

Sperm Hyperactivation and Capacitation Induced By Light Stimuli in Cryopreserved Semen

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Abstract In mammals, such as rabbits, there are some factors involved in a possible fertilization, from complex changes in the membrane of the sperm to obstruction or non-existent of vas deferens, which creates problems in the number and quality of sperm. In this work, we report effects of rabbit sperm motility and capacitation of cryopreserved semen samples under light stimuli. The sperm velocities were correlated with the percentage of capacitated and non-capacitated sperm seen with a fluorescent dye. Consequently, we analyzed the specific correlations between irradiation times, supplied energy and fertility parameters.

Keywords: Sperm capacitation, Fertility, light stimuli

1. INTRODUCTION

Sperm maturation process takes place once sperm gets out of the testis, continues along their passage through the epididymis and finish when they have lost the cytoplasmic droplet and have acquired motility. When sperm arrives at the deferent duct, they are ready to be ejaculated and deposited inside the female's reproductive tract, where they must interact with the oviductal tissue to acquire hyper-motility that will help them reach the liberated oocyte.

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Once they encounter the oocyte, sperm must suffer the acrosome reaction to complete the fertilization process.

When it comes to humans, some studies show that 15% of the couples, in which the fertility status is unknown and have been maintaining unprotected sexual intercourse, will have difficulties to conceive. Approximately in 30% of couples, infertility is due to the male factor, and 20% is a combination of both: female-male; this means that male factor is involved in near 50% of the infertile couples [1,2]. However, there are many options for assisted reproductive treatment, among them, the cryopreservation of semen is a useful procedure to keep the fertilization ability in men with ailing. In the case of non-human animals, the infertility is a serious problem because of pollution factors and environmental stress due to climate change. Increasingly there are more endangered species as result of a drastic decrease in their reproductive capacity due to harmful effects on their metabolism caused by pollutants in the air like nitrogen dioxide, sulfur dioxide and carbon monoxide [14,15,16].

The techniques of sperm freezing have been very useful for assisted reproduction, however, the integrity of the sperm show notable changes after being subjected to a cryopreservation process. The cooling, freezing and unfreeze involved in the cryopreservation, contribute to the structural damages in the sperm, mainly alterations in the plasmatic membrane, acrosome and mitochondria have been reported. As result of this damages, the fertility potential of cryopreserved sperm is reduced [5].

Motility is one of the most important parameters of seminal quality and is evaluated through curvilinear velocity (VCL), average path velocity (VAP), straight line velocity (VSL) and linearity (LIN) [3,5,6]. These parameters increase with sperm capacitation, that is acquired during their journey through the female tract and is characterized by a flagellar movement in which a wide flagellar beating and a lateral head displacement are observed, known as hyperactivation. For achieving artificial fertilization, an *in vitro* sperm capacitation procedure must be performed with the help of culture medium and human albumin supplementation, different hormones, and enrichment medium [9]. Currently, there are several techniques used for *in vitro* sperm capacitation. Viable spermatozoa with acceptable motility obtained with this procedure can be used in assisted reproduction techniques, such as intrauterine insemination, *in vitro* fertilization or intracytoplasmic injection, due to the minimal number of gametes that are required to carry out the reproductive procedures to increase the probability of success [8].

Electromagnetic irradiation is a widely-used tool to stimulate biological systems energetically. Depending on the wave length, dosage, and the state of the irradiated tissue, a laser beam over a tissue can induce an anti-inflammatory

effect, pain reduction, and the acceleration of cell proliferation, among others [5, 7].

In previous studies, different cell types have been exposed to low-energy light radiation, several have used lasers to immobilize spermatozoa, but very few have used it to stimulate them [3]. It has been proven that low-energy laser radiation over spermatozoa can increase the motility of these [5]. The radiation effects are more outstanding when cells or tissues are under stress (due to a lack of oxygen, glucose, among others) [3]. The biological mechanisms of interaction with the low energy laser are not entirely known. It is known; however, that different cell types do not behave the same way when they are irradiated by the same wavelength [5].

It is very recurrent to use a Computer-Assisted Sperm Analysis system (CASA) to determine the effect that a laser beam has on the different motility parameters of the spermatozoa, as it provides higher objectivity, sensitivity, and reliability than the direct observation analysis method [4], since it reduces human error [11]. Conventional CASA systems allow the determination of sperm movement characteristics but do not incorporate dynamic properties such as accelerations, viscosity, and shear stress.

2. METHOD

2.1 Semen donors, housing, and feeding

All animals were kept in individual wired cages (60 × 80 × 40 cm) and were maintained under farm conditions, with natural lighting and temperature conditions. Feed and water were administered *ad libitum*, using metal feeders, giving commercial brand pellets for rabbit maintenance, while automatic nipples provided water. The Institutional Bioethical Committee approved all management conditions.

2.2 Semen extraction and cryopreservation

Semen of 13 sexually matured bucks of 18 months of age, and previously trained, was collected, using an artificial vagina. Semen collection was performed twice a week, during the spring season (March to May). The semen evaluation was performed on all semen samples, but only those that covered the inclusion criteria were incorporated into de semen pool (semen parameters for inclusion criteria: 80% motility or above, 80% viability or above, and less than 20% of morpho-anomalies).

Semen pool was diluted in a 1:6 ratio with a commercial semen diluent, which was prepared as follows: 60% distilled water, 20% egg-yolk and

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20% Triladyl™ (Minitube, Germany). The first step of dilution was done in a 1:1 ratio, having both, semen and diluent tempered at 37°C, then, the rest of the diluent was added after 5 minute periods, dividing the final diluent volume into three equal portions, which were gradually mixed with the semen sample, however, the dilution temperature was gradually lowered to ambient temperature (about 20°C).

Once the final volume was completed, diluted semen was packed into 0.5 cc plastic straws; then they were kept under refrigeration for 40 minutes at 4 °C. After that, cryopreservation was done by exposing semen straws to nitrogen vapor, placing them 5 cm above liquid nitrogen for 10 min, then, straws were plunged into liquid nitrogen and finally kept into cryogenic tanks, for further evaluation.

2.3 Semen thawing

Thawing was carried out by withdrawing the straws from the cryogenic tank and keeping the straws at room temperature for a few seconds. Then, they were placed in a 37 °C water bath, the straws were cut, and the semen was deposited in 1.5 ml plastic tubes, to proceed with the sample irradiation and proper evaluation.

2.4. Semen samples irradiation

A Fermion I continuous wave solid state (CW) laser with a 405 nanometers wavelength and 20 to 100 mW variable power, was used to irradiate the semen samples. In the present study, a power of 70 mW was used, and the samples were divided to be exposed to radiation into two groups: The first group was irradiated for 60 seconds (4.2 joules) and the second group for 90 seconds (6.3 joules). In all cases, 15µL semen samples were placed on glass slides, which were perpendicularly irradiated on an effective 4.5 cm² surface, while being placed inside a thermoregulatory chamber at 37°C. Density energy in each sample irradiated was 15.5 mW/cm². The control group was not irradiated but were subjected to the same experimental conditions as other groups. In contrast, two treatment groups were irradiated, under the same conditions, but samples of 20 µL were placed over a glass slide, inside the chamber. After that, semen evaluation with the CASA software, as well as the capacitation status was carried out.

2.5. Sperm capacitation test

Cryopreservation promotes plasma membrane as well as cytoskeleton changes, which induced what is called cryo capacitation; therefore, the capacitation

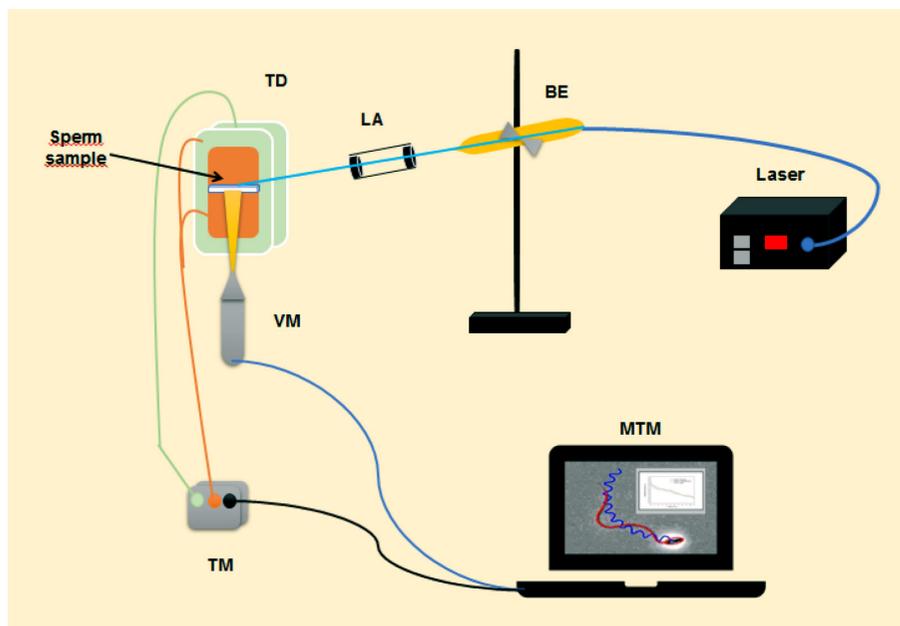


Figure 1: Experimental setup. The sperm samples were placed on a glass slide that was enabled for measuring temperature. The samples were excited by our blue 405 nm laser, and the sperm motion was recorded with a high-resolution video camera. (BE) Beam expander, (LA) Lens array, (MTM) Monitor of temperature and motility, Sperm Simple, (TD) Thermoregulator, Device (TM) Temperature microcontroller and (VM) Video microscope.

status must be evaluated. To do so, the chlortetracycline (CTC) test, was carried out on the frozen-thaw samples, on the control (without irradiation), as well as on the two irradiated semen groups (60 and 90 sec). Following the method described by García-Macedo *et al.*, (2001), with a few modifications. Briefly, one drop of 20 μl of CTC solution (750 μmol CTC, 130 mmol NaCl, 5mmol cysteine and 20mmol tris-Cl, pH7.7) was mixed with the same amount of semen, either irradiated or not. Then, semen-CTC samples were incubated during 30 sec. After that, 2 μl of 12.5% glutaraldehyde in Tris-HCl solution (2 mmol, pH 7.8) were added for fixation. Then, 10 μl of this mixture was smeared on a warmed glass-slide, which was covered with 10 μl of mounting media (PBS-Glycerol 1:1), and were observed under 100X magnification, in an epifluorescence microscope (Nikon, Eclipse 90i, Japan). One hundred cells per group were counted for capacitation status analysis of each experimental group, with three repetitions, classifying the three possible statuses: capacitated (B), non-capacitated (F), and acrosome reacted (AR).

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2.6 Motility analysis

The recorded videos were exported to extract each frame of the videos as a PNG image sequence using the open source software VirtualDub v1.10.4 and the FFMpeg Input plugin. To process and edit the image sequences, we used the open source software ImageJ v1.50i and the MosaicSuite Background Subtractor plugin. The image sequences were processed by applying different options.

We developed a JAVA plugin for ImageJ, similar to the commercial versions of a Computer Assisted Sperm Analyzer (CASA), which provide us information such as Curvilinear velocity (VCL), Straight line velocity (VSL), Average path velocity (VAP), Linearity (LIN) and Balancing (WOB).

3. RESULTS AND DISCUSSION

To carry out the motility study of both fresh and cryopreserved samples, the viscosity was estimated using high-resolution rheometry. The values were in the range of 0.008 and 0.014 Pa.s for the fresh samples, while for the cryopreserved samples a range of 0.16-0.23 Pa.s was obtained as shown in figure 2

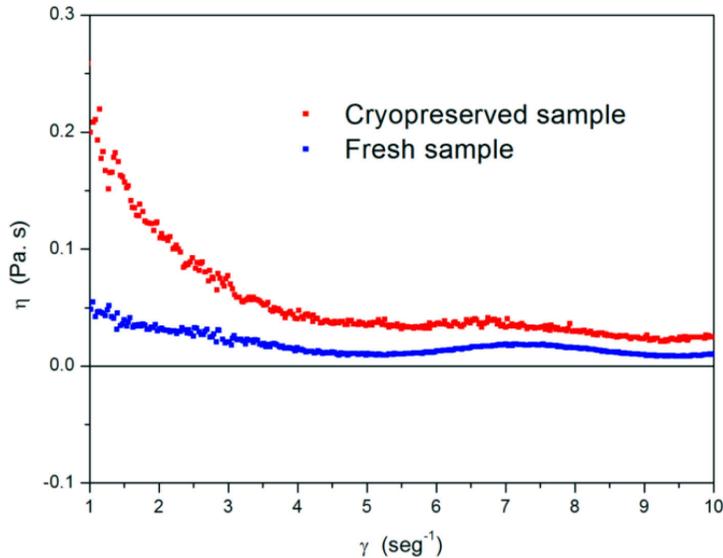


Figure 2: The graph compares the viscosity (η) as function of shear rate (γ) between a sample of fresh sperm (blue squares) and one that was previously cryopreserved (red squares).

The viscosity values were used in the estimation of motility with CASA. Fresh samples were capacitated and analyzed. Table 1 shows the results and a significant increase in velocities was observed, the trajectories are not rectilinear and are in agreement with the normal parameters of motility

Concerning the cryopreserved samples, they were irradiated with a blue laser beam at 4.2 joules and 6.3 joules. We chose a power of 70 mW because this represents a less time of exposure of the sample and therefore a lower sperm stress due to changes in temperature. The results are shown in Table 2, and we can see that the laser light stimulus effectively increases the sperm motility of the thawed samples

Table 1: Motility evaluation results of three Capacitated (C) and Non-Capacitated (NC) fresh sperm samples.

	Sample 1		Sample 2		Sample 3	
	C	NC	C	NC	C	NC
VCL ($\mu\text{m}/\text{sec}$)	64.48 \pm 17.1	54.66 \pm 15.2	72.53 \pm 20.7	61.06 \pm 15.18	68.89 \pm 14.5	57.88 \pm 19.1
VAP ($\mu\text{m}/\text{sec}$)	40.90 \pm 15.4	37.39 \pm 11.6	58.63 \pm 12.5	50.59 \pm 17.4	57.98 \pm 21.2	41.98 \pm 15.7
VSL ($\mu\text{m}/\text{sec}$)	24.38 \pm 9.1	14.31 \pm 6.3	38.95 \pm 5.8	35.66 \pm 11.1	27.96 \pm 8.9	25.78 \pm 9.0
LIN (%)	59.62 \pm 20.6	38.28 \pm 13.1	50.37 \pm 14.2	70.50 \pm 20.7	55.89 \pm 14.9	39.78 \pm 14.5
WOB (%)	63.42 \pm 15.4	68.40 \pm 21.5	51.87 \pm 17.6	84.84 \pm 23.2	59.23 \pm 21.5	47.87 \pm 25.7

Table 2: Evaluation of motility in irradiated thawed sperm samples show that velocities increasing.

	CONTROL	4.2 JOULES	6.3 JOULES	p
VLS ($\mu\text{m}/\text{s}$)	0.8359 \pm 0.23	13.6214 \pm 1.0005	14.3342 \pm 0.71	<0.05
VAP ($\mu\text{m}/\text{s}$)	0.9821 \pm 0.12	18.8359 \pm 0.68	17.8359 \pm 0.34	<0.05
VCL ($\mu\text{m}/\text{s}$)	1.2699 \pm 0.31	22.2184 \pm 1.3003	23.0023 \pm 0.8769	<0.05
LIN (%)	66.5 \pm 1.2	61.32 \pm 1.8	62.3 \pm 1.2	0.003
WOB (%)	77.9 \pm 2.1	84.7 \pm 2.3	73.9 \pm 2.7	0.007

To evaluate the effects of irradiation on capacitation, a CTC test was carried out and results in Table 3 are evidence that the stimulation of laser light in thawed samples favors capacitation

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Table 3: The percentage of spermatozoa non-capacitated (F) and capacitated (B), with three groups of irradiated spermatozoa with their corresponding repetitions C1, C2 and C3.

	NON CAPACITED (F)			CAPACITED (B)		
	Irradiation energy			Irradiación energy		
	0	4.2 Joules	6.3 Joules	0''	4.2 Joules	6.3 Joules
C1	12	13	5	28	53	60
C2	19	9	5	28	66	64
C3	14	12	4	29	56	61
Media=	15	11	5	28	58	62

On the other hand, to evaluate the possible damage due to irradiation a sperm vitality test was performed using an eosin-nigrosin staining. In this study, the membrane of the dead spermatozoa is permeable to the dye, and it is possible to observe the percentage of viable and non-viable spermatozoa. The results of Table 4 show that the sperm viability of the irradiated samples is affected between 15 and 22 % compared to the non-irradiated samples

Similar studies have been reported that irradiation of human sperm with visible light (400-800 nm) and reactive oxygen species (ROS) production is induced in mitochondria, activating the Src and EGFR proteins involved in sperm hyperactivation [19]. In a similar investigation, improve in bull (*Bos taurus indicus*) sperm motility, and acrosome membrane integrity after irradiation with red light (660nm) was proved [5]. Also, treatment with red (660nm) and white (400-800 nm) light was proved to increase motility and fertilization in tilapia sperm, while in ram sperm only red light effects have been observed. Additionally, the use of UV light (294nm) and blue light (360nm) generate a high amount of intracellular ROS which decreased motility and fertility in both species (Zan-Bar *et al.*, 2005).

Table 4: Show the percentage of viable and non-viable spermatozoa for the three groups under study.

Group	Viable spermatozoa (%)	Non-viable spermatozoa (%)
0 Joules	48	52
4.2 Joules	37	63
6.3 Joules	41	59

CONCLUSIONS

With our experiments, we could verify that the irradiation of cryopreserved sperm samples with a blue laser increases their motility and capacitation. This phenomenon could be explained by the fact that the procedure promotes the generation of reactive oxygen species (ROS) and this highly oxidative process increases tyrosine phosphorylation in sperm proteins and promotes such hyperactivation and sperm capacitation.

The increase in sample viscosity of thawed sperm affects their motility and promotes rectilinear trajectories with less oscillation. However, the capacitation and sperm viability are preserved moderately, suggesting that the stimulus of blue light in shorter periods of time could be a technique that contributes to thawing processes, of course without neglecting any careful study of possible DNA damage that complements the present study.

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