human sperm motility. As guessed, without any intervention, fresh spermatozoids would continue losing their motility over time. At the same time, we observed that laser beam had preserved and in some cases improved the motility of irradiated sperms. Consistency/increase of sperm motility even in low extents could improve the chance of pregnancy, and also, it could have beneficial applications in artificial reproductive technology techniques.

There exist numerous studies about the influence of low-level laser on the sperms of human beings and other species which support our findings. Sato et al. [21], using multiple exposure photography, reported significant improvement in the motility of human spermatozoa irradiated with krypton laser red light. Lenzi et al. [22] confirmed the positive effect of laser irradiation on velocity and linearity of human spermatozoa by means of computer-assisted analysis. Iaffaldano et al. [23] showed that He–Ne laser irradiation with energy

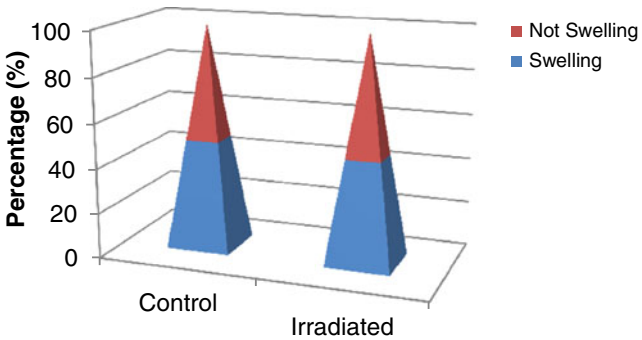

Fig. 7 HOS test results of 19 samples according to the percentage of swelling and non-swelling cells in control and 10 J/cm^2 irradiated groups after 3 h of irradiation

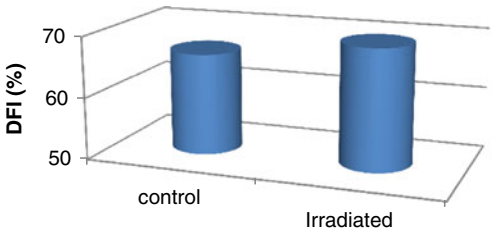

Fig. 8 SCD test results of 19 samples according to the DFI (percentage) in control and 10 J/cm^2 irradiated groups after 3 h of irradiation

doses ranging from 3.24 to 5.4 J/cm^2 increased sperm motility index of stored turkey semen compared to the control group. Carol-Baques et al. [13] found that irradiation of dog sperm with continuous wave diode laser improves motility parameters including average path velocity, linear coefficient and beat cross frequency.

Although all the biological mechanisms of low-level laser therapy are not completely understood, the activation of certain receptors or messengers at a molecular level may explain these biological responses. Several studies have shown that second messengers, such as cAMP, Ca^{2+} and reactive oxygen species (ROS), play key roles in sperm motility. It is well known that bio-stimulation occurs after laser irradiation of isolated mitochondria [24] as well as of cells [25, 26] with an increase in the synthesis of ATP. According to previous studies, irradiating spermatic cells with certain energy doses of laser beam resulted in acceleration of intracellular Ca^{2+} concentration [9, 27]. The Ca^{2+} influx from the extracellular environment is an essential component of the signaling cascade leading to fertilization because it triggers flagellar beating, acrosome reaction, capacitation and many other cell functions [28]. On the other hand, laser beam stimulates ROS generation in cell membrane, cytoplasm and mitochondria [29]. Reactive oxygen species such as superoxide anion, hydrogen peroxide and nitric oxide act as second messengers and consequently regulate sperm capacitation, induce the acrosome reaction and fertilize the oocyte [16].

Effect of low-power laser on human sperm is dependent on different parameters such as semen sample quality, irradiation method, wavelength, output power and radiant exposure. In this study, we changed the energy density doses and measured the motility features at different times while the other laser specifications kept up constant during the experiment. We found that the effect of laser irradiation on sperm motility highly depends on both the fluence of applied laser beam and post-exposure time. Based on our results, the maximum effect appears on doses of 4 and 6 J/cm^2 at the times of 45 and 60 min after irradiation. By passing of time, we observed an inhibitory effect on progressive motility at higher doses. Harrison et al. [17] used two different light sources for irradiating human spermatozoa: a LED cluster of 660 and 850 nm (100–400 J dose) and an 810-nm diode laser (2–4 J dose).

They reported that maximum increase in the sperm motility index came from the mid-range doses at 30 min for LED cluster and from the lowest dose for laser at 30 min post-exposure. Another study by Tadir et al. [18] showed a gradual decrease in sperm velocity following the longer duration of exposure. According to the above-mentioned equation (Eq. 1), at constant output power and aperture size, increasing the irradiation time is equivalent to enhancing the energy density of laser beam. Hence, this study confirmed that applying excessive laser doses on human sperm would degrade its beneficial effects.

The mechanisms by which this occurs can be explained as follows. Although ROS have a fundamental role in sperm physiology, their excess can damage spermatozoa [19]. Furthermore, at high laser doses, more Ca^{2+} influx induces the hyperactivity of Ca^{2+}-ATPase calcium pumps and exhausts the ATP reserves of the cell. This process leads to deceleration of cell pump activity, and the intracellular osmotic pressure explodes the spermatozoa [30].

As the sperm features change during their various stages of growth, we need greater knowledge of the light energy interaction in the spermatozoid. This would enable the right laser wavelength for each cell activity to be optimized or administered more effectively (depending on the specific chromophores).

In our experiment, the results of sperm chromatin dispersion (SCD) test showed that the 830-nm laser irradiation can increase the level of DNA fragmentations in sperm cells, but it was not statistically significant. At the same time, the HOST results revealed that the 830-nm laser irradiation does not improve the functional quality, and this latter was inconsistent with the previous study [13].

In this experiment, we changed only the energy density (radiant exposure) and did not change any other important parameters, such as power density (irradiance). Our results with the 830-nm wavelength cannot be extrapolated to other wavelengths because we already know that the biological response is wavelength-dependent at equal doses [31]. Clinical and experimental studies show that laser therapy has its greatest effects on cells, tissues and organs that are affected by a generally deteriorated condition [32], and we still do not know how stressed or low-quality sperm would react to laser irradiation.

This experiment suggests a wide range of research possibilities. One of these is the usage of more sensitive methods for assessing sperm DNA fragmentation, e.g. TUNNEL test. Monitoring the fructose level in semen as the main energy supply for sperm motility before and after irradiation might be very useful. The other one is economic importance of using low-level laser irradiation to maintain sperm motility for artificial insemination. Another useful application is to determine the maximum delay, between obtaining and irradiating the sample, at which the sperm still reacts positively to the laser irradiation. An interesting study would also be to analyse how sperm would react to other wavelengths.

Conclusion

In conclusion, we showed that there is a correlation between improving the human sperm motility and low-level laser irradiation at certain doses. However, further studies are necessary to broaden the theoretical and practical knowledge of stimulatory effect of laser beam on the fertilizing ability of human sperm. An understanding of the stimulatory effect of light on the fertilizing ability of spermatozoa could lead to novel methods for both contraception and the treatment of infertility in human beings.

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