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ORIGINAL ARTICLE

Does low-level laser therapy on degenerated ovine testes improve post-thawed sperm characteristics?

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Abstract

Low-level laser therapy (LLLT) can modulate redox state of the cell which could be useful to treat testicular degeneration and also prevent injuries by sperm cryopreservation. The aim of this study was to evaluate the effects of LLLT treatment on semen cryopreservation from rams submitted or not to testicular degeneration by testicular insulation. Eleven White Dorper rams were divided into four groups: animals that were not insulated (Control) and not treated (No Laser) ($n = 2$); animals that were not insulated and treated with LLLT ($n = 3$); animals that were insulated and not treated with LLLT ($n = 3$), and animals that were insulated and treated with LLLT ($n = 3$). Testicular insulation was performed using scrotal insulation bags for 72 h. LLLT treatment was 28 J/cm^2 energy, 808 nm of wavelength, and 30 mW of power output, irradiated on testis for 15 days with an interval of 48 h. Three ejaculates from each ram were collected: before insulation, 23, and 59 days after insulation bag removal. Cryopreservation was performed of the third ejaculate. Sperm evaluation was performed before and after cryopreservation considering sperm motility, morphology, acrosomal and plasma membrane integrity, mitochondrial potential, and oxidative stress. As expected, cryopreservation had a negative effect on several sperm motility characteristics and sperm membranes. LLLT treatment did not improve sperm quality from rams submitted to testicular insulation. Thus, testicular insulation and cryopreservation effects on spermatozoa were not attenuated by LLLT in this study.

Keywords Biostimulation · Semen · Cryopreservation · Heat stress · Ram

Introduction

Testicular degeneration (TD) is considered a cause of subfertility and infertility, which is commonly caused by heat stress in mammals [1]. Although its pathogenesis is not fully understood, the increase of cell metabolism occurs during the TD process without proportional rise of vascularization [2]. The consequences are testis hypoxia and production of reactive oxygen species (ROS) [3]. ROS in small amounts are important to sperm capacitation but, in great concentrations, overcome endogenous antioxidant substances produced by testis and cause oxidative stress [4]. This, in turn, promotes sperm plasma membrane peroxidation and DNA fragmentation [4], leading to cellular apoptosis. In humans, testicular heat stress can result in fertilization failure or embryonic death due to sperm abnormalities [5]. These conditions also occur in bovines [6, 7] and ovines [8]. In addition, increase in scrotal and testis temperature and consequent disruption of testicular thermoregulation are responsible for defective

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spermatogenesis, which leads to sperm abnormalities and decrease of progressive sperm motility [7–9]. There is no currently effective treatment for TD. Even though removal of the harmful stimulus and administration of nutraceuticals are the only treatments recommended, neither are very effective in some cases [10, 11].

Moreover, the process of semen cryopreservation is also known to cause an imbalance of ROS [12]. During semen cryopreservation, injuries occur on plasma and acrosome membranes and on sperm cell mitochondria [13–15]. In addition, the semen cryopreservation process can lead to DNA fragmentation and apoptosis of sperm cells [16]. Thus, damage caused by the cryopreservation process, known as cryoinjuries, leads cryopreserved semen to present lower fertility than fresh semen [17]. However, extended storage time of semen is necessary to preserve specific male germline and also improve implementation of assisted reproductive techniques. In human reproduction and, in many cases, in veterinary medicine as a genetic preservative, semen even with poor quality must, however, be cryopreserved. The combination of bad semen quality and cryopreservation may have an even wider negative effect on pregnancy rates.

Low-level laser therapy (LLLT) has been used in many biological systems with analgesic, anti-inflammatory, and biostimulatory functions [18–20]. LLLT modulates various biological processes by increasing mitochondrial activity and ATP synthesis, changing the redox state of the cell [21]. Therefore, LLLT could extenuate the effects of testicular degeneration and, consequently, improve the cryopreservation process of the samples [22]. Thus, with the knowledge that TD causes sperm kinetics and morphology injuries and that cryopreservation can enhance these effects, the aim of the present study was to investigate the effect of testicular LLLT on post-thawed sperm quality of cryopreserved semen of rams presenting poor semen quality prior to cryopreservation.

Materials and methods

Experimental design

This study used 11 White Dorper rams (*Ovis aries*) with an average age of 15.3 ± 2.3 months and weight of 66.3 ± 7.5 kg. The rams were housed in a paddock; hay and concentrate were provided according to National Research Council (NRC, 1998) guidelines. After scrotal trichotomy, all animals that were previously evaluated for semen quality were placed into control and insulated groups. Testicular insulation was performed using three-layer insulation bags on the scrotum for 72 h to induce testicular degeneration. Rams were then homogeneously divided into four treatment groups: animals that were not insulated (Control) and not submitted to LLLT treatment (No Laser) ($n = 2$); animals that were not insulated (Control) and submitted to LLLT treatment ($n = 3$); animals that were insulated and not submitted to LLLT treatment ($n = 3$), and animals that were insulated and submitted to LLLT treatment ($n = 3$).

Insulation bag removal was considered day 0. Semen was collected and evaluated at three time points: before testicular insulation, , and 59 days after insulation bag removal.

For cryopreservation, semen was collected 59 days after insulation bag removal and analyzed before (*in natura*) and after cryopreservation. On day 23, after removal of the insulation bags, LLLT was initiated, with therapy lasting 15 days.

Low-level laser therapy protocol

This study used a gallium-aluminum-arsenide (GaAlAs) laser device with an 808-nm infrared wavelength (Thera Laser®, DCM Equipment, São Carlos, Brazil). Laser energy was applied directly to scrotal surface with a penetration of approximately 5 mm. Treatment protocol was adapted from Taha and Velojerdi [23].

Equipment was set with a power of 30 mW and 28 J of energy per cm^2 [23]. Treatment, performed on six rams (LLLT groups), lasted 15 days with a 48-h interval between applications: 5 J/cm^2 on days 1–2, 4 J/cm^2 on days 3–4, 3 J/cm^2 on days 5–6, and 2 J/cm^2 (total of 28 J/cm^2).

Semen collection and pre-cryopreservation analysis

Semen samples were collected using artificial vagina. Immediately after collection, *in natura* semen was maintained at 37 °C and analyzed considering characteristics of volume (mL), subjective motility (%), concentration, morphology, computer-assisted sperm analysis (CASA), plasma, acrosomal and mitochondrial membrane integrity, and production of ROS.

Subjective motility was assessed between slide and coverslip using phase contrast microscopy (× 100 magnification, Nikon, Eclipse 80i). Sperm concentration was performed by diluting semen 1:1000 in 4% formaldehyde-phosphate buffered saline (PBS), and cells were counted using a Neubauer chamber (× 400 magnification, Nikon, Eclipse 80i). Sperm morphology was analyzed by microscopy of differential interference contrast (DIC), under immersion oil (× 1000 magnification) in wet preparation analyzing 200 cells (Nikon Eclipse, 80i, Tokyo, Japan). Abnormal sperm cells were classified according to Blom [24] in percentage of major, minor, and total defects, as shown in Table 1.

Sperm kinetic parameters were analyzed regarding: total motility (TM, %), progressive motility (PM, %), rapidly progressively motile spermatozoa (RAP, %), straight-line velocity (VSL, μm/s), average path velocity (VAP, μm/s), curvilinear velocity (VCL, μm/s), linearity (LIN, %), amplitude of

Table 1 Classification of morphologically abnormal spermatozoa in major and minor defects adapted from Blom (1973)

Major defects	Minor defects
Acrosome defects	Head defects:
Proximal droplet	Narrow
Head defects:	Giant, short, broad, and small normal
Underdeveloped	Free normal
Tail curled in the head	Abaxial, retroaxial, oblique implantation
Free pathological heads	Simple bent tail
Narrow at base	Distal droplet
Pear-shaped defect	
Small abnormal heads	
Abnormal contour	
Pouch formation	
Double forms	
Midpiece defects: Corkscrew defect, pseudodroplet, tail stump, broken neck, swelling, and others	
Tail defects:	
Strongly coiled or folded tail	
Coiled with distal droplet attached	

lateral head displacement (ALH, μm), beat cross frequency (BCF, Hz), and straightness (STR, %) using Sperm Class Analyzer (SCA®, Microptic SL, Barcelona, Spain). The settings were previously programmed for ram sperm (Table 2).

Plasma membrane integrity, acrosome membrane integrity, and mitochondrial membrane potential were evaluated by fluorescent probes combination protocol: propidium iodide (PI), fluorescein isothiocyanate-conjugated *Pisum sativum* agglutinin (FITC-PSA), and 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl carbocyanine iodide (JC-1) described by Celeghini et al. [25]. This analysis used epifluorescence microscopy (Nikon, model 80i) at × 1000 magnification using a triple filter (D/F/R, C58420), featuring the UV-2E/C (340–380 nm excitation and 435–485 emission), B-2E/C (465–495 excitation and 515–555 emission), and G-2E/C (excitation 540–525 and 605–655 emission). A total of 200 sperm cells were evaluated considering the percentage of cells with plasma membrane integrity (PI), acrosome integrity (AI), high potential mitochondrial membrane (HP), and sperm simultaneously showing plasma and acrosome membrane integrity and high potential of mitochondrial membrane (AIPIHP).

Table 2 Setup of computer-assisted sperm analysis for ovine semen using Sperm Class Analyzer (SCA, Microptic, Barcelona, Spain)

Characteristics	Adjusted to
Number of acquired images	25
Rate of acquired images	24/s
Minimal cell size	3 μm^2
Curvilinear velocity (VCL) for rapid cells	> 75 μm/s
Curvilinear velocity (VCL) for slow cells	< 45 μm/s
Straightness (STR)	> 80%
Linearity (LIN)	< 50%
Temperature	37 °C

Production of reactive oxygen species (ROS) was measured by CellRox Deep Red® fluorescent probe as described by Alves et al. [26]. Two hundred cells were analyzed by epifluorescence microscopy (Nikon, Eclipse 80i) at × 1000 magnification using a triple filter (D/F/R, C58420) concerning the aspect of sperm midpiece. Positive sperm in production of ROS were considered when sperm midpiece was stained in pale or intense red.

Semen cryopreservation

Individual ejaculates were diluted in a commercial egg yolk-based extender BotuBov® (Botupharma, Botucatu-SP, Brazil) with the final concentration of 100×10^6 sperm/mL. Diluted semen, previously identified by ram and treatment number, was packaged in 0.5-mL French straws. Cryopreservation of semen samples was performed using Tetakon TK 3000 controlled-rate cooling machine (TK Technology Ltd., Uberaba-MG, Brazil). The cooling rate was previously set for ram semen. Sperm samples were cooled to 5° at − 0.25 °C/min, and further to − 120 °C at − 20 °C/min. Afterward, straws were immediately immersed in liquid nitrogen (− 196 °C) and stored.

Post-cryopreservation semen analysis

For analysis, two straws per batch from each ram were thawed in a water bath at 37 °C for 30 s. Post-thawed semen was analyzed considering characteristics of sperm morphology, computer-assisted sperm analysis, plasma, acrosomal and mitochondrial membranes and ROS production, as described for pre-cryopreservation analysis.

Statistical analysis

Information from each ram was detailed in spreadsheets for statistical analysis. Research design applied completely randomized factorial design (2 × 2 × 2) with two replications. Factors consisted of three levels: (1) insulation (with or without), (2) laser therapy (treated or not), and (3) cryopreservation (*in natura* or frozen/thawed), comprising a total of eight treatment combinations.

The data were analyzed by the procedures from SAS (SAS Inst. Inc., Cary, NC, USA, version 9.4). Continuous variables were tested using the PROC GLIMMIX according to their

homogeneity, and normality of variances using SAS Guide Data Analysis. Variables that did not follow these assumptions were transformed accordingly, and outliers were removed when necessary. The fixed effects in the model were insulation, laser therapy and cryopreservation, and their interactions. The residual effect was included as a random effect. All results were reported as least squares means, and means were separated using Tukey's test. Means (± SE) were used to describe all response variables. Differences with $p \leq 0.05$ were considered significant and $0.05 < p \leq 0.10$ were designated as a tendency toward a difference for the explanatory variables evaluated.

Results

Table 3 shows the results of the sperm characteristics of *in natura* semen from rams before scrotal insulation and 23 days after insulative bag removal. Note that groups were similar prior to insulation, and 23 days later, total sperm motility and progressive motility decreased in the insulated group. On the other hand, sperm production of reactive oxygen species and major and total sperm defects increased after scrotal insulation characterizing testicular degeneration. Upon confirmation of testicular degeneration, 15-day laser therapy began.

Fifty-nine days after insulation bag removal, no interaction was found for scrotal insulation, sperm cryopreservation, and LLLT, as presented in Tables 4, 5 and 6. There was an exception in Table 5, where interaction was found between cryopreservation and scrotal insulation to percentage of cells producing ROS ($p = 0.0481$). Before cryopreservation, in *in natura* semen, percentage of sperm cells producing ROS in the insulated groups was higher than the percentage in not insulated groups. Regarding post-thawed cryopreserved semen, the percentage of sperm cells producing ROS was higher on not insulated rams treated with LLLT than in not insulated rams and not treated with LLLT and insulated rams treated or not with LLLT (Table 5).

Table 4 demonstrates results of sperm kinetic parameters. The cryopreserved semen decreased in TM ($p = 0.0034$), PM ($p = 0.0008$), RAP ($p = 0.0006$), VSL ($p = 0.0005$), VAP ($p = 0.0004$), VCL ($p = 0.0011$), and LIN ($p = 0.0058$) when compared to *in natura* semen. No scrotal insulation effect was observed on any of the sperm kinetic characteristics. Furthermore, LLLT was not efficient to improve sperm characteristics since no difference was found between groups; only STR was lower ($p = 0.0354$) to LLLT group than the No Laser group.

Moreover, negative effects of cryopreservation were also found on the sperm membranes (Table 5). It was noted that the percentage of cells with intact acrosome membrane ($p = 0.0038$), plasma membrane integrity ($p < 0.0001$), high mitochondrial membrane potential ($p < 0.0001$), and cells with these three characteristics simultaneously ($p < 0.0001$) decreased after cryopreservation. In addition, no effect was noted on scrotal insulation and LLLT on sperm membranes; although, there is a tendency ($p = 0.07$) of interaction of LLLT and cryopreservation on total of cells presenting intact plasma and acrosome membrane and high mitochondrial membrane potential which shows that probably LLLT increase the percentage of these cells independently of scrotal insulation.

Table 6 shows the effects of scrotal insulation, cryopreservation, and LLLT on abnormal sperm. We observed that the percentage of minor sperm defects were higher ($p = 0.011$) in the insulated group than control group. However, no effect was found for cryopreservation and LLLT.

Discussion

On the present study, rams were submitted to scrotal heat stress in order to induce testicular degeneration and injuries to semen quality. Once induced injuries to semen quality, the rams were treated with LLLT on testis and semen was collected, analyzed, and cryopreserved with the intention of evaluating the effects of LLLT on cryopreservation process.

Table 3 Mean ± SE of total motility (TM), progressive motility (PM), sperm-producing reactive oxygen species (ROS), major sperm defects (MJD), minor sperm defects (MID), and total sperm defects (TD) from ovine *in natura* semen collected before experimental insulation and 23 days after insulation in Control and Insulation groups. *P* value for effects of groups

Sperm characteristics	Before insulation		*p* value	After insulation (23 day)		*p* value
	Control	Insulation		Control	Insulation	
TM (%)	85.65 ± 5.08	84.47 ± 5.47	0.89	68.66 ± 3.87[a]	51.60 ± 2.99[b]	0.01
PM (%)	53.82 ± 4.69	55.1 ± 6.27	0.87	49.60 ± 2.71[a]	37.12 ± 2.35[b]	0.02
ROS (%)	1.2 ± 1.2	0.59 ± 0.42	0.62	0.25 ± 0.14[b]	1.00 ± 0.14[a]	0.01
MJD (%)	13.2 ± 3.03	10.5 ± 3.16	0.56	24.87 ± 11.83[b]	64.50 ± 10.58[a]	0.04
MID (%)	4.8 ± 1.05	6.59 ± 2.1	0.49	3.75 ± 2.23	3.00 ± 1.99	0.8
TD (%)	18 ± 3.31	17.09 ± 2.57	0.83	28.62 ± 10.06[b]	67.50 ± 9.00 [a]	0.02

[a, b] Different superscript lower case letters on the same line indicates statistical difference between treatments ($p<0.05$)

Table 4 Mean ± SE of total motility (TM), progressive motility (PM), rapid cells (RAP), straight-line velocity (VSL), average path velocity (VAP), curvilinear velocity (VCL), linearity (LIN), amplitude of lateral head displacement (ALH), beat cross frequency (BCF), and straightness (STR) in *in natura* and post-cryopreserved semen collected from rams submitted (Insulation) or not (Control) to testicular insulation and treated (LLLT) or not (No Laser) with low-level laser therapy. *p* value for effects of cryopreservation (CRY), insulation (INS), and low-level laser therapy (LLLT) and interactions effects

Sperm Characteristics	*in natura*				Post-cryopreservation				*p* value						
	Control		Insulation		Control		Insulation		Treatment effects			Interaction effects			
	No Laser	LLLT	No Laser	LLLT	No Laser	LLLT	No Laser	LLLT	CRY	INS	LLLT	INS vs LLLT	INS vs CRY	LLLT vs CRY	INS vs LLLT vs CRY
TM (%)	62.9 ± 14	53.9 ± 14.6	62.3 ± 0.9	47.8 ± 11	26.1 ± 9	33.9 ± 13.3	27.5 ± 10.3	28.8 ± 7.6	0.0034	0.75	0.66	0.71	0.92	0.32	0.98
PM (%)	52.7 ± 12.3	39.6 ± 15.6	50.2 ± 3.3	38.9 ± 12.1	16.9 ± 4.6	20 ± 7.8	11.9 ± 3.6	17.4 ± 6.6	0.0008	0.70	0.57	0.88	0.87	0.24	0.98
RAP (%)	52.3 ± 13.3	44.1 ± 13.6	48.9 ± 6.4	40 ± 12.7	16.1 ± 6.8	22 ± 8.7	10.7 ± 2	16.3 ± 6.3	0.0006	0.51	0.84	0.97	0.90	0.31	0.99
VSL (μm/s)	116.7 ± 20.2	94.9 ± 5.0	100.5 ± 17.5	98.8 ± 10.5	73.3 ± 7.8	75.5 ± 1.6	57.3 ± 10.5	61.1 ± 9.0	0.0005	0.20	0.59	0.51	0.58	0.37	0.57
VAP (μm/s)	124.9 ± 22.2	110.8 ± 2.2	109.2 ± 19	111.4 ± 7.4	79.6 ± 10.7	87.7 ± 2.3	66.5 ± 11.2	71.8 ± 8.7	0.0004	0.20	0.96	0.69	0.68	0.45	0.57
VCL (μm/s)	136.1 ± 19.3	121.3 ± 4.4	115.9 ± 19	120.5 ± 7.2	90 ± 14.4	103.4 ± 2.5	81.6 ± 10.8	84.6 ± 9.1	0.0011	0.16	0.85	0.79	0.85	0.43	0.38
LIN (%)	85.4 ± 2.8	78.5 ± 5.5	86.4 ± 1.1	81.7 ± 5.0	82.2 ± 4.5	73 ± 1.4	69.2 ± 4	71.5 ± 3.4	0.0058	0.37	0.12	0.24	0.11	0.67	0.41
ALH (μm)	2.6 ± 0.2	2.3 ± 0.3	1.9 ± 0.2	2.1 ± 0.2	2.1 ± 0.4	3.0 ± 0.1	2.7 ± 0.4	2.5 ± 0.4	0.14	0.36	0.50	0.59	0.27	0.43	0.09
BCF (Hz)	10.6 ± 0.4	8.7 ± 0.4	9.0 ± 0.2	9.9 ± 0.9	9.3 ± 0.6	9.7 ± 0.5	8.2 ± 0.4	8.8 ± 0.6	0.18	0.17	0.94	0.09	0.31	0.23	0.13
STR (%)	93.5 ± 0.5	85.7 ± 4.6	92.0 ± 0.1	88.2 ± 3.9	92.5 ± 2.6	86.1 ± 1.8	85.6 ± 1.9	84.4 ± 2.7	0.21	0.37	0.0354	0.28	0.27	0.63	0.88

Testicular degeneration was induced by scrotal insulation that is a usual technique performed by heat stress simulation that can be performed differently according to stimulus duration and intensity and has been a good model to study testicular degeneration [6]. Damage to sperm morphology [27], decline of motility parameters [27], and ultrastructural damage [28] are expected after TD. In the present study, the significant effect of TD was noted at 23 days after scrotal insulation bag removal, characterized by reduction of total and progressive motility, increase of oxidative stress, and major and total sperm defects.

Arman et al. [27] insulated rams for 16 h a day for 21 days, evaluating *in natura* and frozen/thawed semen until 21 days after the start of insulation. They found that this intermittent protocol caused not only the reduction in motility characteristics in *in natura* semen, but also in frozen/thawed semen. Frozen semen had worse results of motility parameters than *in natura*, showing that insulation could make the sperm cell less able to support damage by cryopreservation. In our study, we observed that cryopreservation was deleterious technique to sperm cells. Regardless of efforts to reach the optimal cryopreservation method and using proven safe and effective techniques, sperm damage is unavoidable [29]. Intracellular and extracellular water changes are one of the main causes of cryopreservation injuries, called cryoinjury [30], and also of sperm damage during rewarming. Formation of intracellular and/or extracellular ice crystals associated with osmotic shock are the main principles of cryoinjury [14, 31–33].

Motility is one of the parameters that is most affected by cryopreservation [34]. In spite of that, ram semen has a relatively high proportion (40–60%) of preserved motility after thawing [35], as observed in the results of this study. Even so, the motility parameters except ALH, BCF, and STR decreased after cryopreservation (Table 4), which was similar to those reported by Cruz Junior et al. [28] in rams. Other traits affected by cryopreservation were plasma and acrosome membrane integrity and mitochondrial potential. The results of Salamon and Maxwell [36] demonstrated that only 20–30% of the cells remained biologically undamaged. In other words, 20–30% of the cells had intact plasma membrane, intact acrosome, and high mitochondrial potential after thawing. In our study, only 8% of cells remained biologically undamaged.

Concerning ROS production, there was an interaction (p = 0.0481) between cryopreservation and insulation on sperm cells producing ROS. *In natura* semen had a greater percentage of sperm cells producing ROS in insulated group than control group. This is expected considering that insulation causes an increase in testicular temperature, resulting in an increase of cell metabolism and, consequently, ROS increase [37]. CellROX Deep Red®, the probe used in this study, could only detect hydroxyl and peroxide present on viable cells. In fact, sperm cryopreservation process increases production of ROS. However, after cryopreservation, only the group of rams

Table 5 Mean ± SE of acrosome integrity (AI), plasma membrane integrity (PI), high mitochondrial potential (HP), sperm plasma and acrosome membranes integrity and high mitochondrial potential (AIPIHP), sperm-producing reactive oxygen species (ROS) in *in natura* and post-cryopreserved semen collected from rams submitted (Insulation) or not (Control) to testicular insulation and treated (LLLT) or not (No Laser) with low-level lasertherapy; *p value* for effects of cryopreservation (CRY), insulation (INS), and lasertherapy (LLLT) and interactions effects

Sperm characteristics	*in natura*				Post-cryopreservation				*p* value			*p* value			
	Control		Insulation		Control		Insulation		Treatment effects			Interaction effects			
	No Laser	LLLT	No Laser	LLLT	No laser	LLLT	No Laser	LLLT	CRY	INS	LLLT	INS vs LLLT	INS vs CRY	LLLT vs CRY	INS vs LLLT vs CRY
AI (%)	63.8 ± 18.3	58.5 ± 16	77.7 ± 2.9	54.5 ± 14.1	28.8 ± 5.8	35.5 ± 14.7	28.5 ± 11.6	41.2 ± 2.3	0.0038	0.67	0.80	0.73	0.90	0.19	0.50
PI (%)	62.5 ± 16.5	57.7 ± 13.8	75.7 ± 3.8	48.6 ± 13.7	8.5 ± 1.5	18.3 ± 9	13.3 ± 6.2	14.5 ± 2.6	< 0.0001	0.86	0.47	0.29	0.91	0.15	0.64
HP (%)	56 ± 18.5	56 ± 11.4	72.5 ± 5.8	34.2 ± 9	5.3 ± 3.3	10.5 ± 5.3	9.7 ± 4.7	10 ± 5.2	< 0.0001	0.96	0.19	0.09	0.71	0.09	0.18
AIPIHP (%)	53.3 ± 17.8	50.2 ± 12.9	68.7 ± 4.9	31.6 ± 8.2	5 ± 3.5	8.8 ± 4.5	8.2 ± 3.8	9.5 ± 4.8	< 0.0001	0.98	0.16	0.14	0.77	0.07	0.20
ROS (%)	3.2 ± 0.1^{b}	2.4 ± 1.8^{b}	6.7 ± 0.9^{a}	7.8 ± 3.1^{a}	2.3 ± 1.8^{b}	7 ± 3.1^{a}	2.7 ± 1.4^{b}	2.7 ± 0.7^{b}	0.37	0.41	0.40	0.63	0.0481	0.47	0.29

Different letters on the same line indicates statistical difference ($p < 0.05$)

that were not submitted to scrotal insulation and treated with LLLT presented higher percentage of cells producing ROS. Rams that were not submitted to scrotal insulation and not treated with LLLT and rams that were submitted to scrotal insulation and treated or not with LLLT presented lower percentage of ROS production after cryopreservation. This observation was probably caused by an effect of semen extender that was probably able to neutralize the ROS produced by sperm cells in the groups except to control rams that were treated with LLLT. On this group, probably the cells were producing higher amounts of ROS in response to cryopreservation process, and semen extender was not able to neutralize them. In fact, one of attributes of LLLT is increases mitochondrial activity.

LLLT did not improve semen cryopreservation from rams submitted or not to insulation, but demonstrated a tendency ($p = 0.07$) to improve the percentage of cells with acrosome and plasma membranes integrity and high mitochondrial membrane potential (AIPIHP). The effects observed on semen characteristics, except from STR, were only due to cryopreservation and/or testicular degeneration.

There are many studies regarding LLLT applications in wound healing [19], cell proliferation and regeneration [19, 20], and antimicrobial [38] and analgesic effect [18]. However, few studies exist on laser application on the testis. Studies reported that LLLT irradiated directly on spermatozoa increases in *in natura* sperm motility in humans [39] and dogs [40] and can induce sperm capacitation and reduction of sperm damage [41]. Fernandes et al. [42] used an aluminum-gallium-indium phosphide (AlGaInP) laser before freezing bull semen samples and obtained a higher percentage of cells with plasma and acrosome membrane integrity after thawing. Dobrin et al. [43] used Ne-He laser, but the exposition was performed after thawing ram semen, and found an improvement of motility, mitochondrial function, and integrity of plasma membrane. These data corroborate with those from Ibrahim et al. [44] that found sperm progressive motility increase, viability, osmotic resistance, and acrosome integrity after LLLT treatment. However, in the studies mentioned above, laser irradiation was performed directly on the sperm cell, which means that LLLT effect was not evaluated on spermatogenesis on these studies.

Bermudez et al. [45] evaluated the effect of LLLT on rat testis and observed an increase of germ cell DNA content after laser irradiation (28 J/cm^2). In another study, Bermudez et al. [46] found that the same dose increased the percentage of DNA on spermatogonia, primary spermatocytes, and in some elongated spermatids, following one cycle of seminiferous epithelium and also shortly after exposure to LLLT. Our group previously used an energy dose of 28 J/cm^2 in ram testis and reported increase on area of seminiferous epithelium on seminiferous tubules, but observed increased of sperm damage as well [22]. On the best of our knowledge, the present study is

Table 6 Mean ± SE of sperm with major defects (MJD) and sperm with minor defects (MID) in *in natura* and post-cryopreserved semen collected from rams submitted (Insulation) or not (Control) to testicular insulation and treated (LLLT) or not (No Laser) with low-level laser therapy; *p value* for effects of cryopreservation (CRY), insulation (INS), and lasertherapy (LLLT) and interaction effects

Sperm characteristics	*in natura*				Post-cryopreservation				*p value*				*p value*			
	Control		Insulation		Control		Insulation		Treatment effects				Interaction effects			
	No Laser	LLLT	No Laser	LLLT	No Laser	LLLT	No Laser	LLLT	CRY	INS	LLLT	INS vs LLLT	INS vs CRY	LLLT vs CRY	INS vs LLLT vs CRY	
MJD (%)	5.3 ± 3.8	7.8 ± 2.9	8.2 ± 0.3	12.8 ± 5.2	13 ± 9.5	8.5 ± 3.8	5.8 ± 2.2	6.8 ± 1.1	0.99	0.93	0.73	0.49	0.14	0.34	0.76	
MID (%)	8.5 ± 3.5	9.5 ± 1.6	22.8 ± 7.2	44 ± 13.5	12.5 ± 6.5	12.5 ± 1.3	11 ± 3.5	26 ± 8.2	0.29	0.011	0.10	0.11	0.10	0.74	0.81	

the first one evaluating the effect of LLLT performed on testis under the thawed semen.

Conclusion

On the present study, scrotal insulation was efficient to induce sperm quality injuries. However, cryopreservation effects on sperm cells were not attenuated by LLLT in testis at energy of 28 J/cm^2, 808 nm of wavelength, and 30 mW of power output in the present study. Even though, LLLT presented a tendency in the increase of the percentage of AIPIHP cells after cryopreservation seems to increase the integrity of plasma and acrosome membrane and potential of mitochondrial membrane. Therefore, further studies to investigate the best protocol of LLLT for treatment of testicular degeneration and improving frozen/thawed sperm parameters are still needed.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval The experiment agrees with ethical principles in animal research adopted by "Ethic Committee in the Use of Animals" of the School of Veterinary Medicine and Animal Science of University of São Paulo, protocol number 2467/2012.

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