European Journal of Biology and Biotechnology
www.ejbio.org

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

Adrian Emmanuel Iglesias Reyes, María de Lourdes Juárez Mosqueda, Oscar Gutiérrez Pérez, Alda Rocío Ortíz Muñiz, Jesús Alberto Guevara González, Abel E. Villa Mancera, Armando Gómez Vázquez, Pedro Sánchez Aparicio, Carlos J. Bedolla Cedeño, and Alejandro Córdova Izquierdo

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; the best percentage of viability were T5 with 51.41%, there was a significant difference with T3 and 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.

Published Online: July 22, 2022

ISSN: 2684-5199

DOI: 10.24018/ejbio.2022.3.4.295

A. E. I. Reyes
Metropolitan Autonomous University, Xochimilco Unit, Mexico City.
M. de Lourdes Juárez Mosqueda
National Autonomous University of Mexico, Mexico City.
O. Gutiérrez Pérez
National Autonomous University of Mexico, Mexico City.
A. R. Ortiz Muñiz
Autonomous Metropolitan University, Unit Iztapalapa, Mexico City.
J. A. Guevara González
Autonomous Metropolitan University, Xochimilco Unit, Mexico City.
A. E. V. Mancera
Meritorious Autonomous University of Puebla, Mexico City.
A. Gómez Vázquez
Juarez Autonomous University of Tabasco, Mexico City.
P. Sánchez Aparicio
Autonomous University of the State of Mexico, Mexico City.
C. J. Bedolla Cedeño
Michoacán University of San Nicolás de Hidalgo, Michoacán, Mexico City.
A. Córdova Izquierdo*
Autonomous Metropolitan University, Xochimilco Unit, Mexico City.
(e-mail: acordova@correo.xoc.uam.mx)

****Corresponding Author***

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

semen that has been spread in liquid form and used the same day, for stored at 15-20 °C for 1 to 5 days. 85% of all inseminations are carried out on the day of harvest or the next day. Virtually all AI with semen stored in liquid is used to produce market pigs. Frozen pork semen has been commercially available since 1975, both in pellet and straw form. However, less than 1% of all inseminations are carried out with frozen and thawed semen, most of the time after export from one country to another and mainly for the purpose of improving the genetic base of a particular country or herd (Johonson *et al.,*2000).

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, lysine, 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; Tvrdá *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 Izquierdo, *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 Izquierdo, *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 $300X10^6$/straw, and then placed at 5 cm of liquid nitrogen vapors for 20 minutes and stored in 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) (Johonson, *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 eosin-blacksin 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

Treatments	Composition	Motility (%)	Viability (%)	NAR (%)
T1	α-tocoferol (4mg/ml)	37.33[a]	48.86[abc]	92.93[b]
T2	Quercetina (25µM)	36.53[a]	49.40[abc]	93.03[ab]
T3	Quercetina (50µM)	33.28[ab]	46.28[c]	94.21[ab]
T4	Quercetina (100µM)	38.06[a]	48.13[abc]	93.70[ab]
T5	α-tocoferol + Quercetina (4mg/ml+25µM)	39.44[a]	51.41[a]	94.13[ab]
T6	α-tocoferol + Quercetina (4mg/ml+50µM)	36.03[a]	50.70[ab]	93.93[ab]
T7	α-tocoferol + Quercetina (4mg/ml+100µM)	34.50[ab]	47.10[bc]	93.93[ab]
T8	Control	28.53[b]	45.60[c]	94.90[a]

[a-c] 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 + Quercetin (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 mobility, 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.

Acknowledgement

CONACYT is thanked for supporting the first author as a fellow with registration number 624422, making this research possible; to the Support Program for Research and Technological Innovation Projects (PAPIIT) of the General Directorate of Academic Personnel Affairs (DGAPA) of the UNAM with the PAPIIT grant IN219717 and to Dr. Oscar Gutiérrez for the facilities to obtain the semen that is used for this study.

Conflict of Interest

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

References

Caldevilla M, Ferrante A, Pendola C, Miragaya M. (2016) Comparación del efecto de dos curvas de congelación de semen porcino. Resultados preliminaries. *Spermova*, 2(6), 112-118. Spanish.

Olivo Zepeda IB, Conejo Nava J, Flores Padilla JP, Núñez Anita RE, García Luna F, Val Arreola D, et al. (2017). Evaluación de dos protocolos de refrigeración previos a la congelación del semen de porcino, *Actas Iberoamericanas en Conservación Animal,* 195-200. Spanish.

Williams S, Fernández V, Gavazza M, Marmunti M, Zeinsteger P, Prenna G. (2015) Congelación se semen porcino: resultados y avances en la técnica. *Analecta Vet,* 3(1), 17-25. Spanish.

Yan Z, Dinghui D, Yu C, Yuan L, Ming Z, Guangbin Z, et al. (2017). Criopreservation of boar sperm induces differential microRNAs expression. *Cryobiology.*

Johonson LA, Weitze KF, Fiser P, Maxwell WM. (2000). Storage of boar semen. *Anim Reprod Sci,* 62(1-3), 143-72.

Marcial Cumpa G, Joel Pomahuali. (2009). Evaluación comparativa del ácido ascórbico y del tocoferol sobre la fertilidad del semen de gallo. *Anales científicos UNALM,* 70, 27-33. Spanish.

Flores Solano C, Meléndez C, Carmona G, Márquez Y, Mendoza C, Villanova L. (2017). Actividad de la superóxido dismutasa cu/zn en plasma seminal e integridad de la membrana plasmática en semen de cerdo (Sus scrofa doméstica) conservado con melatonina. *Gaceta de Ciencias Veterinaria,* 22(29, 37-42.

Tae Hee K, In Suh Y, In Chul P, Hee Tae C, Jong Taek K, Choon Keun P, et al. (2014). Effects of quercertin and genistein on boar sperm characteristics and porcine IVF embyo developments. *Journal of Embryo Transfer,* 141-8.

Seifi Jamadi A, Kohram H, Zare Shahneh A, Ansari M, Macías Garcias B. (2016). Quercetin Ameliorate Motility in Frozen-Thawed Turkmen Stallions Sperm. *Journal of Equine Veterinary Science*, 45, 73-77.

Pérez Aguirre burualde MS, Fernández S, Córdoba M. (2012). Acrosin activity regulation by protein kinase C and tyrosine kinase in bovine sperm acrosome exocytosis induced by Lysophosphatidylcholine. *Reproduction in Domestic Animals*, 47(6), 915-920.

Silva CB, Cajueiro FP, Silva PC, Soares PC, Guerra MP. (2012). Effect of antioxidants resveratrol and quercetin on in vitro evaluation of frozen ram sperm. *Theriogenology*, 77(8), 1722-6.

Gibb Z, Mayordomo TJ, Morris LH, Maxwell WM, Grupen CG. (2013). Quercetin improves the postthaw characteristics of cryopreserved sex-sorted and nonsorted stallion sperm. *Therigenology*, 79(6), 1001-1009.

Silva CB, Arruda CP, Silva SV, Souza HM, Guerra MP. (2016). High resveratrol or quercetin concentrations reduce the oscillation index of frozen goat semen. *Arq. Bras. Med. Vet. Zootec,* 68(5).

Tvrdá E, Tusimova E, Kovácik A, Páal D, Libova L, Lukac. (2016). Protective effects of quercetin on selected oxidative biomarkers in bovine spermatozoa subjected to ferrous ascorbate. *Reprod Domest Anim,* 51(4), 524-537.

Afshin Seifi J, Ejaz A, Mahdi A, Hamid K. (2017). Antioxidant effect of quercetin in an extender containing DMA or glicerol on freezing capacity of goat semen. *Crybiology*, 75, 15-20.

Avdatek F, Yeni D, Inanc ME, Cil B, Tuncer BP, Türkmen R, et al. (2017). Supplementation of quercetin for advanced DNA integrity in bull semen criopreservation. *Andrologia*.

Chae MR, Kang SJ, Lee KP, Choi BR, Kim HK, Park JK, et al. (2017). Onion (*Allium cepa L.*) peel extract (OPE) regulates human sperm motility via protein kinase C-mediated activation of the human voltaje-gated proton channel. *Andrology*, 5(5), 979-989.

Restrepo Betancur G, Duque Cortés JE, Rojano Benjamín A. (2016). Efecto de la quercetina, la L-ergotioneina y la pentoxifilina en el semen equino posdescongelado. *Rev Salud Anim,* 38(3). Spanish.

Martínez Flórez S, González Gallego J, Culebras JM, Tuñon MJ. (2000). Los flavonoides: propiedades y acciones antioxidantes. *Nutricion Hospitalaria,* XVII(6), 271-278. Spanish.

Córdova Izquierdo A, Pérez Gutiérrez JF, Méndez Hernández W, Villa Mancera AE, Huerta Crispín R. (2015). Obtención, evaluación y manipulación del semen de verraco en una unidad de producción mexicana. *Revista Veterinaria.* 26(1), 69-74. Spanish.

Westendorf P, Richeter L, Treu H. (1975). Zur tiefgefrierung von ebersperma Laborund Besamungsergebnisse mit dem Hulsengerger Pailletten-Verfahren. *DTW Dtsch Tierarztl Wochenschr,* 261-267. Deutsch.

Córdova Izquierdo A, Oliva JH, Lleó B, García Artiga C, Corcuera BD, Pérez Gutiérrez JF. (2006). Effect of different thawing temperatures on the viability, in vitro fertilizing capacity and chromatin condensation of frozen boar semen packaged in 5 ml straws. *Animal Reproductive Science,* 92(1-2), 145-54.

Gutiérrez Pérez O, Juárez Mosqueda ML, Uribe Carvajal S, Trujillo Ortega ME. (2009). Boar spermatozoa cryopreservation in low glicerol/trehalose enriched freezing media improves cellular integrity. *Cryobiology*, 58(3), 287-92.

Córdova Izquierdo A, Córdova MS, Córdova Jiménez CA, Pérez Gutiérrez J, Martín Rillo S. (2005). Congelación de semen de verraco en dos tipos de pajillas y capacidad fecundante in vitro de los espermatozoides. *CIENCIA Ergo-Sum,* 12(3), 271-274. Spanish.

Iglesias Reyes A, Guevara González J, López Díaz O, Guerra Liera J, Huerta Crispín R, Sánchez Sánchez R, et al. (2019). Evaluación de la técnica modificada de tinción Giemsa en la valoración acrosomal de espermatozoides de mamíferos. *Abanico Veterinario,* 9(1), 1-8. Spanish.

Moretti E, Mazzi L, Terzuoli G, Bonechi C, Lacoponi F, Martini S, et al. (2012). Effect of quercetin, rutin, naringenin and epicatechin on lipid peroxidation induced in human sperm. *Reproductive Toxicology,* 34(4), 651-657.

Seifi Jamadi A, Kohram H, Shahneh AZ, Ansari M, Macías García B. (2016). Quercetin Ameliorate Motility in Frozen-Thawed Turkmen Stallions Sperm. *Journal of Equine Veterinary Science*, 45, 73-77.

Johinke D, Graaf SP, Bathgate R. (2014). Quercetin reduces the in vitro production of H2O2 during chilled storage of rabbit spermatozoa. *Animal Reproduction Science,* 151(3-4), 208-219.

Ranawat P, Kaushik G, Saikia UN, Pathak CM, Khanduja KL. (2012). Quercetin impairs the reproductive potential of male mice. *Andrologia,* 45(1),56-65.

Córdova Izquierdo A, Torres Trejo DI, Iglesias Reyes AE, Guerra Liera JE, Huerta Crispín R, Villa Mancera AE, et al. (2019). *Valoración reproductiva del toro.* https://www.researchgate.net/publication/331701226_Valoracion_reproductiva_del_toro Spanish.