



EFFECT OF THE ADDITION OF SEMINAL PLASMA, VITAMIN E AND INCUBATION TIME ON POST-THAWED SPERM VIABILITY IN BOAR SEMEN

[EFECTO DE LA ADICIÓN DE PLASMA SEMINAL, VITAMINA E Y TIEMPO DE INCUBACIÓN EN LA VIABILIDAD POST-CONGELAMIENTO DEL ESPERMA EN SEMEN DE VERRACO]

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SUMMARY

The objective of the study was to evaluate the effect of seminal plasma (SP), vitamin E (VE), and incubation time on sperm viability of post-thawed boar semen. Thirty six ejaculates were used and allocated to four treatments: T1, semen + BTS (Belstville Thawing Solution) + 10% SP; T2, semen + BTS + 200µg/ml VE; T3, semen + BTS + 10% SP + 200µg/ml VE; T4, semen + BTS (control). Motility (MOT), intact acrosomes (IA), membrane integrity (MI) and mitochondrial activity (MA) were evaluated, at 0 and 30 min after thawing. A split plot design was used and the data analyzed using a mixed model analysis of variance. There was a significant effect of SP and VE on IA and MI ($P < 0.05$) but not on MOT and MA ($P > 0.05$). There was significant effect of incubation time on MOT (21.3 and 27.9%) and IA (46.0 and 36.0%), at 0 and 30 min post-thawing ($P < 0.05$). It is concluded that the vitamin E, seminal plasma or the combination of both, affected the thawed sperm traits here studied. Incubation time increased motility and intact acrosome percentage but did not affect the membrane integrity and mitochondrial activity.

Key words: semen; thawing, boar; viability; seminal plasma; vitamin E.

RESUMEN

El objetivo del estudio fue evaluar el efecto del plasma seminal (PS), Vitamina E (VE) y tiempo de incubación sobre la viabilidad espermática de semen de verracos después de su congelamiento. Treinta y seis eyaculados fueron usados y asignados a cuatro tratamientos: T1, semen + BTS (Solución de postcongelamiento Belstville) + 10% PS; T2, semen + BTS + 200µg/ml VE; T3, semen + BTS + 10% PS + 200µg/ml VE; T4, semen + BTS (control). La motilidad (MOT), integridad de acrosomas (IA), integridad de membrana (IM) y la actividad mitocondrial (AM) se evaluaron a los 0 y 30 min después del congelamiento. Se utilizó un diseño en parcelas divididas y los datos se analizaron mediante un análisis de varianza para modelos mixtos. Se encontró efecto significativo de PS y VE sobre IA y IM ($P < 0.05$) pero no sobre MOT y AM ($P > 0.05$). Hubo efecto significativo de tiempo de incubación sobre MOT (21.3 y 27.9%) y IA (46.0 y 36.0%), a los 0 y 30 min postcongelación ($P < 0.05$). Se concluye que la vitamina E, plasma seminal o la combinación de ambos afectaron las características seminales aquí estudiadas. El tiempo de incubación incremento la motilidad y el porcentaje de acrosomas intactos, pero no afecto la integridad de la membrana y la actividad mitocondrial.

Palabras clave: semen; descongelamiento; viabilidad; plasma seminal; vitamina E.

INTRODUCTION

There are different reproductive technologies used in the pig industry in order to improve production. Among them it can be mentioned artificial insemination using refrigerated or frozen semen. However, fertility using frozen semen is low. It has been shown that cryopreserved spermatozoa have a premature ageing effect characterized by changes with

negative effects on fertility. This is associated with structural and functional changes in the spermatozoa during the frozen process, specifically during the thaw process, such as: sudden changes in temperature, osmolarity and pH, which decreases sperm viability. Similarly there is a reduction in motility and sperm fertility associated with an excessive formation of reactive oxygen substances (ROS), which increase the membrane peroxidation. Different additives have been

tested to minimize the spermatozoa damage (Bailey, 2008). Vadnais *et al.* (2005) and Yoval (2009) found that the use of 10% seminal plasma, added in various stages of the cryopreservation process increases the resistance of the spermatozoa to cold shock, when sperm cells are incubated in this medium. Vitamin E (α -tocopherol) is a fat-soluble antioxidant that acts stopping the propagation of the lipid peroxidation of the membrane during freezing (Breininger *et al.*, 2005), and improving motility of spermatozoa (Ucet *et al.*, 2010). Most of the works on boar seminal plasma have been done during freezing; however, few of those have studied its effect during thawing. The aim of this study was to evaluate the effect of the addition of 10% seminal plasma, 200 μ g/ml Vitamin E and both additives to the thawing medium, as well as the effect of incubation time on post-thawed sperm viability of pigs.

MATERIALS AND METHODS

Semen samples of six boars of the PIC line, 2 to 4 years old, used as widgets in farms were obtained; all boars were under the same management and feeding regimen. Each boar was collected once per week with the gloved hand technique, obtaining a total of 36 samples of the rich fraction. The semen samples were placed in a water bath at 37°C and the macroscopic characteristics such as color and aspect were evaluated by direct observation of the samples. Semen volume was measured using a graduated sterile flask of glass, previously moderated in a water bath at 37°C. Subsequently the samples were diluted 1:1 in BTS commercial diluent (Belstville Thawing Solution) and deposited in a refrigerator until its transfer to the laboratory (Hernández *et al.*, 2007a). Before freezing, the samples were again evaluated based on microscopic characteristics, including: individual motility (IM), sperm concentration, morphology and acrosome integrity. The samples suitable for freezing where those with a progressive IM \geq 80%, a spermatid concentration \geq 100 x 10⁶ sperm/ml, a percentage of abnormalities \leq 10% and AI \geq 90% (Caballero *et al.*, 2007).

Semen evaluation

For individual motility assessment, a drop of semen was placed in a slide and observed at a 400 X magnification. The results obtained were expressed as percentage according to the technique described by Hernández *et al.* (2007a). Spermatozoa concentration was evaluated taking a sample of diluted semen in a physiological saline solution (1:100) of methylene blue and 1% formaldehyde, and with the help of a Pasteur pipette a drop of semen was taken and placed in the Burker chamber. Spermatozoa were counted under a microscope at 400 X, and the results were expressed as the number of sperms per millimeter of

semen. The sperm morphology was determined by placing a drop of semen in a solution of 1% formaldehyde, using the technique of Pursel *et al.* (1975). A volume of 0.5 ml of the mix was taken and placed in a slide and the sperm morphology determined in a phase contrast microscope at 1000 X. For each sample, 100 sperms were counted, and the results expressed as percentage of normal spermatozoa. The percentage of intact acrosomes was evaluated from a solution of 1% formaldehyde (v/v), taking a drop of this solution and placing it on a slide. Smears were fixed and observed with a microscope at 1000 X, counting a total of 100 spermatozoa in the same smear. Spermatozoa with intact acrosomes were those that presented a dark sharp form. The results were expressed in percentage.

Semen Freezing

The freezing of semen was carried out using the sperm cryopreservation protocol described by Thurston *et al.* (1999). After previous evaluation of semen, samples were placed in a refrigerator at 16°C for 3 h, and then placed in a Falcon tube and centrifuged at 800 X g for 15 min at 16°C, using a refrigerated centrifuge (Hermle, Germany). The tubes were taken into a cold room at 16°C and the supernatant was retired using a plastic pipette. LEY diluent (80% sterile water, 8.49 g lactose, 20% egg yolk and 30 μ l of Kanamycin) was added to the semen pellet, until it reaches a concentration of 1.5 x 10⁶ sperms per milliliter. The tubes with semen were submerged in a container with water at 16°C and then cooled at 5°C, for 2 h, in a refrigerator. Thereafter the samples were transported back to the cold room, where it was added a second diluent, LEYGO (92.5% LEY, 6% glycerol and 1.5% Orvus ES Paste) to reach a final concentration of 1x 10⁶ spermatozoa per milliliter. Then the samples were full packed using an automatic semen packing machine (IMV, France), with each straw containing a final concentration of 500 x 10⁶ spermatozoa. The straws were placed on a stainless grill of steel, inside a container with liquid nitrogen so they were suspended at 5 cm from the surface of the nitrogen. After 20 min of exposition to the nitrogen vapor, the straws were immersed and stored in baskets properly identified inside of thermos with liquid nitrogen at -196°C, where they remained until their evaluation.

Collection of the seminal plasma

The seminal plasma (SP) used in the treatments was obtained from three complete ejaculates of a stud of boars classified as good freezers, based on their seminal characteristics after freezing: \geq 40% individual motility and \geq 75% normal acrosomes (Hernández *et al.*, 2007a). Each semen sample was centrifuged 3 times at 3800 X g during 15 min at 17°C (Thurston *et al.*, 1999), and the supernatant was eliminated from the

pellet and deposited in Falcon tubes mixing the three samples; then they were evaluated under the microscope to ensure the total absence of sperm cells; the SP was stored at -20°C until its use.

Thawing

The straws were thawed placing them in a water bath at 37°C for 20 seconds, shaken gently and the contain deposited in Falcon tubes with BTS. SP and vitamin E, VE (Sigma-Aldrich). For each treatment three straws were used, and after thawing they were added BTS (1:1) medium and placed in a test tube deposited in a water bath at 37°C, obtaining a final volume per treatment of 3 ml (1.5 ml semen + 1.5 ml BTS). Four final treatments were established: T1: semen + BTS + 10% SP; T2: semen + BTS + 200µg/ml VE; T3: semen + BTS + 10% SP and 200 µg/m VE and T4: semen + BTS (control). The samples were kept in a water bath and evaluated at 0 and 30 min.

Motility and intact acrosomes were evaluated according to Hernández *et al.* (2007a). For the evaluation of the mitochondria membrane integrity the Harrison and Vickers (1990) technique was used. For the evaluation of the mitochondrial activity Rhodamine123 was utilized. Several dilutions were made in a vial to obtain 1 ml volume of semen with a sperm concentration of 30×10^6 spermatozoa, then moved to a dark room and added 6 µl of the above-mentioned staining, and incubated in an oven at 37°C for 10 min; when this time elapsed the sample was moved back to a dark room and an aliquot of 6 µl was taken and evaluated with an epifluorescence microscope (Olympus Japan) with a 400 X magnification. One hundred cells were counted. Active spermatozoa were those that emitted green fluorescence at the middle piece. The membrane integrity was evaluated by the fluorescence technique using propidium iodide (PI); 30 µl of diluted semen was taken and placed in a vial containing 2 µl of the mentioned stain. The samples remained incubated in an oven at 37°C for 15 min, and after that the sample was moved back to the black room and 4 µl was taken and observed in a fluorescent microscope at 100 X. One hundred cells were counted considering as integral cells those that did not emit red coloration in the head. Those that emitted a red coloration were considered with membrane damage. The results for both indicators were expressed in percentages.

Design and statistical analysis

A split-plot design was used and data were analyzed using mixed models (MIXED) procedures (SAS, 2002). To achieve normal distribution, the data from all the sperm characteristics were transformed using the square root function. The statistical model included the fixed effects of treatment (4), week (6), time (2),

time x treatment interaction, the random effect of boar, boar x week interaction (error a), treatment within boar x week interaction (error b) and the residual term. When the outcomes were significant, treatment means were compared using Tukey multiple rank test. Values were considered significant when $P < 0.05$. To determinate the significance of the sources of variation transformed data were used, but the non-transformed means are provided.

RESULTS

Significant effect of treatments was observed for acrosome and membrane integrity. For acrosome integrity a better performance was observed for the control compared with T1 and T3 ($P < 0.05$) but it was similar to T2 (Table 1). For membrane integrity all treatments were higher than the control ($P < 0.05$) but there were no differences among them ($P > 0.05$). On the other hand, no significant effect of treatment for motility traits and mitochondrial activity was found ($P > 0.05$). A significant effect of post-thawed incubation time on motility was found. Motility increased 6% ($p < 0.05$) from 0 to 30 minutes. However, this trend was not observed for acrosome integrity, which decreased 10% ($P < 0.05$). Similarly no significant effect of time on membrane and mitochondrial activity was found (Table 2). Treatment x time interaction had a significant effect ($P < 0.008$) on MA. Also significant effect of boar, week and the interaction of both was found for all the evaluated traits. However, in this study only the effects of time and treatment are discussed

Table 1. Effect of treatments on motility (MOT), intact acrosomes (IA), membrane integrity (MI) and mitochondrial activity (MA) of boars.

Treatment	N	MOT (%)	IA (%)	MI (%)	MA (%)
T1	72	24.02 ^a	39.57 ^{bc}	53.54 ^a	56.41 ^a
T2	72	24.23 ^a	42.28 ^{ab}	52.00 ^a	57.34 ^a
T3	72	26.11 ^a	35.67 ^c	53.00 ^a	58.00 ^a
T4	72	24.09 ^a	46.47 ^a	44.67 ^b	52.00 ^a
EE		1.01	1.30	1.52	1.68

^{a,b,c}Different letters between treatments indicate significant differences ($P < 0.05$); EE = Standard error.

DISCUSSION

The lack of significant differences between treatments, in this study, on sperm motility agrees with Vadnais *et al.* (2005) results, who also did not find any effect on this variable using different SP treatments during the thawed boar semen process. Likewise, Okasaki *et al.* (2009) did not find effect of the addition of 10% and 20% SP on boar semen motility after 6 hours of

incubation. On other hand, Satorre *et al.* (2007) found a significant improvement in sperm motility with the addition of VE to the freezing medium. Peña *et al.* (2003) evaluated two levels of VE in two fractions of the ejaculate and observed that the motility observed significantly improved in comparison with the control group. Hernández *et al.* (2007b) reported a significant improvement in sperm motility with the addition of SP to the boar semen freezing medium. Garcia *et al.* (2010) found a significant effect of treatment ($P<0.001$) on motility, when post-thawed porcine semen was incubated with 10% SP at 0 and 60 min (48 and 28%, respectively). Similar results using VE were reported by Breininger *et al.* (2005). It is important to mention that the subjective evaluation process of motility is not a reliable indicator of the quality of the sperm after thawing. There are discrepancies between the results reported by various authors and the obtained in this study. One of the possible explanations is the reliability of the results, characterized by sensitivity and the specificity of method of evaluation (optic microscope). When this subjective method is used, it is estimated 30- 60% variation compared with objectives methods, such as Computer Assisted Sperm Analysis, CASA (Budworth *et al.*, 1988; Amman, 1989; Peña *et al.*, 2003). CASA has high levels of accuracy and reliability when compared with other methods of sperm classification (Verstengen *et al.*, 2002).

Table 2. Effect of incubation time (minutes) of thaw semen on motility (MOT), intact acrosomes (IA), membrane integrity (MI) and mitochondrial activity (MA) of boars.

Time	N	MOT (%)	IA (%)	MI (%)	MA (%)
0	144	21.32 ^a	46.00 ^a	50.50 ^a	56.76 ^a
30	144	27.92 ^b	36.00 ^b	51.02 ^a	55.07 ^a
EE		0.48	0.94	0.70	0.83

^{a,b}Different letters between times indicate significant differences ($P<0.05$); EE= Standard error.

Changes in the acrosomal integrity before freezing are associated with the premature cryocapacitation making the sperm incapable of fertilizing the eggs. Garner *et al.* (1995) and Silva and Gadella (2006) mentioned that the acrosomal status is one of the principal parameters in the evaluation of the viability and functionality of the membrane. In this study, treatments showed a significant effect on the percentages of intact acrosomes getting higher values with the addition of VE and with the control. In previous studies higher values for intact acrosomes were observed with the use of VE added to the freezing diluents of pigs (Uc *et al.*, 2010). Hernández *et al.* (2007b) reported a significant effect on the

percentage of intact acrosomes when they added SP during the freezing boar semen process. The results of this study suggest that the dose of Vitamin E (α -tocopherol) was enough to inhibit the effects of lipid peroxidation by enhancing the acrosomal integrity in the thawed semen. This could be explained because α -tocopherol protects the spermatozoa of detrimental effects caused by the oxidative stress associated to the thawing process. It has been expressed that VE inhibit the lipid peroxidation by removing the free radicals before they act on the fatty acids of the sperm plasma membrane contributing to the maintenance of sperm viability (Merkies *et al.*, 2003; Breininger *et al.*, 2005; Ordoñez, 2008).

After thawing, it is important that the spermatozoa keep the integrity of the plasma membrane, because the sperm depends on this to successfully fertilize the oocyte (Hafez, 1996; Neil, 2006). In this study, the membrane integrity was significantly higher with the addition of VE, SP and the mixing of both additives compared with the control treatment. Perez *et al.* (2001) incubating fresh ovine semen with two treatments (VE and VE+SP) found a decrease in viability when VE was used; however, when they incubated the semen with both VE and SP, they found a significant increase in viability compared with the control group (41.2% and 25%). Nevertheless, their results were lower than the observed in this study (53%). SP provides proteins and antioxidants substances (like the enzyme superoxide dismutase enzyme, peroxidase, catalase, vitamin C and E), which protects the plasma membrane; however, the content of vitamin E is not enough in oxidative stress situations, as it occurs during thawing. Thus, the addition of the mixture of SP and vitamin E in the diluent of thawing could increase the protective effect in the sperm cells. Furthermore, during the thawing process, the temperature cause changes in the lipid phase, resulting in an increment in the membrane fluency (Holt and North, 1984; Parks and Graham, 1992), which causes the loss of decapacitant proteins before the spermatozoa are deposited in the female reproductive tract, causing the premature cryocapacitation. However, there are reports which mention that the addition of SP to the incubation medium of the sperm could prevent and reverse this state (Vadnais and Roberts, 2007).

As mentioned before, the plasma and acrosomal membranes of the spermatozoa are considered the main sites of the damage caused by the thermal, mechanical and chemical stress during thawing. For this reason, the interaction between the sperm and the surrounding medium, it is a crucial factor that could affect the properties of the mentioned surface. The addition of SP, after a stress situation, can reverse the damages caused to the membrane, since it has been determinate that specific components of SP, especially

decapacitant proteins, are absorbed in the surface of the sperm, preventing the changes required for capacitation and acrosomal reaction, and/or inhibiting the traduction pathways signals that initiate those events. In this way contributes to the stability of the membrane, of the phospholipids and of its cytoskeleton, maintaining sperm viability (Garcia *et al.*, 2006; Colas *et al.*, 2009).

In this study, no significant differences between treatments for mitochondrial activity were found. Similar results were obtained by Yoval *et al.* (2009), who evaluated different concentrations of SP on mitochondrial activity and also noted that there were no differences between different concentrations of SP. In the fresh semen the evaluation of mitochondrial activity is an objective way to quantify the amount of damage on the sperm midpiece, because this organelle is the site where the oxidative metabolism is executed. There is few works that measure the effects caused by thawing on mitochondrial activity.

One of the structures that suffer more damage during the cooling and freezing processes is the midpiece of the spermatozoa (Bailey *et al.*, 2000); it is probable that the inclusion of different additives to the thawing medium, add various components (like some proteins) in enough proportion, that can join the midpiece and interact with the membrane making it more resistant and protect it from the harmful effects of freezing. The freezing of semen mostly produce an increase in the concentration of reactive oxygen substances. The oxidative stress caused by these, cause malfunctions in the mitochondria, which can be prevented or contracted by the presence of antioxidants agents in the diluents. This suggests that in this study the additives used in the thawing process had enough concentration of antioxidants, because the mitochondrial activity in the studied treatments did not decrease.

The observed gradual increase of motility with time can be attributed to the temperature at which the samples were incubated. It is probable that freezing temperatures affect the proteins phosphorylation, preventing the fast progressive motility immediately after freezing, being necessary some time, during the incubation process at 37°C, to acquire rapid progressive motility. Similar results have been reported by Medina-Robles *et al.* (2008) when incubating thawed boar semen.

In contrast with motility, the percentages of intact acrosomes in this study decreased significantly with incubation time from 0-30 minutes (46% - 36%). Similar effects on the acrosomal integrity were reported by Breininger *et al.* (2005), who evaluated different concentration of Vitamin E, and observed a decrease in the acrosomal integrity with incubation

time from 4 to 37°C. Fernandez *et al.* (2007) reported similar results after 2 hours of incubation with VE, to temperatures of 37°C. Similar results were reported by Soler *et al.* (2003), who evaluated the effect of the incubation on thawing without any additive, and they observed a significant decrease in acrosomal integrity, after 120 min of incubation. On the other hand, Okasaki *et al.* (2009) found significant effect when adding different concentrations of SP in thawed boar semen, incubated for 3 hours, reporting a decrease in the percentages of losses of the acrosomal integrity, after 120 minutes of incubation. Dominguez-Rebolledo *et al.* (2010) evaluated the effect of VE in thawed semen of deer, reporting that the addition with this antioxidant maintain higher percentages of acrosomal integrity in conditions without stress and with oxidative stress (media with 100 µM of Fe²⁺+ 500 µM ascorbate) evaluated at 0, 2 and 4 h after incubation. Pettit and Buhr (1998) and Medeiros *et al.* (2002) mentioned that one of the major damages to the sperm cell during the cryopreservation occurs at the acrosomal membrane level, resulting in damage to the actin protein, which contributes to the structural stability of the acrosomal membrane and post-acrosomal, and as the mobility of the sperm flagellum, affecting the sperm viability.

In this study there was no significant effect of time on the membrane integrity and mitochondrial activity. Dominguez-Rebolledo *et al.* (2010) reported in thawed deer semen, that when they added Vitamin E, for an incubation period of 4 hours, the values of the mitochondrial activity remained high and the values for membrane integrity decreased over time. Michael *et al.* (2009) evaluated the effect of the addition of VE to the freezing media of dog semen and observed a significant decrease in the membrane integrity. The differences between motility, acrosome integrity, membrane integrity and mitochondrial activity suggest that the addition of SP and Vitamin E to the thawed media and the incubation time, affected the intracellular components and/or membrane required for the normal function of the spermatozoa (Breininger *et al.*, 2005).

CONCLUSIONS

The addition of Vitamin E, seminal plasma or both, affected directly the post-thawed sperm characteristics, and incubation time modified the motility and percentage of intact acrosomes, whereas it maintained the membrane integrity and the mitochondrial activity.

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