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# Inovação e Pluralidade na Medicina Veterinária 3

Alécio Matos Pereira
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(Organizadores)

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# Inovação e pluralidade na medicina veterinária
# 3


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*Ciências Agrárias e Multidisciplinar*

# APRESENTAÇÃO

A diversidade das áreas de conhecimento favorece ao leitor o melhor entendimento dos mais variados assuntos na atualidade relacionados a ciência animal e suas particularidades.

O livro abrange diversos temas importantes relacionados a saúde animal e humana, reprodução animal, sanidade. Sendo divididos em volume II composto por 16 capítulos e volume III com 17 capítulos. Nestes foram descritos relatos, experimentos e revisões no âmbito nacional e internacional. Que contém informações concisas que proporcionaram ao leitor uma visão clara e completa de todo conteúdo abordado.

No volume II e III, são abordados assuntos como a ocorrência de parasitas em pescados, anestesia em pacientes cardiopatas, deficiência de cobre e zinco em pequenos ruminantes, medicina, epidemiologia, forragicultura, equideocultura, áreas da medicina veterinária e zootecnia.

O ambiente aquático se torna propício para o surgimento de várias doenças parasitárias. Estes podem gerar riscos à saúde animal e na população humana consumidora de pescados.

A (MDM) Associação Médicos do Mundo *World Doctors*, é uma iniciativa privada e filantrópica que tem como objetivo promover atendimento humanitário a pessoas e animais em situação de vulnerabilidade social, fornecendo atendimento médico e social.

Na produção de volumosos a estacionalidade é um fator recorrente em vários sistemas de produção animal. Principalmente na região Nordeste, que apresenta irregularidade das chuvas ao longo do ano e pode haver períodos de estiagem. E para amenizar as perdas produtivas é a utilização das técnicas de conservação de forragem, que favorece na disponibilidade de alimento durante todo o ano.

Deste modo, a diversidade de assuntos abordados nos volumes II e III apresentam capítulos com pesquisas, relatos, objetivos e resultados, desenvolvidos por diferentes pesquisadores, professores e estudantes de pós-graduação. Como uma maneira de evidenciar a pesquisa cientifica como uma fonte importante para auxiliar na atualização de estudantes e profissionais.

Alécio Matos Pereira
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## SUMÁRIO

# CAPÍTULO 13

# EFFECT OF THE ADDITION OF L-CARNITINE AND PYRUVATE ON BOAR SEMEN CRYOPRESERVATION

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**ABSTRACT:** The conventional freezing protocol for boar semen lasts around 8 hours and requires heavy equipment. Results in fertility and litter size obtained using frozen-thawed porcine semen are very different from those obtained with natural service or artificial insemination using cooled semen. The aim of this study was to evaluate freeze-thawing of boar semen comparing the traditional slow method to a rapid curve of temperature descent, using two cryoprotectants (glycerol and dimethylformamide) in presence of an antioxidant and an energy substrate (L-carnitine and pyruvate). Methods: Samples were re-diluted with 5% DMF; 3% glycerol; 5% DMF + L-carnitine and pyruvate and 3% glycerol + L-carnitine and pyruvate. Kinematic parameters, sperm viability and sperm acrosome

status and membrane functional integrity simultaneously (CB/HOS) were evaluated. Results: No significant differences (p>0.05) were observed between slow or rapid curves, or between cryoprotectants, nor between presence or absence of L-carnitine and pyruvate for any of the motility, CB/HOS patterns or live acrosome-reacted, and dead acrosome-intact sperm (FITC-PNA/PI) evaluated post-thaw. However, a significant increase (p< 0.05) in live acrosome-intact sperm was observed with the rapid curve and in dead acrosome-reacted sperm was observed with the slow curve. Conclusion: Either glycerol or dimethylformamide could be used as cryoprotectants and the addition of L-carnitine together with pyruvate to the freezing media did not make a difference to the quality of thawed porcine semen.
**KEYWORDS:** Dimethylformamide, glycerol, L-carnitine, pyruvate, boar semen.

## 1 | INTRODUCTION

Semen cryopreservation is a biotechnology that has been used for many years to preserve genetic material, enable genetic improvement and increase reproductive efficiency of highly valuable boars. Basic components of extenders have been the same over the past 35 years and include egg yolk, glycerol (Pursel and Johnson, 1975), a carbohydrate and detergent substances. Glycerol is the most utilized permeable cryoprotectant to freeze semen in many species, including boars (Barbas and Mascarenhas, 2009). Different concentrations of glycerol have been used in mammals, varying from 2 to 10%. Several studies have demonstrated that the most suitable glycerol concentration to freeze boar semen varies from 2 to 4 % (Westendorf *et. al.,* 1975; Holt WV, 2000). Nevertheless, it is known that glycerol can induce chemical and osmotic toxic effects and changes in the sperm membrane lipids (Fiser and Fairfull, 1984; Fahy GM, 1986). For this reason, other cryoprotectants such as amides have been assayed. Amides have a lower molecular weight and higher water solubility, so they reduce intracellular ice crystal formation, producing minimum toxicity and less osmotic damage (Ashwood-Smith MJ, 1987).

Boar sperm membranes contain a high proportion of polyunsaturated fatty acids, which can suffer lipid peroxidation during cryopreservation (Cerolini *et. al.,* 2001). The increased production of reactive oxygen species during cryopreservation has been associated with the reduction of motility and fertility of thawed semen (Chatterjee *et. al.,* 2001; Aitken *et. al.,* 2014). This damage could be minimized with the addition of antioxidants to the extender prior to the freezing process (Großfeld *et. al*., 2008). Carnitine modulates several sperm functions such as, fatty acid oxidation, acetyl-CoA/free CoA ratio and the use of pyruvate and lactate as energy substrates. In addition, it has a powerful antioxidant effect since it reduces lipid availability to peroxidation (Matalliotakis *et. al.,* 2000) and increases the activity of antioxidant enzymes such as catalase, superoxide dismutase and glutathione peroxidase; leading to improvement of sperm motility (Lisboa *et al*. 2014). Pyruvate acts as hydrogen peroxide scavenger and it can be used as antioxidant in extenders to avoid

peroxidative damage ([Bilodeau et al. 2002), improving semen quality (equine: Goncalvez da Silva 2008; bovine: Korkmaz et al., 2017).

The conventional freezing protocol for boar semen lasts around 8 hours and requires heavy equipment (refrigerated centrifuges and programmable freezers, among others) (Saravia et al., 2010; Rodriguez-Martinez and Wallgren, 2011). Therefore, development of easier and practical freezing protocols to implement “on field” would be useful.

The aim of this study was to apply a rapid freezing curve for cryopreserving boar semen using glycerol and dimethylformamide. Also, the addition of pyruvate and L- carnitine to minimize sperm damage during cryopreservation was studied. The final objective was to develop a useful protocol that could be easily applied on field.

## 2 | MATERIALS AND METHODS

### 2.1 Animals and location

The study was approved by the Institutional Committee for Use and Care of Laboratory animals (CICUAL) of the Faculty of Veterinary Sciences of the University of Buenos Aires (Protocol Nº 2014/17). The present work was carried out at the Faculty of Veterinary Sciences of the University of Buenos Aires, Argentina. Nine (n= 9) males clinically healthy, ranging between 1.5 and 3 years of age were included in the study. Eighteen ejaculates were obtained (n=9; r=2) using the gloved hand technique (King *et al.,* 1973).

### 2.2 Sperm cryopreservation

For every ejaculate the following two freezing curves were evaluated simultaneously: rapid and slow. In all cases, the straws remained in the liquid nitrogen tank for at least 2 weeks before being thawed in circulating water at 37 ºC for 1 minute.

All chemicals and reagents were obtained from Sigma Chemical Company, St Louis, Mo.

#### *2.2.1 Rapid curve*

Samples were diluted in Androstar® plus (Minitüb, Germany). Afterwards, they were centrifuged 15 minutes at 800 g and the pellet was rediluted to 300 x $10^6$ sperm/ml in: 1) 5% DMF, lactose 11 %, 20 % egg yolk, 0.5 % Equex®; 2) 3 % glycerol, lactose 11 %, 20 % egg yolk, 0.5 % Equex®; 3) 5 % DMF, lactose 11 %, 20 % egg yolk, 0.5 % Equex® + 50 mM L-carnitine and 10 mM pyruvate and 4) 3 % glycerol, lactose 11 %, 20 % egg yolk, 0.5 % Equex® + 50 mM L-carnitine and 10 mM pyruvate. The samples were equilibrated for 30 minutes at room temperature, after loading in 0.5 ml straws, placed in plastic gobelets inside a bronze canister filled with a mixture of ethanol-acetone 1:1 (Figure 1). A rapid

freezing curve was carried out according to Miragaya et al., 2001. Briefly, the canister was held 6 cm above the liquid nitrogen level in a 10 litres nitrogen tank, monitoring the temperature descent with a digital thermometer from room temperature to -15 ºC (descent at a rate of 10-12 ºC/min) and then to -120 ºC (at a rate of 25 - 40 ºC/min). Finally, straws were plunged into liquid nitrogen at -196 ºC.

#### *2.2.2 Slow curve*

The slow freezing curve was performed as described by Westerndorf et al. (1975) with modifications (Caldevilla et al., 2016). Semen was placed at 17 ºC for 2 h and then centrifuged at 800 g for 15 minutes. The pellet was rediluted with the same 4 extenders used in the rapid curve. Samples were stabilized for 2 h at 5 ºC and loaded in 0.5 ml straws. The straws were placed on a rack, inside a styrofoam box, 5 cm above liquid nitrogen level for 20 minutes. Finally, the straws were plunged into liquid nitrogen at -196°C.

### 2.3 Post-thaw sperm assessment

The following seminal characteristics were evaluated after thawing: kinetic parameters, viability, acrosome integrity, membrane function and acrosome status.

#### *2.3.1 Evaluation of kinetic parameters*

Kinetic parameters were evaluated using a computerized system, ISAS v1 (Proiser®, Valencia, Spain). The following parameters were analyzed: the total sperm motility and progressive motility (%), CLV curvilinear velocity (µm/sec), SLV straight line velocity (µm/seg), VTM medium path velocity (µm/seg), ALH lateral head displacement (µm) and BCF beat cross frequency (Hertz).

#### *2.3.2 Evaluation of sperm viability and acrosome integrity evaluation.*

The staining technique combines Fluorescein Isothiocyanate –*Arachis hypogea* agglutinin (FITC-PNA), a lectin that selectively binds the outer acrosomal membrane, with Propidium Iodide (PI) for viability. Briefly, samples were incubated at 37 °C for 10 min with FITC-PNA. Then, 2µl of PI were added and incubated for another 10 min at 37 °C. Finally it was evaluated using an epifluorescence microscope (Figure 2).

#### *2.3.3 Evaluation of membrane function and acrosome status*

Coomassie blue (CB) was used on semen samples previously subjected to a hypoosmotic swelling (HOS) test (Ferrante *et al.,* 2017). Briefly, sperm were subjected to a

HOS according to Vazquez *et al.,* (1997) modified: samples were incubated for 10 minutes at 37 ºC in fructose- sodium citrate, washed with PBS centrifuging at 600g for 3 minutes. The pellet was re-diluted in 2 % paraformaldehyde, incubated at a room temperature during 4 minutes. A second centrifugation was performed (600 g for 3 minutes), repeating the procedure twice. The pellet was then re-diluted in 300 $\mu$l of PBS and a drop was placed on a slide, within a circle marked with a Dako Pen®, and dyed 5 minutes with CB (Figure 3).

### 2.4 *Statistical Analysis*

Data were analyzed using a factorial design of 3 factors with 2 levels each, blocking the males. Therefore, an analysis of variance was performed on data with a normal distribution and otherwise a Kruskal Wallis test was performed.

## 3 I RESULTS

### 3.1 Post-thaw sperm kinetic parameters

No significant differences were observed ($p > 0.05$) in motility of thawed sperm neither between the rapid and slow curves, nor between DMF and glycerol nor in the presence or absence of L-carnitine and pyruvate. No significant differences ($p > 0.05$) were observed in the kinetic parameters CLV, SLV, VTM, ALH, BCF, between the rapid and slow curves, neither between DMF and glycerol, nor between the presence or absence of L-carnitine and pyruvate in the extenders. (Table 1)

### 3.2 Post-thaw sperm viability and acrosome integrity

When comparing sperm parameters within each freezing curve separately, no significant differences were observed ($p > 0.05$) in the FITC-PNA/PI patterns between DMF and glycerol, nor with the addition of L-carnitine and pyruvate when using the rapid curve (Table 2). No significant differences were observed ($p > 0.05$) in FITC-PNA/PI patterns, between DMF and glycerol, nor with the L-carnitine and pyruvate addition using the slow curve (Table 3).

When comparing the rapid vs. the slow freezing curve, the percentage of live spermatozoa with intact acrosomes was significantly higher ($p < 0.05$) in the rapid curve ($24.5 \pm 1.9$) compared to the slow curve ($21.2 \pm 0.2$), while the percentage of dead sperm with reacted acrosomes was significantly lower ($p < 0.05$) in the rapid curve ($58.3 \pm 0.8$) than in the slow curve ($62.3 \pm 0.5$).

### 3.3 Post-thaw sperm membrane function and acrosomal status

No significant differences ($p > 0.05$) were observed in either of the HOS/CB patterns in thawed samples, neither between curves nor between cryoprotectants, nor in the presence

or absence of L-carnitine and pyruvate (Table 4).

## 4 I DISCUSSION

The cryopreservation method used for boar semen has been used for a long time. It is a gradual process that lasts between 3 to 5 h in short protocols, and 8 to 10 h in longer protocols (Saravia et al., 2010). The present study proposes the use of a rapid freezing curve that decreases the length of the protocols that are currently used. In addition to the short time that it takes to carry out the freezing curve, another important advantage is that it may be used “on field”, on any pig farm, without the necessity of transporting either the boars or the semen samples, or having a programmable freezer.

Although glycerol has historically been the accepted cryoprotectant used to freeze boar semen (Crabo and Einarsson, 1971; Pursel and Johnson, 1974), the concentration added to the freezing extender is essentially limited by its toxicity (Almlid and Johnson, 1988; Holt WV, 2000). Therefore, in different species, other cryoprotectants have been evaluated (equine: Squires et al., 2004; dog: Lopes et al., 2009; cattle: Oh et al., 2012). In fertility trials with frozen semen, a significant improvement in fertility was obtained using DMF compared to glycerol (equines: Squires et al., 2004; Vidament et al., 2002; rabbits: Hanada et al., 1980; fish: Ogier De Baulny et al., 1999). In the present study, the sperm parameters assessed after cryopreservation of boar semen with glycerol did not show significant differences compared to dimethylformamide. On the contrary, better results have been reported regarding to boar sperm motility, viability, integrity and membrane function when using a lactose-egg yolk extender with 3% glycerol, compared to one with dimethylformamide (Malo et al., 2012). This data agrees with Kim et al., (2011) who observed that when using dimethylacetamide and dimethylsulfoxide as cryoprotectants, motility and membrane integrity were lower in comparison to the results obtained when freezing with 3% glycerol. However, other authors have observed that the replacement of glycerol with amides (5% dimethylformamide or dimethylacetamide), in the extender resulted in an increase in motility and membrane integrity in thawed boar semen (Bianchi et al., 2008, Hwang et al., 2009). Differences between the results obtained in the previously mentioned studies and ours might be related to the different freezing protocols that were implemented and variations in the extender´s composition.

It has been reported that the addition of antioxidants to freezing extenders increases post-thaw sperm quality and the reproductive efficiency after artificial insemination (Großfeld y col., 2008). However, in the present study the addition of L-carnitine to the freezing extenders did not improve post-thaw sperm quality in boars. On the contrary, Sariozkan et al. (2014) preserved rabbit semen at 5 ºC for 24 h with the addition of L-carnitine observing that, at 12 and 24 h, sperm motility and membrane function were better compared to the control group; showing a decrease in acrosome damage at 24 h. Similarly, in Angora goats,

the addition of antioxidants like carnitine reduced the percentage of sperm with abnormal acrosomes and the total sperm abnormalities; although there was no improvement in post-thaw sperm motility versus the control group (Bucak et al., 2010a). In bulls, the addition of L-carnitine to the semen freezing extender, improved sperm motility, reduced morphological abnormalities and DNA damage in thawed samples (Bucak et al., 2010b). Aliabadi et al. (2017) showed that the addition of L-carnitine did not improve the post-thaw motility of human sperm however; there was an improvement in plasma membrane and acrosome integrity. Fattah et al. (2017) reported that the addition of L-carnitine to Beltsville medium improved sperm quality in rooster thawed semen; while the addition of this component to the freezing extender improved sperm motility in cats thawed epididymal sperm (Manee-in et al., 2014). The addition of pyruvate to extenders improved semen quality in bovines (greater sperm motility and percentages of intact membrane and acrosome, and lower percentages of DNA fragmentation) and sperm motility in equines (Goncalvez da Silva, 2008; Korkmaz et al., 2017). In boars, the addition of a mixture of antioxidants containing pyruvate, catalase and mercaptoethanol to the extender, which included glycerol or DMF as cryoprotectants, did not improve semen quality (Buranaamnuay et al., 2011). Similarly, in the present study, the addition of pyruvate to the extenders did not improve the assessed sperm parameters. Differences in the results obtained between these studies and the ones performed in bovine and equine might be attributed to a different sperm response to exogenous antioxidants between species and/or different freezing protocols. Considering the results obtained in other species, it would be interesting to evaluate other antioxidant´s concentration in order to assess if better results are accomplished.

The currently used protocol to freeze boar semen involves many steps and is time-consuming (lasting around 8 hours to process an ejaculate). It also requires expensive equipment as refrigerated centrifuges and programmable freezers. Thus, it becomes unattractive for routine use and unpractical to implement on field. A simplified procedure, which requires around 3.5 hours, has been developed (Saravia et al., 2010). However, the mentioned technique involves the use of a programmable freezer. In the present study, a rapid freezing curve without the need of sophisticated equipment has been evaluated. As the assessed seminal characteristics did not show differences between the traditional slow curve and the studied rapid curve, the last one would be useful to implement on field.

There is a great variety of methods to evaluate the acrosome, but most are complicated or are expensive and require specialized equipment. It is possible to simultaneously evaluate acrosome status and sperm viability with FITC-PNA combined with PI, as it was done in the present study. The limiting factor of the technique is the need for a fluorescence microscope, which is rarely found in a laboratory under field conditions. In contrast, the CB stain is an effective and inexpensive method to evaluate sperm acrosomes in mammals. This is the first study that evaluated the acrosome status of boar sperm using the CB stain on samples previously subjected to the HOS test, with the objective to simultaneously

evaluate the acrosome and plasma membrane function. This combination of techniques successfully evaluated these two important parameters and can easily be incorporated into the routine breeding soundness evaluation on any pig farm in the field, without the need for an epifluorescent microscope and expensive fluorochromes.

In conclusion, it is possible to use a rapid freezing curve that takes only a few minutes to cryopreserve boar semen, using less equipment and at a reasonable cost. It can be used in the field or on any pig farm, without the need of transporting semen samples or boars, and only a few laboratory equipment are required. Both cryoprotectants, glycerol and DMF, similarly preserved boar semen, and the addition of 50 mM of L- carnitine and 10 mM of pyruvate to the extender was unable to improve post-thaw sperm parameters in boars.

## CONFLICTS OF INTEREST

None of the authors have any conflicts of interest to declare.

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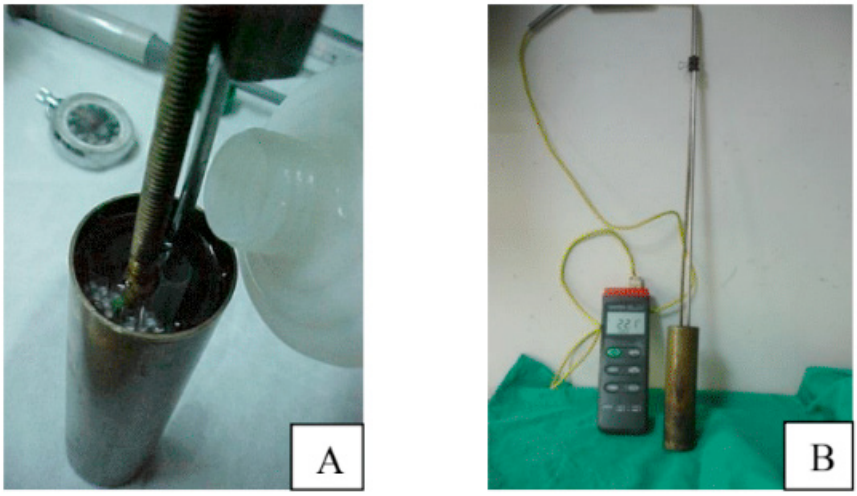


Figure 1. Straws inside bronze canister with ethanol-acetone (A) and a digital thermometer to control the temperature descent (B).

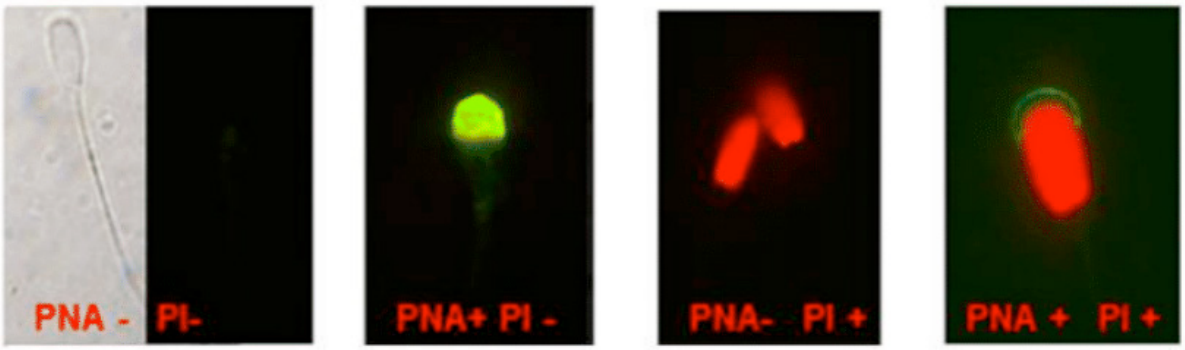


Figure 2. Staining patterns observed using FITC-PNA/PI. PNA-: sperm with an intact acrosome; PNA+: sperm with a reacted acrosome; PI-: live sperm, with intact membrane; PI+: dead sperm, with damaged membrane.

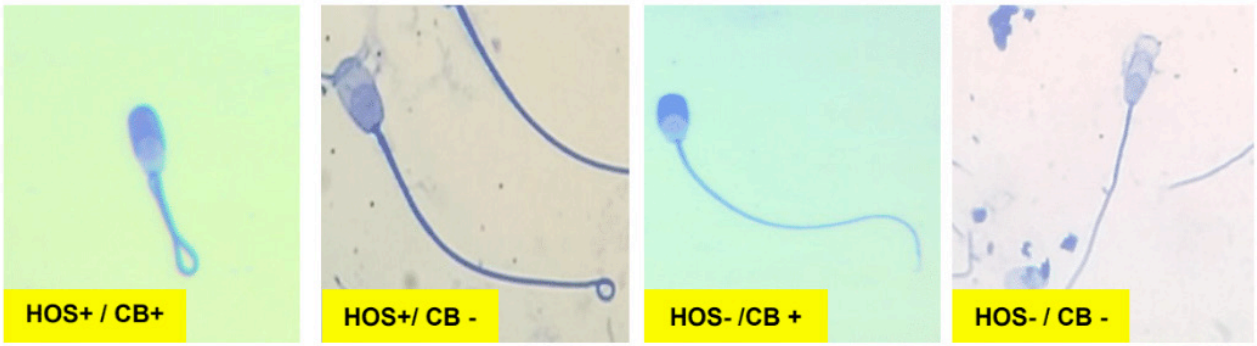


Figure 3. Patterns observed using the HOS/ Coomassie blue stain. HOS+: sperm with functional membrane; HOS-: sperm with non-functional membrane; CB+: sperm with an acrosome; CB-: sperm without an acrosome.

| Freezing Curve | Antioxidant | Cryoprotectant | Progressive motility [%] | Total motility [%] | Curvilinear velocity [µm/s] | Straight line velocity [µm/s] | Medium path velocity [µm/s] | Lateral head displacement [µm] | Beat Cross frequency [Hz] |
|---|---|---|---|---|---|---|---|---|---|
| rapid | without carnitine | glycerol | 12.5 ± 6.6 | 22.3 ± 10.0 | 32.8 ± 11.0 | 12. 4 ± 5.3 | 17.1 ± 6.0 | 1.5 ± 0.9 | 4.1 ± 2.3 |
| rapid | with carnitine +pyruvate | glycerol | 11.0 ± 5.6 | 21.8 ± 9.5 | 31.7 ± 12.5 | 12.5 ± 5.5 | 17.2 ± 6.0 | 1.5 ± 1.0 | 13.1 ± 2.1 |
| rapid | without carnitine | DMF | 11.3 ± 5.6 | 18.4 ± 7.0 | 30.0 ± 10.5 | 11.5 ± 4.4 | 15.5 ± 5.3 | 1.5 ± 0.9 | 4.1 ± 2.7 |
| rapid | with carnitine +pyruvate | DMF | 11.0 ± 6.0 | 19.5 ± 7.7 | 29.8 ± 8.9 | 10.2 ± 5.0 | 14.9 ± 5.6 | 1.5 ± 1.0 | 3.5 ± 1.9 |
| slow | without carnitine | glycerol | 12.6 ± 7.0 | 21.8 ± 12.0 | 36.9 ± 14.0 | 14.7 ± 6.5 | 19.3 ± 7.0 | 1.6 ± 1.0 | 5.3 ± 3.3 |
| slow | with carnitine +pyruvate | glycerol | 12.3 ± 5.7 | 22.0 ± 4.5 | 32.2 ± 14.0 | 13.7 ± 5.5 | 17.8 ± 6.5 | 1.5 ± 0.9 | 4.3 ± 3.9 |
| slow | without carnitine | DMF | 13.0 ± 7.4 | 24.3 ± 9.0 | 32.0 ± 9.0 | 13.6 ± 2.9 | 17.7 ± 3.2 | 1.4 ± 0.9 | 4.1 ± 2.3 |
| slow | with carnitine +pyruvate | DMF | 10.7 ± 6.6 | 18.7 ± 6.5 | 33.1 ± 14.0 | 11.1 ± 4.6 | 17.3 ± 7.5 | 1.3 ± 1.0 | 2.8 ± 1.7 |

Table 1. Evaluation of kinetic parameters. Data are expressed as mean ± SD.

DMF: dimethylformamide

| Freezing Curve | Antioxidant | Cryoprotectant | PNA- Pi- | PNA + Pi- | PNA- Pi + | PNA+ Pi + |
|---|---|---|---|---|---|---|
| rapid | without carnitine | glycerol | 23.2 ±10.0 | 2.4 ± 2.0 | 15.5 ± 7.0 | 59.0 ± 12.0 |
| rapid | with carnitine + pyruvate | glycerol | 25.0 ± 10.0 | 2.5 ± 2.0 | 15.1 ± 8.0 | 57.5 ± 14.7 |
| rapid | without carnitine | DMF | 22.8 ± 7.5 | 2.9 ± 2.0 | 15.3 ± 6.8 | 59.0 ± 9.0 |
| rapid | with carnitine + pyruvate | DMF | 27.1 ± 11.0 | 3.2 ± 2.7 | 12.0 ± 6.0 | 57.8 ± 13.0 |

Table 2. Evaluation of sperm viability and acrosome integrity using a rapid curve. Data are expressed as mean ±SD.

| Freezing Curve | Antioxidant | Cryoprotectant | PNA- Pi- | PNA + Pi- | PNA- Pi + | PNA + Pi + |
|---|---|---|---|---|---|---|
| slow | without carnitine | glycerol | 21.3 ± 8.4 | 2.8 ± 2.0 | 14.4 ± 7.8 | 61.4 ± 11.6 |
| slow | with carnitine + pyruvate | glycerol | 21.5 ± 7.0 | 2.8 ± 2.0 | 14.0 ± 7.8 | 61.7 ± 9.8 |
| slow | without carnitine | DMF | 21.0 ± 7.2 | 3.2 ± 2.8 | 13.6 ± 7.5 | 62.3 ± 8.9 |
| slow | with carnitine + pyruvate | DMF | 21.1 ± 9.5 | 2.9 ± 2.0 | 13.7 ± 5.6 | 62.3 ± 10.9 |

Table 3. Evaluation of sperm viability and acrosomal integrity using a slow curve. Data are expressed as mean ±SD.

DMF: dimethylformamidePNA-/PI-: live sperm with intact acrosomes. PNA+/PI-: live sperm with reacted acrosomes. PNA-/PI+: dead sperm with intact acrosomes.PNA+/PI+: dead sperm with reacted acrosomes.

| Freezing Curve | Antioxidant | Cryoprotectant | CB+ H+ | CB - H+ | CB + H- | CB - H- |
|---|---|---|---|---|---|---|
| rapid | without carnitine | glycerol | 18.8 ± 5.4 | 6.6 ± 5.0 | 9.2 ± 2.6 | 65.4 ± 5.9 |
| rapid | with carnitine+ pyruvate | glycerol | 18.0 ± 5.3 | 7.4 ± 6.2 | 9.4 ± 3.2 | 65.2 ± 5.3 |
| rapid | without carnitine | DMF | 14.9 ± 5.3 | 7.2 ± 4.9 | 8.4 ± 3.2 | 69.5 ± 6.4 |
| rapid | with carnitine+ pyruvate | DMF | 19.3 ± 5.6 | 6.6 ± 3.8 | 10.6 ± 3.9 | 63.6 ± 6.9 |
| slow | without carnitine | glycerol | 17.7 ± 7.5 | 8.2 ± 4.3 | 9.6 ± 4.2 | 64.5 ± 8.8 |
| slow | with carnitine+ pyruvate | glycerol | 20.2 ± 7.4 | 7. 8 ± 5.6 | 10.7 ± 2.6 | 61.3 ± 8.4 |
| slow | without carnitine | DMF | 19.6 ±8.8 | 7.2 ± 5.1 | 8.7 ± 4.8 | 64.6 ± 12.6 |
| slow | with carnitine+ pyruvate | DMF | 18.3 ± 9.0 | 7.4 ± 6.0 | 10.2 ± 4.3 | 64.1 ± 10.0 |

Table 4. Evaluation of membrane function and acrosomal status. Data are expressed as mean ±SD.

DMF: dimethylformamideCB + H+: sperm without acrosome. Hypoosmotic test positiveCB -H+: sperm with acrosome. Hypoosmotictest positive. CB + H-: sperm without acrosome. Hypoosmotic test negative. CB -H-: sperm with acrosome. Hypoosmotic test negative

# ÍNDICE REMISSIVO

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## T



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