# The use of low-level laser therapy (LLLT) to enhance human sperm parameters

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## Abstract

Low-lever laser irradiation is believed to bioactivate biological cells through the upregulation of cytochrome c oxidase in the electron transport chain, thereby increasing adenosine triphosphate (ATP) production as well as reducing reactive oxygen species generation. As asthenozoospermia and oxidative stress impacts male fertility negatively, the aim of this study was to determine the effect of low-level laser therapy (LLLT) on human sperm function as well as the possibility of ameliorating the damage caused to spermatozoa during cryopreservation. Washed sperm samples were irradiated for 8 seconds with a 660nm red laser, at a power density of 750 $mW/cm^2$ leading to a 6J dosage. Sperm motility and viability were measured 30 and 60min after irradiation. Fresh sperm samples exposed to LLLT had a greater mean total-, progressive- and fast progressive motility compared to their respective controls, measured 30 and 60min after irradiation. The same result was obtained when comparing the viability of irradiated samples to the control samples. The increase in motility and viability was, however, not significant using ($p>0.05$). Differential exposure prior to- and post cryopreservation showed that irradiating spermatozoa preliminary to cryopreservation significantly increased fast progressive motility, when compared to the control ($P<0.001$). The most significant difference was observed when comparing the group irradiated preliminary to cryopreservation with the group which received irradiation 60min prior to- and immediately after cryopreservation ($P<0.0001$). Progressive motility was significantly higher in the group irradiated 60min prior to cryopreservation ($P<0.001$) as well as the group irradiated 60min prior to- as well as immediately before cryopreservation ($P<0.0001$) when compared to the group irradiated immediately before cryopreservation. Viability analysis showed a significant difference when the group irradiated immediately before cryopreservation and immediately after thawing was compared to the group irradiated 60min prior to cryopreservation and immediately after thawing ($P<0.001$). Based on our findings, spermatozoa exposed to LLLT had a trend of having a higher mean motility and viability than their respective controls. LLLT also proved useful in increasing fast progressive motility in cryopreserved spermatozoa.

# 1. Introduction

Low-level laser therapy uses red- and near infrared light with wavelengths ranging from 600-1000nm to bioactivate biological tissue, increasing cellular metabolism and decreasing local inflammatory conditions (Tafur and Mills., 2008). Light at these wavelengths have photochemical properties with potent anti-inflammatory potential leading to increased cellular proliferation, replication and energy production (Peplow *et al.*, 2010; Manchini *et al.*, 2014). Numerous studies report that the application of LLLT induce increased ATP production, energy metabolism and a decrease in reactive oxygen species (ROS) (Koppers *et al.*, 2008). The mechanism of action is thought to be associated with photo acceptors connected to cytochrome c oxidase in the mitochondrial electron transport chain, directly affecting energy metabolism. Increased oxidative stress lead to the binding of nitric oxide to cytochrome c oxidase in the mitochondrial electron transport chain, thereby inhibiting cellular respiration and ATP production. Low-level laser exposure induces uncoupling of the nitric oxide bound to cytochrome c oxidase, allowing oxygen to return and normal electron transport function to continue (Tafur and Mills., 2008). Numerous other studies suggest that exposure to LLLT additionally up-regulate mitochondrial function and excessive opening of $Ca^{2+}$ channels to achieve hyperactivation. Increased intracellular $Ca^{2+}$ concentration, due to a $Ca^{2+}$ influx, stimulate flagellar beating and the acrosome reaction, both needed to penetrate an oocyte in order to achieve fertilization (Breitbart *et al.,* 1996; Cohen *et al.*, 1998; Shahar *et al.,* 2011; Krasznai *et al.,* 2006). Tiina I. Karu (2012) suggested that the light absorbed from the laser source by cytochrome type b, activates the redox reaction of the cytochrome, enhancing electron transport chain function and cellular respiration. These characteristics enabled LLLT to make a major contribution to the field of regenerative medicine, internal medicine, immunology, rheumatology, orthopaedics, dermatology, neurology and sports medicine (Ohshiro *et al.*, 1988).

Similar to other cell types, human spermatozoa also respond to LLLT with significant changes in sperm energetics (Koppers *et al.*, 2008; Matson *et al.*, 1995; Tafur and Mills, 2008; Karu, 1989; Karu, 1999). Enhanced sperm energetics due to increased ATP consumption lead to enhanced tail movement of spermatozoa, ultimately improving the motility of spermatozoa. (Haung *et al.,* 2009).

Sperm motility is one of the most important characteristics contributing to the potential of a spermatozoon to reach and fertilize an oocyte (Salman *et al.*, 2013). Increased ATP production induced by the application of LLLT to dog spermatozoa enhanced spermatozoon tail movement, which is the single most important organelle when aiming to increase motility (Corral-Baques *et al.*, 2005). Cryopreservatives limit the damage caused during cryopreservation, but also have a toxic effect on sperm. Cryopreservatives cause protein denaturation, membrane destabilization, mitochondrial damage and morphological changes, ultimately decreasing the motility and viability of spermatozoa. It was previously reported that LLLT enhanced the

survival rate and quality of cryopreserved bull sperm as it elevated energy supply and decreased ROS, as a result of improved intramitochondrial oxygen restoration (G. Fernandes *et al.*, 2015).

The specific energy dosage used to irradiate spermatozoa is important as it can have one of three effects: 1. Insufficient dosage will have no effect. 2. Sufficient dosage will have a stimulatory effect. 3. Excessive dosage will have an inhibitory, and possible detrimental, effect (Hawkins and Abrahamse, 2006). Salman *et al.* (2013), used an 830nm Gallium-Aluminum-Arsenide (GaALAs) laser to irradiate human sperm with 0 (control), 4, 6, and 10 J/cm². With the assistance of computer-aided sperm analysis (CASA) they measured motility after laser exposure. Their results showed constant or slight increase in motility while that of the control group decreased over time. A significant increase in motility was noted at doses of 4 and 6 J/cm². Optimal motility was measured 45 and 60min after exposure. Their findings showed that the higher energy dose of 10J/cm² had no effect and in some cases a negative effect, decreasing the motility when compared to the control.

In another study performed by Harrison and co-workers, the motility of human sperm was increased after exposure to two different light sources, i.e.: light-emitting diode (LED) (660 and 850nm) with a 100-400J dose and a Diode laser (810nm) with a 2-4J dose. The maximum effect was observed when using mid-range doses with the LED and the lowest dose with the diode laser, 30min after exposure (Harrison *et al.*, 2008).

A gradual decrease in motility was noted after exposing human spermatozoa for a prolonged time, leading to a detrimental high energy dosage (Tadir *et al.*, 1991). Firestone *et al.* (2011), tested the effect of LLLT on sperm motion and DNA damage. A 50mW/ cm² (1.5 J/cm²) laser system with a 905nm wavelength was used to irradiate human sperm for 30 seconds. Normozoospermic (n=10), astenozoospermic (n=12) and oligo-astenozoospermic (n=11) sperm samples were assessed at 30 and 90min post exposure. FACS Calibur flow cytometer analysis showed no DNA damage compared to controls and a significant increase in motility 30min post exposure, with no difference observed 90min post exposure. Turkey semen irradiated with 3.69J/cm² before cryopreservation was reported to have increased quality compared to control groups (Jaffaldano *et al.*, 2005). They also reported increased viability and energy charge after irradiation.

Literature show inconsistencies in protocols used to irradiate spermatozoa with differing output powers, wavelengths, irradiation time and time of analyses leading to different effects on spermatozoa. The main aim of this study is to determine the effect of LLLT on human spermatozoa in vitro while establishing a standardised protocol for optimal treatment. The use of LLLT in combination with cryopreservation was also assessed to determine if LLLT can improve motility and viability of cryopreserved human spermatozoa.

## 2. Materials and methods

### 2.1. Semen samples and preparation

Semen samples (n=12) from a healthy population of men with unknown fertility status were used. The donor population consisted of students attending the Faculty of Medicine and Health Sciences and were between 18 – 24 years of age. Semen samples were collected by masturbation after 3 days of abstinence according to the World Health Organization (WHO) criteria (WHO, 2010).

After collection the semen samples were placed in an incubator (Heal Force® Smart Cell CO2, Nison™, Shanghai, China) to allow liquification for 30min (5% $CO_2$ enriched at 37°C). A double wash procedure was performed to remove the seminal plasma and cellular debris. In brief an equal amount of HAMS (Human Albumin Serum [Sigma Chemicals Co., St Louis, MO, USA]) was added to each sample and centrifuged at 1500rpm for 5min. Thereafter the supernatant was removed, the pellet resuspended in HAMS and centrifuged again (1500rpm, 5min). The supernatant was removed once more and the pellet resuspended in HAMS – BSA (Bovine Serum Albumin [Roche Diagnostics GmbH, Mannheim, Germany]) to a sperm concentration of $40 \times 10^6$/ml.

### 2.2. Laser source and irradiation procedure

The BioFlex® Therapist Professional system was used to irradiate the semen samples with either an array or a laser probe.

A pilot study was performed to determine the optimal energy dosage, exposure time, exposure technique (array or probe) and time of analysis after exposure when the sperm is at an optimal energy state.

Both the laser array and the single diode laser probe were tested. In brief an Eppendorf tube containing the washed sperm sample was surrounded with the flexible array by folding the array around the centrally positioned Eppendorf tube. The LD-R 100 red laser probe was used to irradiate the sample by manually placing the hand-held laser probe directly against the side of the Eppendorf tube containing the washed semen sample. Moving the probe 1cm up and down during irradiation ensured direct exposure of the laser to the entire volume of the washed sperm sample. A laser physicist from the Department of Physics, Stellenbosch University, was consulted to determine the exact energy dosage per exposure time. Based on the outcome of the pilot study as well as the better accuracy of exposure and energy exposure determination, the $DUO^{+240}$ laser array was removed from the study as more significant and reliable results were obtained using the LD-R red laser probe. The LD-R red laser probe settings and specifications is illustrated in Table 1. The exposure time needed to achieve 6J was calculated to 8sec using the following formula:

$$Irradiation\ time = \frac{(energy\ density)}{Output\ power} \times aperture\ size$$

## 2.3. Study design

The experiment was split into two phases. Phase one focused on the effect of LLLT on the quality of fresh sperm and Phase two focussed on the effect of LLLT on cryopreservation.

The washed sample was split into four equal volumes (mL) and aliquoted in Eppendorf tubes ($40 \times 10^6$/ml) and placed in a Digital dual dry bath (Labnet AccuBlock™, Edison, NJ USA) at 37°C for the duration of the experiment. The samples were treated as indicated in Fig 1.

Motility and viability for all four samples used in Phase one were measured 30 and 60min after laser exposure. After the final measurement Phase two was initiated continuing with the same samples. One control and one probed sample was probed for 8s and the four samples were cryopreserved. After 24 hours of cryopreservation, samples were thawed and each of the 4 tubes was split into two, one of which was exposed to 8s of laser therapy after the thawing proses. These 8 Samples, of which 1 did not receive any exposure to the laser (control) and the other 7 receiving laser exposure at a different time prior to- and post cryopreservation, was now used to measure motility and viability 60min after thawing.

## 2.4. Cryopreservation

A commercially available cryoprotectant (SpermFreeze™, Fertipro, Belgium) was used for cryopreservation of the sperm samples and was always allowed to reach room temperature prior to mixing with the sperm samples in a 7:10 ratio. The sperm samples (1mL) were placed in cryotubes and the Fertipro Spermfreeze (0.7mL) were added in a drop wise fashion while swirling gently. The mixed samples were left for 10min at room temperature to stabilise, whereafter they were placed in a container with liquid nitrogen just above the level of the liquid nitrogen in the vapours for 15min. The samples were subsequently submerged in the liquid nitrogen and stored until thawing.

The samples were removed from liquid nitrogen and thawed by placing the samples in a beaker containing water at room temperature for 5min. The content was then transferred to Eppendorf tubes, inserted in the incubator and allowed to heat to 37°C for 15min.

### 2.5. Motility analysis

Sperm motility parameters were determined with CASA, using the Sperm Class Analyser ™ (SCA, Microptic, Spain). The parameters analysed included Motility, Progressive motility (PR), Rapid progressive (type A), Medium progressive (type B), Non-progressive (type C) and Immotile (type D). During the preparation the sample was diluted to achieve a concentration of 30-40milion cells/ml and a volume of 2µl was loaded onto a 20 µm Leja 8 chamber slide (Leja slide (LJ; 20-µm depth; Leja®

Products B. V., Nieuw-Vennep, the Netherlands). Slides and samples were kept at 37°C, by means of heating stages, during all steps of the procedure. At least 5 fields were analysed randomly on each slide to ensure that a good representation of the entire sample were obtained. The settings of the SCA® system are listed in Table 2.

All measurements were performed by the same individual to eliminate variation.

## 2.6. Viability

A dye-exclusion method was used to measure cell vitality. The sperm sample was mixed with Eosin and nigrosine stains (Sigma-Aldrich, St Louis, MO, USA) in a 1:2:3 ratio and 10µL of the mixture was transferred to a glass microscope slide after which an evenly distributed smear was made. The slides were allowed to air-dry for 8 hours and subsequently mounted using DPX mounting medium (Dako, CA, USA). The slides were analysed with normal brightfield microscopy at 60X magnification. Cells that stained pink were regarded as dead, while cells that remained white were regarded as viable. A total number of 200 cells were counted and the number of viable cells were expressed as a percentage.

## 2.7. Statistics

The Kolmogorov–Smirnov test for normality was used to assess whether the data was parametrically distributed, after which an unpaired t-test or Man Whitney test was utilized where necessary to test differences between control and laser treated samples. All measurements were expressed as the mean ± SEM (standard error of the mean). For Phase 2 of the experiment, a two-way ANOVA was used to compare changes in the samples due to different sequences of laser pre- and post-cryopreservation. Statistical analysis was performed using GraphPad Prism version 5.03 for Windows (GraphPad Software, San Diego California USA). Statistical significance was set at $P<0.05$.

# 3. Results

## 3.1. Phase 1

All n=12 samples irradiated with LLLT had a greater total-, progressive- and fast progressive motility as well as viability, compared to the control groups, measured 30 and 60min after exposure (Table 3). However, differences in Phase 1 was not significant using a confidence interval of 95%. (Fig. 2,3,4 & 6).

Analysis performed 30min post exposure showed the irradiated group (37,39±5,460) had a higher total motility when compared to the control group (31,52±5,832) as shown in Fig. 2A. The same results were obtained when comparing the progressive- and fast progressive motility for the LLLT groups to the respective control groups as shown in Fig. 2B and 2C. Exposure to LLLT did not affect the viability of spermatozoa, with no difference between the control group (69,42±2,503) and the LLLT group (69,92±2,647) 30min post exposure (Fig. 2D).

A greater difference in motility and progressive motility was observed in the samples analysed 60min post exposure (Table 3). The LLLT group (38,31±5,808) had a higher total motility when compared to the untreated control group (29,96±5,855) after 60min as shown in Fig. 3A. The progressive motility measured 60min post exposure showed similar results when the LLLT group (28,12±5,211) was compared to the control (21,71±5,137) (Fig. 3B). Fast progressive motility was slightly higher in the treated group (6,483±1,524) compared to the control (4,591±1,205) shown in Fig. 3C. Viability analyses showed that the LLLT group (72,42±2,343) had more viable cells compared to the control group (69,08±2,586) as shown in Fig. 3D.

Side by side analysis of the LLLT parameters, respectively measured 30 and 60min post exposure, showed that total motility was higher in the groups measured 60 min after exposure (38,31±5,808) compared to groups measured 30min after exposure (37,39±5,460) (Fig. 4A). The same effect was observed when comparing the progressive motility as shown in Fig. 4B. Fast progressive motility, however, was slightly lower in the group measured 60min post exposure (6,483±1,524) compared to the group measured 30min post exposure (6,504±1,356), shown if Fig. 4C. Viability compared between these two groups showed a higher percentage of viable sperm cells in the 60min post exposure group (72,42±2,343) compared to the 30min post exposure group (69,92±2,647) (Fig. 4D). All the associated control group had a lower total-, progressive- and fast progressive motility as well as viability analysed after 60min compared to groups analysed after 30min. This show that motility and viability of the control groups decreased over time, while all LLLT groups, except fast progressive motility, increased over time (Table 3).

A representative photomicrograph of the motility tracks of two representative samples are shown in Figure 5. These photomicrographs represent a visual comparison between the movement of spermatozoa in controlled samples and LLLT treated samples analysed 30 and 60min after exposure respectively.

## 3.2. Phase 2

Irradiation of spermatozoa at different stages during the cryopreservation process led to significant changes in progressive motility, fast progressive motility and viability. The mean ± SEM for all differentially exposed samples, grouped from 1 to 8 are illustrated Table 4.

No significant changes in total motility was observed between the 7 differentially exposed samples and 1 control sample (Fig. 6A). As shown in Fig. 6B, progressive motility had a significant difference of $p<0.0001$ when comparing the control group, irradiated prior to cryopreservation (group 3) (3,483±0,8538) to the group initially irradiated, irradiated again after 60min and cryopreserved (group 7) (12,99±1,900). A significant difference of $P<0.001$ was also seen when group 3 was compared to the group irradiated and cryopreserved after 60min (group 5) (10,97±1,668). As shown in Fig. 6C, exposing spermatozoa to LLLT had to the most significant effect on fast progressive motility, with the highest mean achieved by the control group, irradiated after 60min, cryopreserved and thawed (group 3) (10,07±1,330). This group had the most significant difference ($P<0.0001$) when compared to the group irradiated, cryopreserved after 60min, thawed and irradiated again after thawing (group 6) (1,948±0,5348). A significant value of $p<0.001$ was also observed when comparing group 3 to the control (group 1) (3,231±1,240), the group only irradiated after thawing (group 2) ( 2,187±0,5125), the group irradiated after 60min and thawed after cryopreservation (group 5) ( 2,645±0,7846) and finally the group initially irradiated with a repeated irradiation before and after cryopreservation (group 8) (1,439±0,4391).

Analysis of the viability of differential exposure prior to- and post cryopreservation showed a significant difference of $p<0.001$ when comparing the group initially irradiated, cryopreserved and irradiated again after thawing (group 6) (28,67±2,843) to the group irradiated immediately prior to- and post cryopreservation (group 4) (18,58±2,917) as shown in Fig. 6D.

## 4. Discussion

Motility is the most important parameter contributing to the ability of a sperm cell to reach, penetrate and deliver its nuclear content to an oocyte in order to achieve fertilization (Salman *et al.*, 2013). LLLT has proven to increase human sperm motility by uncoupling nitric oxide bound to the cytochrome c oxidase enzyme, inhibiting the function of the electron transport chain and thus ATP production (Tafur and Mills., 2008). Exposing LLLT to cytochrome c oxidase at different doses and time intervals can be used to modulate the function of this enzyme (Albuquerque-Pontes *et al.*, 2014).

During Phase one of the study, we evaluated the effect of LLLT on fresh semen to test if irradiation to human spermatozoa could possibly increase the motility and viability of these cells in vitro. Analysis 30 and 60min after irradiation showed no significant increase in motility parameters measured with CASA, when comparing the LLLT samples to the control samples. The same results were obtained for the viability of the spermatozoa measured with the dye-exclusion method (Fig.2 & Fig.3). However, the mean of all motility and viability measurements was higher in the LLLT group compared to their respective control groups, as shown in Table 3. The mean of the LLLT groups increased over time, measured at 30 and 60min, while the control samples had a lower mean after 60min compared to 30min. This might show a trend in the LLLT maintaining viability and motility over time. Direct comparison between the 30 and 60min exposure groups, however, did not show a significant difference(p>0.05). As mentioned by Tuner and Hode (2002), LLLT will have the greatest effect on cells affected by a deteriorated state, subjected to oxidative stress. Since the donors used for the experiment was students in their reproductive age, it can be assumed that they had a relatively healthy sperm quality, compared to older, more inactive people, whose sperm quality might have had a more substantial effect after exposure to LLLT.

Lubart *et al.* (1997) suggested that additionally to nitric oxide uncoupling, LLLT stimulates calcium to connect to the spermatozoon plasma membrane, promoting better cell maintenance. Cryopreservation is known to decrease motility and viability of sperm, by inducing damage to the plasma membrane, mitochondria and alterations in morphology (Celeghini *et al.*, 2008). Progressive- and fast progressive motility as well as the viability of the cryopreserved spermatozoa was improved by the application of LLLT. Progressive motility analysis showed that irradiation 60min prior to- and immediately prior to cryopreservation (group 7), had the most beneficial effect on progressive motility in cryopreserved spermatozoa (Fig. 6B). Samples irradiated immediately before cryopreservation has shown to have a significant increase in fast progressive motility compared to all other samples except group 8. (Fig. 6C).

Finally, we analysed the difference in the number of viable spermatozoa in the samples 60min after removing the frozen sample from liquid nitrogen and thawing. The only significant difference (p<0.001) in viability was found when comparing the

group irradiated, cryopreserved after 60min and irradiated again after thawing (group 6) to the group irradiated immediately before and after cryopreservation (group 4). Fig. 6D.

To conclude our study, LLLT may improve the quality of spermatozoa, however, further research is needed, as there is a lack of knowledge surrounding this topic. Inconstancies in the techniques used and time of analysis as shown in previous studies suggest that a better understanding of the precise mechanism of action is needed. The condition of the spermatozoa used during the study may also affect the outcome of treatment. Fresh spermatozoa from healthy adults was not significantly affected by exposure to LLLT, while cryopreserved spermatozoa, subjected to damage by the cryopreservation process and cryopreservatives showed significant improvement with the application of LLLT. Concerning the data obtained from our study, LLLT may have beneficial effects, improving and preserving cryopreserved spermatozoa. This technique may prove to be useful in improving fertility rates in reproductive technologies, by improving stored, cryopreserved spermatozoa.

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# Figures and tables

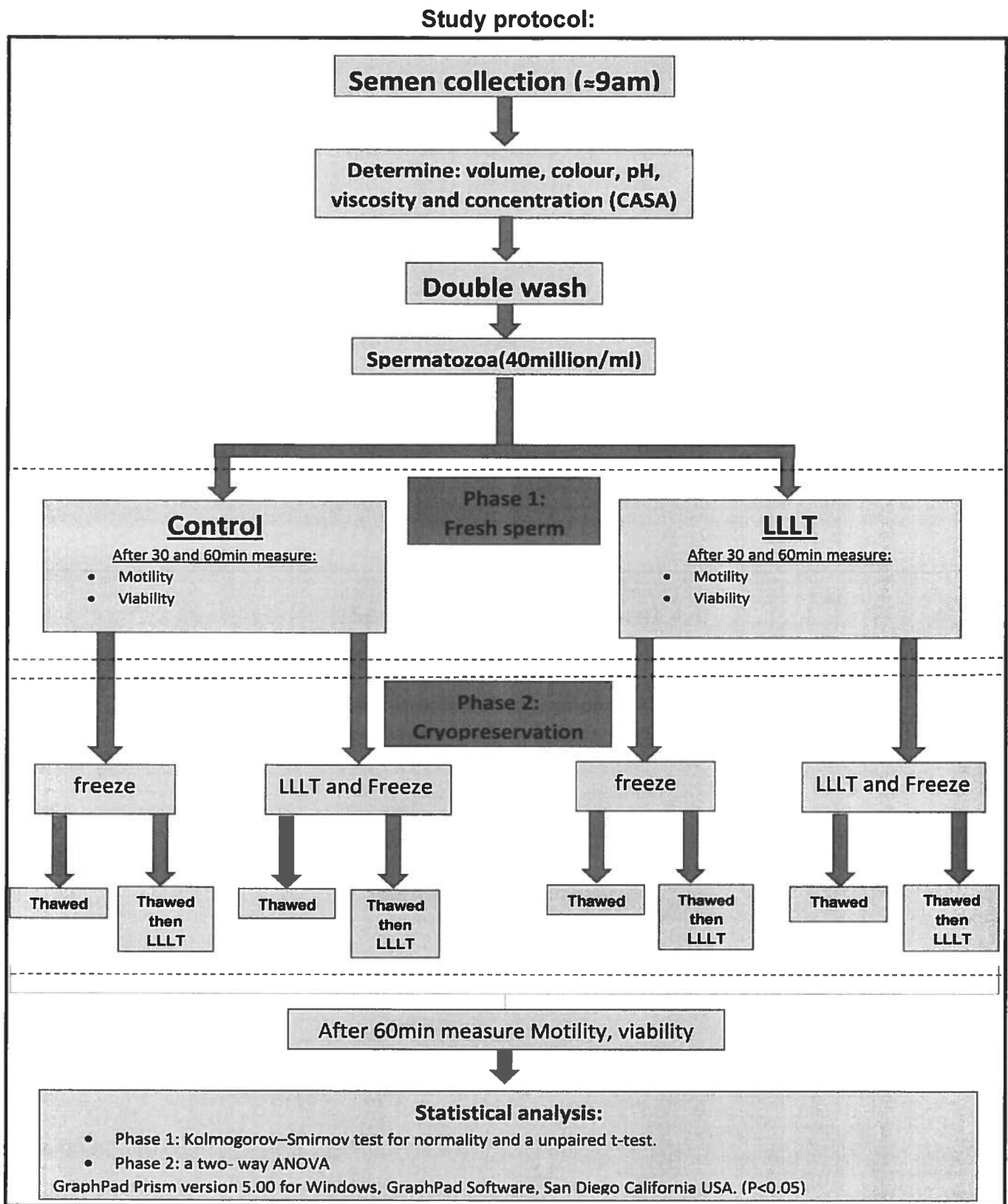# Figures:

**Study protocol:**



Figure 1. Shows the protocol used in Phase one and Phase two of this study.

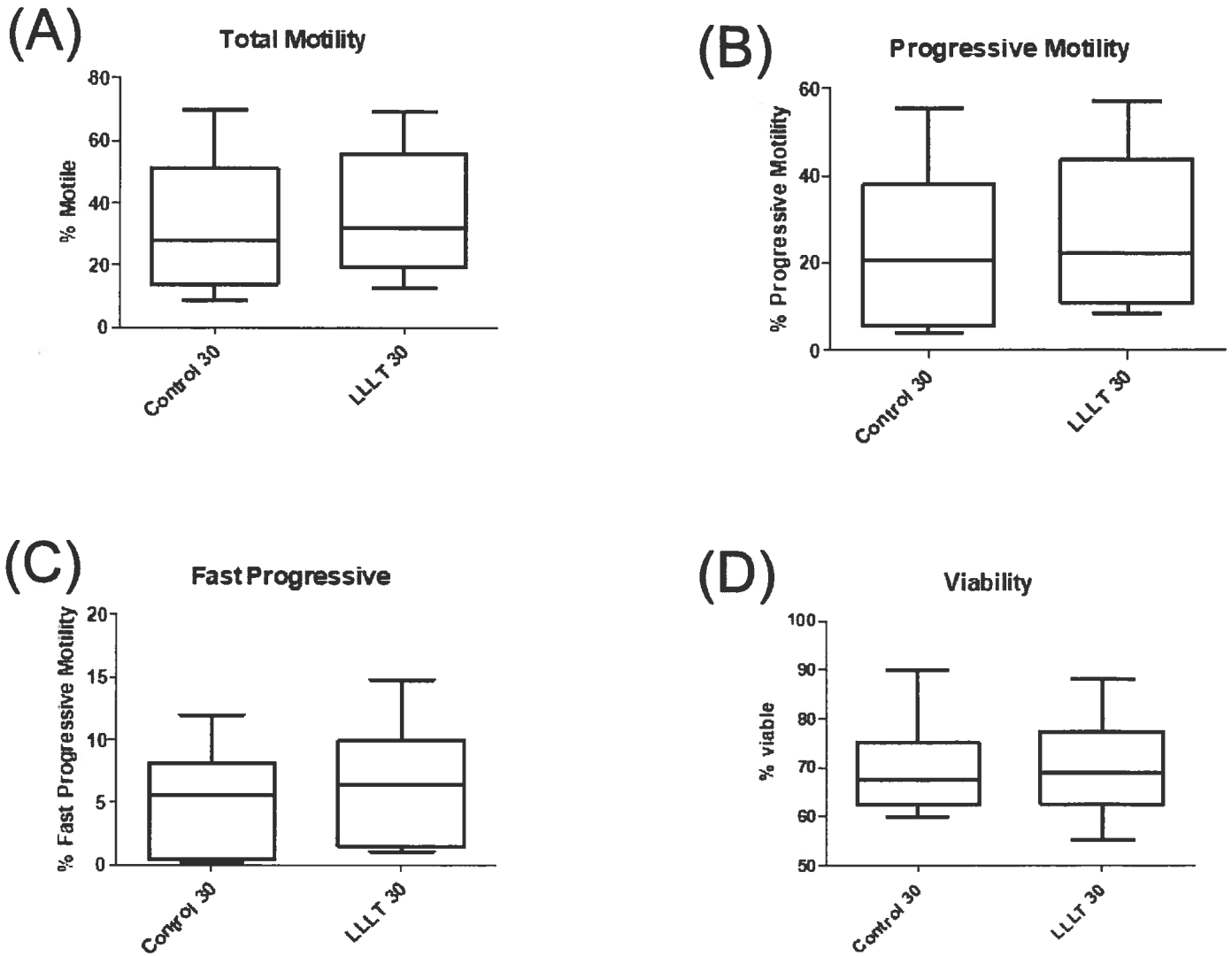**Phase 1:** The effect of LLLT on the motility and viability of fresh semen samples.



Figure 2. Show the difference in total motility(A), progressive motility(B), fast progressive motility (C) and viability (D) between the LLLT group, exposed to 6J of irradiation for 8 seconds, and the associated control group, 30min post exposure.

Note: * represent p value = <0.01, ** represent p value = <0.001, *** represent p value = <0.0001.
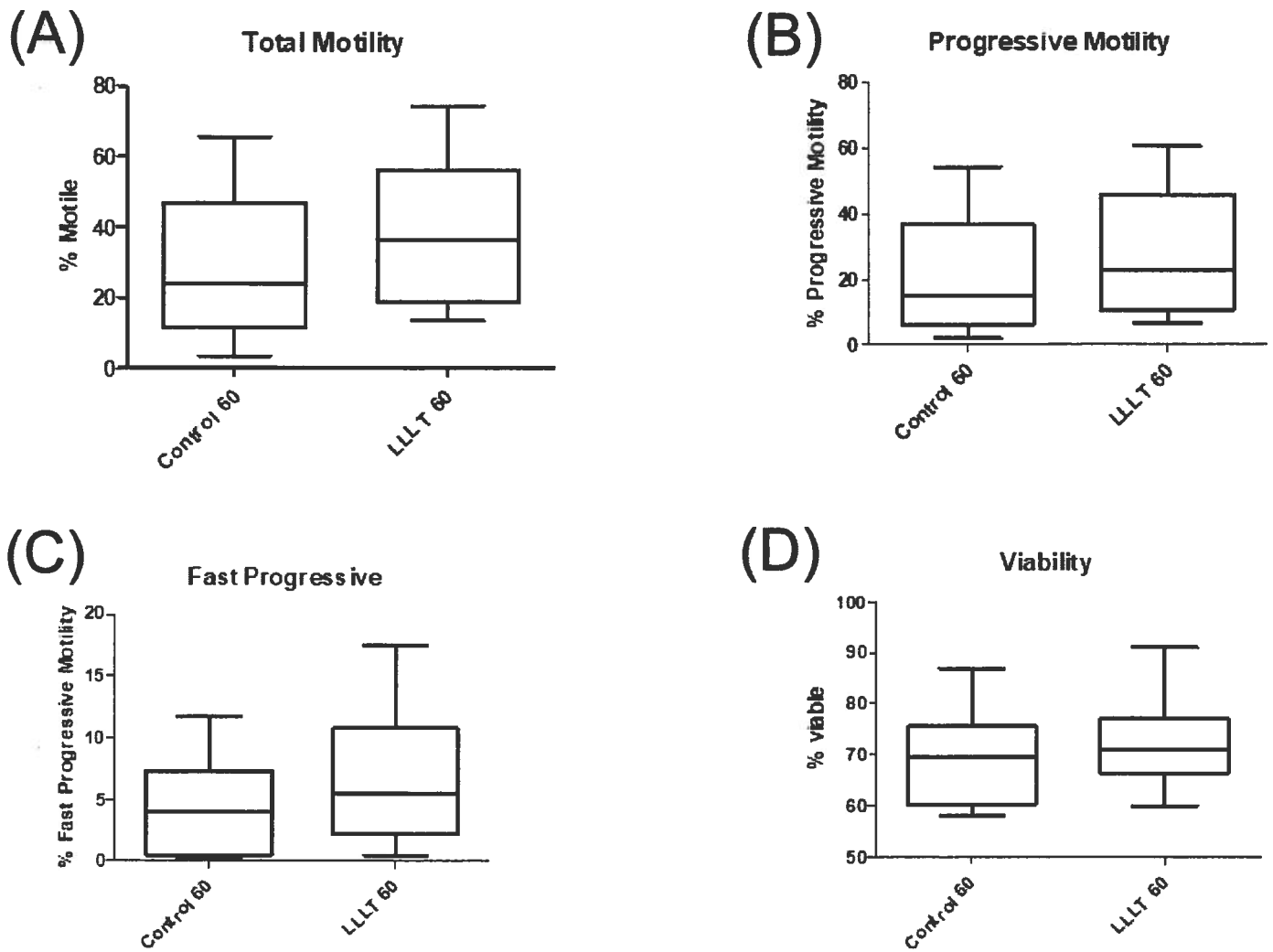
Figure 3. Show the difference in total motility(A), progressive motility(B), fast progressive motility (C) and viability (D) between the LLLT group, exposed to 6J of irradiation for 8 seconds, and the associated control group, 60min post exposure.

Note: * represent p value = <0.01, ** represent p value = <0.001, *** represent p value = <0.0001.
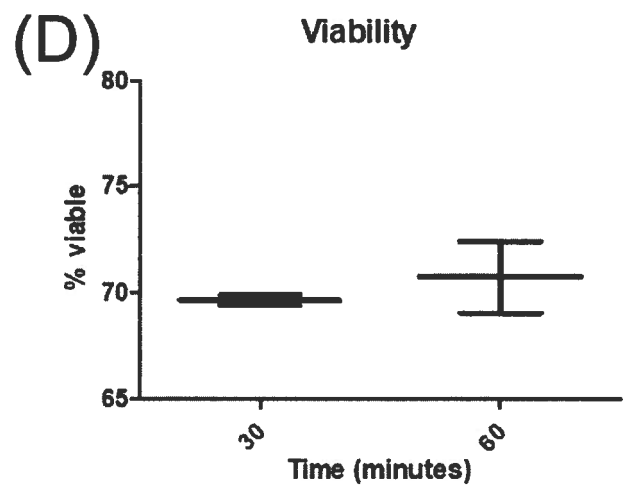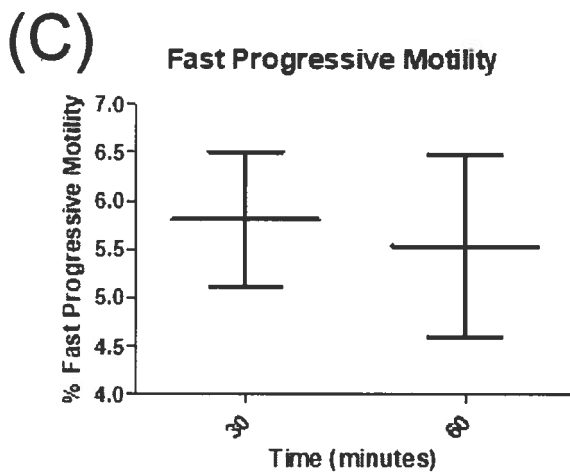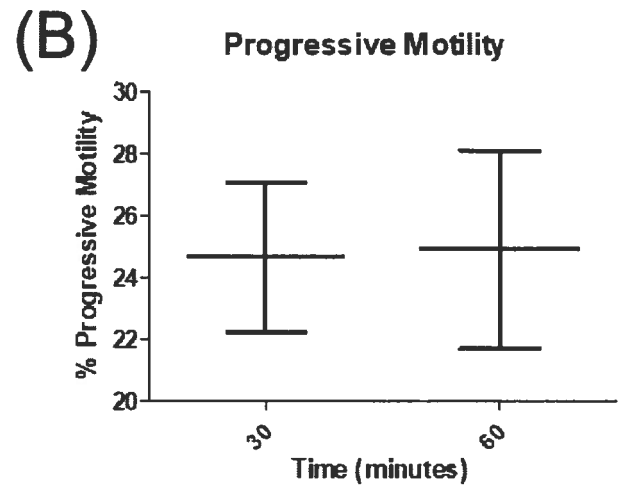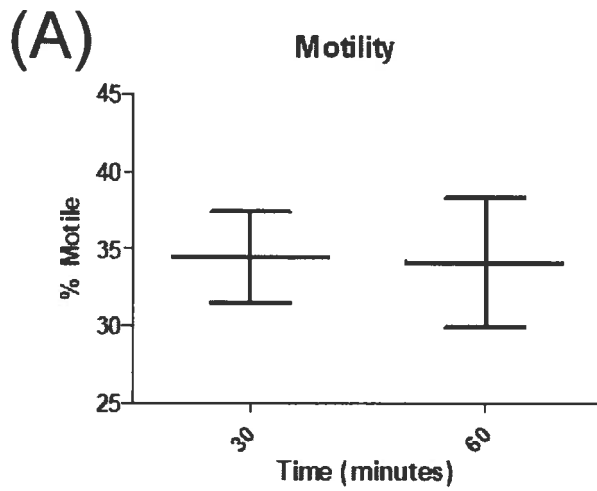
Figure 4. Show the difference in total motility(A), progressive motility(B), fast progressive motility (C) and viability (D) between the LLLT groups, exposed to 6J of irradiation for 8 seconds, and respectively analysed 30 and 60min post exposure

Note: * represent p value = <0.01, ** represent p value = <0.001, *** represent p value = <0.0001.

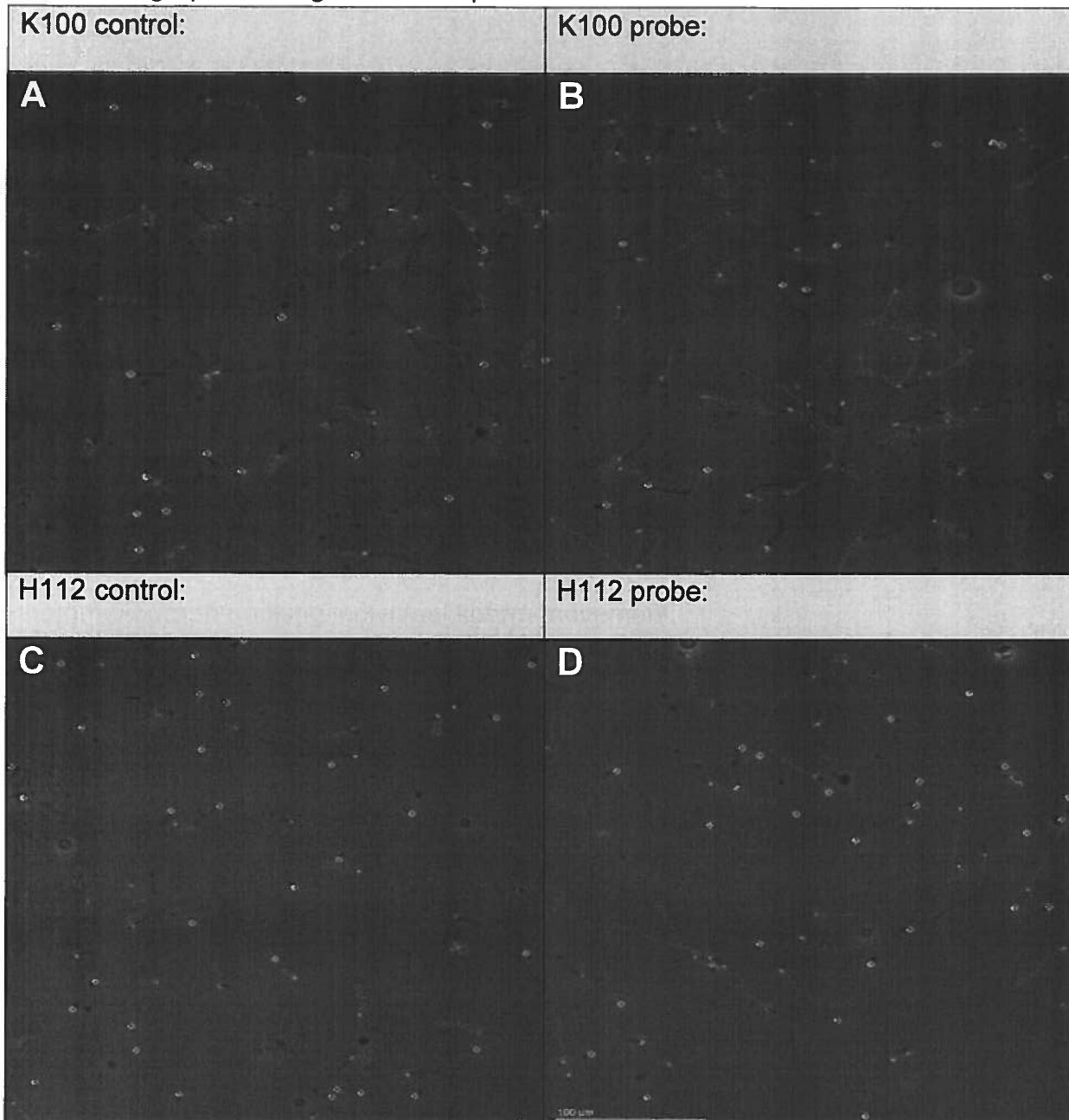Photomicrograph tracking individual sperm movement.



Figure 5. Photomicrograph tracking sperm movement through the CASA system.
Top (A and B): Analysis performed 30 minutes post exposure.
Bottom (C and D): analysis performed 60 minutes post exposure.
Right panel: samples irradiated with 6J for 8 sec.
Left panel: corresponding control groups for the two random samples.

**Phase 2:** Effect of LLLT on motility and viability of cryopreserved spermatozoa.



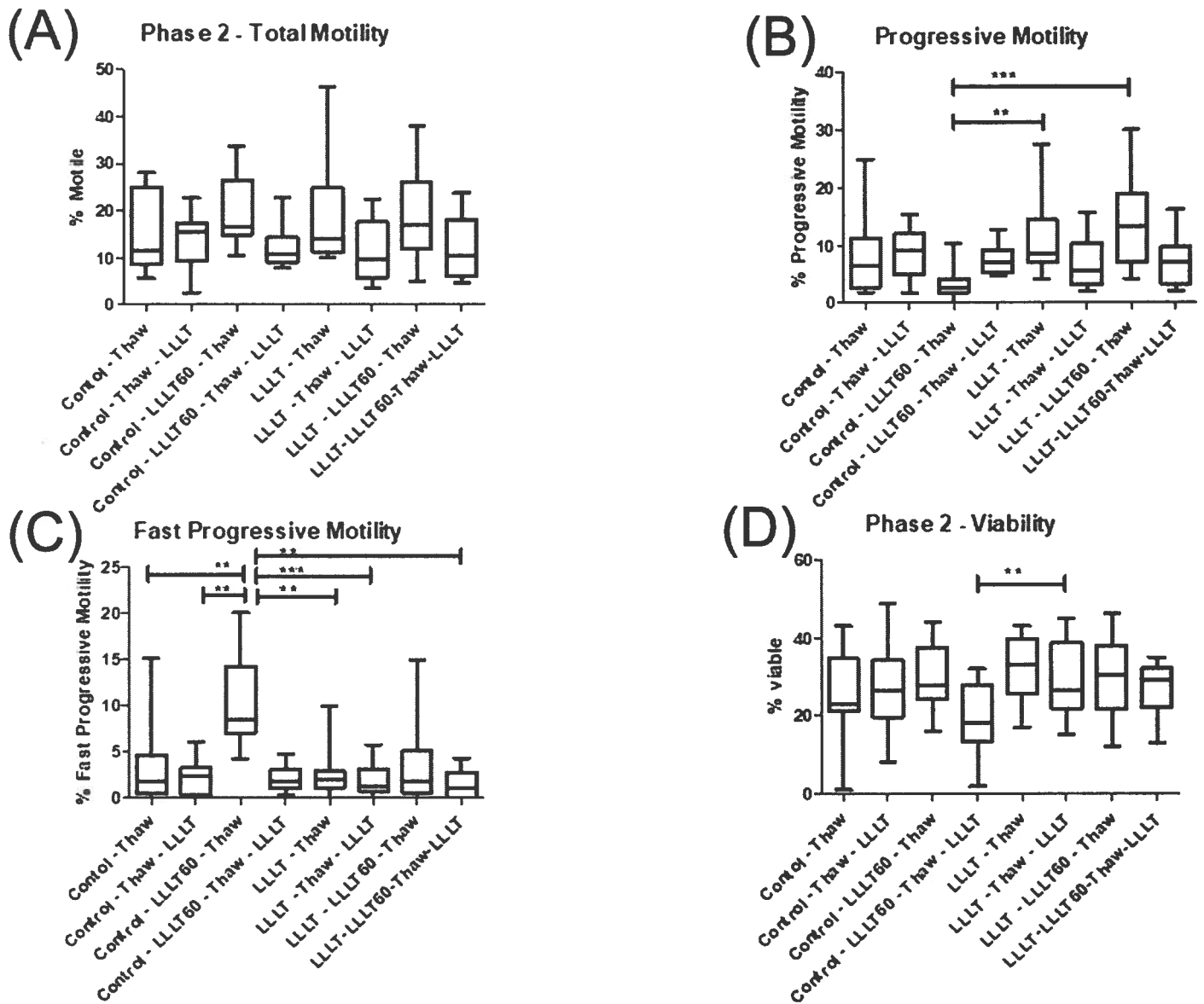Figure 6: Show the difference in total motility (A), progressive motility (B), fast progressive motility (C) and viability (D) between differentially exposed semen samples, prior to- and post cryopreservation. All measurement was taken 60 minutes after the cryopreserved samples was thawed and incubated.

Note: * represent p value = <0.01, ** represent p value = <0.001, *** represent p value = <0.0001.

# Tables:

Table 1. Technical specification and settings used to irradiate semen samples with LD-R 100 red laser probe.

| LD-R 100 red   laser probe | | |
|---|---|---|
| Parameter | Units | LD-R 100 |
| Effective laser class | | 3B |
| Optical power | mW | 75 |
| Surface area | Cm² | 0.10 |
| Power density | mW/Cm² | 750.0 |
| Number of diodes | | 1 |
| Type | | Laser diode |
| Wave setting | | Continuous |
| Time | Seconds | 8 seconds |
| Dinstance from subject | cm | 0 |

Table 2. Camera settings for the SCA® system

| | Parameter | Setting |
|---|---|---|
| **Acquisition control** | Acquisition mode | Timed |
| | Exposure time | 19900 |
| | Time base | 20 μs |
| | Exposure time | 995 |
| | Acquisition frame rate | 50 FBS |
| **Counter and timer controls** | Time duration Raw | 4095 |
| **Analog controls** | Gain | 435 |
| | Black level | 168 |
| | Balance ration | 1.20313 |
| **Microscope** | Magnification | X40 |
| | Filter | Green |
| | Phase | 1 |

**Phase 1: Fresh spermatozoa exposed to LLLT vs control, 30 and 60min post exposure.**

| Time of Analysis after irradiation | Parameter measured | LLLT | Control | Group with greatest mean |
|---|---|---|---|---|
| 30min | Total motility (%) | 37,39±5,460 | 31,52±5,832 | LLLT |
| | Progressive motility (%) | 27,09±4,895 | 22,26±4,965 | LLLT |
| | Fast progressive motility (%) | 6,504±1,356 | 5,114±1,233 | LLLT |
| | Viability (%) | 69,92±2,647 | 69,42±2,503 | LLLT |
| 60min | Total motility (%) | 38,31±5,808 | 29,96±5,855 | LLLT |
| | Progressive motility (%) | 28,12±5,211 | 21,71±5,137 | LLLT |
| | Fast progressive motility (%) | 6,483±1,524 | 4,591±1,205 | LLLT |
| | Viability (%) | 72,42±2,343 | 69,08±2,586 | LLLT |

Table 3. Show the mean total-, progressive- and fast progressive motility as well as the viability of the samples exposed to LLLT as well as their respective controls. Analysis was performed 30 and 60min after irradiation, using the CASA system. Results are expressed as mean ± standard error of the mean (SEM).

**Phase 2: Differentially exposed spermatozoa during cryopreservation.**

| Treatment group | Parameters measured | | | |
|---|---|---|---|---|
| | Total motility (%) | Progressive motility (%) | Fast progressive motility (%) | Viability (%) |
| 1. Control - Thaw | 15,27±2,446 | 9,159±1,900 | 3,231±1,240 | 25,17±3,494 |
| 2. Control - Thaw - LLLT | 13,71±1,809 | 8,948±1,269 | 2,187±0,5125 | 27,17±3,314 |
| 3. Control - LLLT60 - Thaw | 19,96±2,195 | 3,483±0,8538 | 10,07±1,330 | 29,42±2,491 |
| 4. Control - LLLT60 - Thaw - LLLT | 12,21±1,204 | 7,483±0,6995 | 2,093±0,3757 | 18,58±2,917 |
| 5. LLLT - Thaw | 18,36±3,051 | 10,97±1,668 | 2,645±0,7846 | 32,67±2,310 |
| 6. LLLT - Thaw - LLLT | 11,43±1,819 | 6,895±1,349 | 1,948±0,5348 | 28,67±2,843 |
| 7. LLLT - LLLT60 - Thaw | 18,52±2,815 | 12,99±1,900 | 3,204±1,195 | 29,58±3,059 |
| 8. LLLT-LLLT60-Thaw-LLLT | 12,03±1,867 | 7,274±1,258 | 1,439±0,4391 | 27,00±2,071 |

Table 4. Show the mean total-, progressive- and fast progressive motility as well as the viability of samples differentially exposed prior to- and post cryopreservation. Analysis was performed 60min after thawing, using the CASA system. Treatment groups are labelled from 1 - 8, one control group and 7 LLLT groups, exposed at different times prior to- and post cryopreservation.