Effect of the Addition of α-Tocopherol and Quercetin as Antioxidants to the Diluent, in the Freezing of Boar Semen on Sperm Quality

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ABSTRACT

During seminal freezing, sperm undergo oxidative stress, reducing their motility, viability, and acrosomal integrity. To prevent these damages, antioxidants have been added at the time of seminal freezing. The objective was to assess the antioxidant effect of the combination of α-tocopherol with quercetin, added to the diluent for the freezing of boar semen. The semen of boars of the Pietrain and York/Pietrain breeds was frozen in 0.5 ml straws, before freezing α-tocopherol in a concentration of 4 mg/ml (T1), quercetin in concentrations of 25, 50 and 100 μM (T2, T3 and T4), α-tocopherol + Quercetin in concentrations of 4 mg/ml + 25 μM, 4 mg/ml+50 μM and 4 mg/ml + 100 μM (T5, T6 and T7) and the control group (T8) without antioxidant. The straws were frozen in liquid nitrogen for 7 days and thawed at 42 °C for 12 seconds. 5 repetitions were performed analyzing motility, viability, and NAR. the results were analyzed using a completely randomized design in factorial arrangement comparing the means with a Tukey test. The best percentage of motility was for T5, T4 and T1 with 39.44, 38.06 and 37.33%, respectively, there was a significant difference with T8; and the best NAR percentages were for T8 with 94.90%, with a significant difference for T1. In conclusion, the addition of α-tocopherol and quercetin separately or in combination protects the motility, viability, and NAR of spermatozoa from frozen-thawed boar semen.

Keywords: Antioxidants, frozen, pork, semen, quercetin, α-tocopherol.

I. INTRODUCTION

Semen freezing is a technique that has made it possible to preserve genetic material for a long time, allowing the semen to be transported long distances and having access to the genetic material of the best reproductive males (Caldevilla et al., 2016; Olivo-Zepeda, et al., 2017).

Artificial insemination with fresh or refrigerated semen in pigs has made it possible to improve the pig genetic profile of developing countries, however, since the first artificial insemination (AI) was carried out with frozen boar semen in 1970, the results Fertility and prolificacy have been lower (10-20% and 1-2 piglets) than those obtained with natural breeding or artificial insemination with fresh or refrigerated semen, so that currently more than 99% of AIs are performed using semen refrigerated and only 1% of AI procedures use frozen semen (Caldevilla et al., 2016; Olivo-Zepeda, et al., 2017; Williams et al., 2015; Yan et al., 2017). Therefore, the use of AI with frozen semen in commercial farms has been limited to selection programs, transport of doses over long distances, conservation of populations of interest due to their low quantity and in some cases, tests carried out by groups of research (Williams et al., 2015).

The use of preserved semen for AI in pigs has tripled in the last 15 years. More than 99% of the approximately 19 million inseminations performed worldwide are performed with...
Porcine sperm is very sensitive to low temperatures, (<15 °C), since sperm membranes are rich in polyunsaturated fatty acids (AGP) and, unlike other species, contain less cholesterol, reducing the membrane potential mitochondria and increasing the permeability of the plasma membrane, making the sperm cell sensitive to reactive oxygen species (ROS) produced during the process of obtaining cellular energy, which react with AGP, causing oxidative damage mediated by the lipoperoxidation process (LP), causing biochemical and functional damage to the sperm during the freezing process, reducing mobility, viability and transport in the female genital tract, altering its fertilizer potential (Caldevilla et al., 2016; Olivo-Zepeda, et al., 2017; Williams et al., 2015; Marcial-Gumpa et al., 2009; Flores Solano et al., 2017).

While sperm are sensitive to oxidative stress, they are also equipped with an antioxidant system in seminal plasma and cytoplasm, which are harmoniously integrated to counteract the toxic effects of free radicals (RL), chemically binding to ROS to neutralize them, playing a protective role for cells and delaying oxidation (Williams et al., 2015; Marcial-Gumpa et al., 2009; Flores Solano et al., 2017). However, it has been observed that throughout the cooling curve of the freezing process and during thawing, the concentration of antioxidant enzymes decreases, leaving the spermatozoa exposed to the action of RL (Flores Solano et al., 2017).

In order to provide greater post-thaw survival, various studies have used different antioxidants of an enzymatic and non-enzymatic nature (Vitamin C and E, melatonin, butyl hydroxytoluene (BHT), catalase, EDTA, lysis, cysteine, propolis, MDPA, a mixture of reduced glutathione and oxidate and L-carnitine) added to the diluent as additives, in order to reduce the deleterious effect of ROS during sperm preservation, preventing loss of motility, viability and integrity of sperm DNA during freezing and increasing fertility (Flores Solano et al., 2017; Tae-Hee et al., 2014; Seifi et al., 2016). Among these antioxidants is quercetin, which has been used as an additive in the diluent for the preservation of human, rodent, bovine, ovine, porcine, goat, canine and equine semen (Tae-Hee et al., 2014; Seifi et al., 2016; Perez Aguirre et al., 2012; Silva et al., 2012; Gibb et al., 2013; Silva et al., 2016; Tvrđá et al., 2016; Afshin-Seifi et al., 2017; Avdatek et al., 2017; Chae et al., 2017), nothing that it reduces the OLP of sperm during freezing, prevents their premature training before artificial insemination, improves motility and reduces DNA fragmentation of sperm (Tae-Hee et al., 2014; Avdatek et al., 2017; Restrepo et al., 2016). Also, in other types of cells it has been observed that it has synergistic effects with other antioxidants such as vitamin E and C, since it protects against oxidation and photooxidation, acting particularly against the superoxide radical, delaying peroxidation (Martines-Flórez et al., 2000).

This research aimed to assess the antioxidant effect of the combination of quercetin with α-tocopherol, added to the diluent for freezing boar semen.

### II. MATERIALS AND METHODS

#### A. Semen Collection

10 ejaculates were collected from two boars of the pure German pietrain and york/pietrain breeds located at the Center for Teaching, Research and Extension in Swine Production (CEIEPP) of the Faculty of Veterinary Medicine and Zootechnics of the National Autonomous University of Mexico (UNAM). Ejaculates were obtained with the gloved hand technique described by Córdova Izuierdo, et al., (2015). The stallions were between 1.5 and 3.5 years old. The collected semen was diluted 1: 1 (v: v) in the commercial diluent BTS® (KUBUS, S.A.) for transport to the laboratory. Progressive motility, viability and acrosomal integrity (NAR) were evaluated and only semen with motility greater than 80% was frozen.

#### B. Semen Freezing

Semen freezing was carried out following the protocol of Westendorf, et al., (1975), with some modifications of Córdova Izuierdo, et al., (2006) and Gutiérrez Pérez et al., (2009). The semen was centrifuged at 800 g for 10 min, the supernatant was removed and the cooling diluent (A) (Dextrose, Distilled water, Egg yolk and Gentamicin) was placed, the samples were placed at 15 °C for 90 minutes, to then put them at 5 °C where they were kept for 120 minutes. Then the freezing diluent (diluent B (Dextrose, Distilled water, Egg yolk, Gentamicin and Glycerol)) was placed in two times with an addition difference of 20 min at each time. The diluents were placed in a 1:1 ratio. After time, the treatments were poured into 0.5 ml straws at a concentration of 300X10⁶/straw, and then placed at 5 cm of liquid nitrogen at -196 °C. After 7 days of freezing, the treatments were thawed at 42 °C for 12 seconds (24) and tests of progressive motility, viability and NAR were immediately carried out.

#### C. Addition of Antioxidants

The antioxidants used were α-tocopherol (4 mg/ml), quercetin (25, 50 and 100 µM), and α-tocopherol in combination with quercetin (4 mg/ml + 25 µM, 4 mg/ml + 50 µM and 4 mg/ml + 100 µM) (Sigma-Aldrich®), which were added to diluent A and mixed in a vortex for 2 seconds, to later add diluent A to the sperm package and mixed with pipetting.

#### D. Semen Evaluation

Sperm concentration: It was evaluated with the technique of Gutiérrez Pérez et al., (2009), diluting 25 µL of semen in 500 µL of 0.1% newt, and a drop was placed in the Neubauer chamber. For the sperm count per ml, the following formula was used: no. of sperm cells counted (21) (10000) (Johnson, et al., 2000).

Progressive motility: It was evaluated according to Gutiérrez Pérez et al., (2009), in which a 10 µL drop of semen is deposited on a slide and covered with a coverslip.
(previously tempered) and the evaluation is carried out at 40 magnifications (40X), where 100 are counted sperm and the percentage that moved in a rectilinear manner is calculated.

Viability: It was determined by the method of Gutiérrez Pérez et al., (2009) with cosin-blackson staining, where a 1: 2 dilutions of the stain and semen was made, to later be scanned along a slide and allowed to dry at room temperature for 15 minutes. 100 sperm were counted at 40X magnification, and the percentage of live sperm was calculated.

NAR: It was carried out according to the technique of Iglesias-Reyes et al., 2019, placing a 10 µL drop of semen and sweeping it on a slide, once dry it was fixed with 96° ethyl alcohol and stained with Giemsa solution for 25 minutes. Then it was rinsed with distilled water, allowed to dry and evaluated at one hundred magnifications (100X) with the help of immersion oil. 200 cells were counted and those with the apical border of the intensely stained sperm were considered as spermatozoa with intact acrosome.

E. Statistical Analysis

The results obtained were analyzed with the JMP statistical package, using a completely randomized design in factorial arrangement, with a comparison of means using the Tukey test. Differences were considered significant for a probability of (P <0.05).

III. RESULTS

Table I shows the motility results of the thawed straws, significant differences (P<0.05) were obtained between treatments T1, T2, T4, T5, T6 and T8. For the case of viability, significant differences (P<0.05) were obtained between treatments T5 and T3, T8. And the results obtained from NAR, had significant differences (P<0.05) between treatments T8 and T1.

### TABLE I: RESULTS OF THE STATISTICAL ANALYSIS OF THE MOTILITY, VIABILITY AND NAR OF THE THAWED SPERM

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Composition</th>
<th>Motility (%)</th>
<th>Viability (%)</th>
<th>NAR (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>α-tocopherol (4mg/ml) + Quercetina (25µM)</td>
<td>37.33&lt;sup&gt;a&lt;/sup&gt;</td>
<td>48.86&lt;sup&gt;a&lt;/sup&gt;</td>
<td>92.93&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>T2</td>
<td>Quercetina (25µM)</td>
<td>36.53&lt;sup&gt;a&lt;/sup&gt;</td>
<td>49.40&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>93.03&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>T3</td>
<td>Quercetina (50µM)</td>
<td>33.28&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>46.28&lt;sup&gt;c&lt;/sup&gt;</td>
<td>94.21&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>T4</td>
<td>Quercetina (100µM) + α-tocopherol + Quercetina (100µM) + α-tocopherol</td>
<td>38.06&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>48.13&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>93.70&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>T5</td>
<td>Quercetina (4mg/ml+25µM) + α-tocopherol</td>
<td>39.44&lt;sup&gt;a&lt;/sup&gt;</td>
<td>51.41&lt;sup&gt;a&lt;/sup&gt;</td>
<td>94.13&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>T6</td>
<td>Quercetina (4mg/ml+50µM) + α-tocopherol + Quercetina (4mg/ml+100µM) + α-tocopherol</td>
<td>36.03&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>50.70&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>93.93&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>T7</td>
<td>Control</td>
<td>34.50&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>47.10&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>93.93&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>T8</td>
<td>Control</td>
<td>28.53&lt;sup&gt;b&lt;/sup&gt;</td>
<td>45.60&lt;sup&gt;b&lt;/sup&gt;</td>
<td>94.90&lt;sup&gt;b&lt;/sup&gt;</td>
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</table>

<sup>ab</sup> They indicate a significant difference with a P <0.05

IV. DISCUSSION

In Table I it can be seen that the best motility results obtained were from the treatments containing antioxidants (T5, T4, T1, T2 and T6) 39.44, 38.06, 37.33, 37.33 and 36.03% respectively, with a significant difference (P<0.05) with the control treatment (T8); of these treatments, the one that obtained the best results was the combination of α-tocopherol + Quercetina (4mg/ml + 25µM) (T5). These results coincided with those obtained in viability, since the best treatment was T5 (51.41%), with a significant difference (P<0.05) with T3, T7 and T8 (46.28, 47.10 and 45.60%, respectively).

Although there are no records of the effect of quercetin or a combination of quercetin with α-tocopherol on the motility and viability of frozen-thawed pig sperm, it has been observed that quercetin prevents lipoperoxidation, neutralizing and inhibiting development of free radicals, and interacts with α-tocopherol to enhance the antioxidant capacity of the latter and while quercetin is oxidized, it can be recycled interacting with acerbate and glutathione; on the other hand, during seminal freezing where there is a peroxidation process that induces losses of motility, viability and changes in sperm metabolism, α-tocopherol protects the sperm plasma membrane against lipoperoxidation, caused by exposure to molecular oxygen, acting as an antioxidant by having the ability to transfer a phenolic nitrogen to a free peroxide radical of a peroxidized polyunsaturated fatty acid reducing oxidative damage to sperm membranes induced by freezing, interrupting the destructive chain reactions of lipoperoxidation and avoiding possible damage cellular (Williams et al., 2015; Marcial-Cumpa et al., 2009; Moretti et al., 2012; Afshin-Seifi et al., 2016).

This type of interaction has also been demonstrated with the combination of other antioxidants such as α-tocopherol and vitamin C, which has been observed that there is a regeneration of α-tocopherol during a process of reduction of ascorbic acid, which in turn once it is reduced by NADH, to later follow two routes, reacting again with α-tocopherol or reacting with another free peroxide radical, protecting the structure of the sperm membranes (Marcial-Cumpa et al., 2009). This coincides with (Restrepo Betancur et al., 2016), which demonstrated a favorable effect on progressive motility, curvilinear speed and mean speed in post-thaw chilled equine sperm added with quercetin; Avdatek et al., (2017) also mentions that quercetin densities between 50 and 100 µM are particularly effective to protect sperm motility, as could be observed in the experiment of Afshin-Seifi et al., (2017), which obtained better motility and viability results in semen from goat added with lower concentrations of quercetin (10 µM); Also with fresh bovine semen (Tvrdá et al., 2016) it demonstrated that there are protective effects of quercetin on the motility and viability of spermatozoa when they are incubated for a period of 24 hours, which coincides with the results obtained in this investigation. However, in other studies where quercetin was added in the conservation of semen of rabbits, horses, sheep, bovine and mice (Silva et al., 2012; Gibb et al., 2013; Avdatek et al., 2017; Johinke et al., 2014; Ranawat et al., 2012), no significant differences were obtained in the characteristics of sperm movement, with respect to the control group, for which (Williams et al., 2015; Ranawat et al., 2012) they comment that the effect of quercetin depends on the dose administered to the semen, as well as on the species in which it is being administered, since quercetin acts in a different way by enzymes that are directly or indirectly linked to the maintenance of sperm motility. However, it should be noted that, despite having a significant
difference in these results, the motility and viability values of T5 are not much higher in percentage with respect to the other treatments, which is why the treatments still they cannot be used commercially as an alternative to artificial insemination.

Regarding the results obtained from NAR, they differed slightly with those obtained in motility and viability, since the control group (T8) was superior to T1 with significant differences (P<0.05), however, it did not differ significantly with the treatments where Quercetin or quercetin combined with α-tocopherol (T2, T3, T4, T5, T6 and T7) were added (Table I). However, all the results obtained from NAR can be considered acceptable, since they are higher than the minimum percentage (80%) (Córdova Izquierdo et al., 2019) required for artificial insemination, just as they were higher than the results obtained in other studies where semen was frozen. of pigs with and without antioxidants, obtaining percentages from 10% (Williams et al., 2015) to 84.75% (Gutiérrez Pérez et al., 2009).

The lower NAR values obtained in the other studies may be due to the fact that the oxidative stress that occurs during the cooling of the semen, triggers the lipoperoxidation process, causing the pig sperm membrane to be more permeable, these changes in the fluidity of The membrane could affect the trans-membrane movement of Ca2 + (which is essential in the training process but harmful during storage) by stimulating the calcium-dependent processes associated with the training (Johonson et al., 2000; Restrepo Betancur et al., 2016). However, the use of antioxidants such as quercetin or resveratrol in preserved semen affects the release of intracellular calcium by inhibiting Ca2+ -ATPase, which slows down training, preventing premature training and acrosomal reaction during storage; and it has even been observed that quercetin has had higher values in the maintenance of NAR compared to other antioxidants (catalase and cysteine)(Silva et al., 2012; Gibb et al., 2012).

V. CONCLUSIONS

In conclusion, the addition of quercetin and α-tocopherol separately or in combination protects the motility, viability and NAR of thawed pig sperm.

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CONFLICT OF INTEREST

Authors declare that they do not have any conflict of interest.

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