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# Olive oil as an alternative to boar semen cryopreservation¤

## *Aceite de oliva como una alternativa para la criopreservación de semen suino*

## *O azeite de oliva como uma alternativa para a criopreservação do sêmen suíno*

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### Abstract

The aim of this study was to evaluate different concentrations of olive oil for cryopreservation of boar semen. A total of 21 eyaculates of 18 males with motility ≥70% were used. For freezing, the semen was diluted in treatments: control (Cont–egg yolk), "Koroneiki" oil in egg yolk at 0.25% (A025), 0.50% (A050), 0.75% (A075) and 1.0% (A10). Straws with $5.10^7$ sperm/ml were frozen and stored in liquid nitrogen. Thawing was done at 37ºC for 30 seconds and motility, mitochondrial functionality, DNA integrity, oocite penetration rate and the number of sperm per oocite (in the *in vitro* penetration trial) were evaluated. The variables were compared using the Kruskal-Wallis test. Even though no statistical difference was found between control and treatments containingolive oil ($p>0.05$), the treatment with 0.25% oil concentration showed tendences towards sperm protection, outperforming the controls in mitochondrial functionality and preservation of the fertilizing capabilities in the *in vitro* penetration trial. This is why, olive oil could represent an alternative to help cryopreservation of boar semen.

### Key words

*Antioxidants, in vitro penetration trial, mitochondrial functionality.*

### Resumen

El objetivo de este estudio fue evaluar diferentes concentraciones de aceite de oliva para la criopreservación de semen de verraco. Se utilizaron 21 eyaculados de 18 machos con motilidad igual o superior al 70%. Para la congelación, el semen se diluyó en los tratamientos: control (Cont) (lactosa yema), 0,25% (A025); 0,50% (A050); 0,75% (A075) y 1,0% (A10) de aceite de la variedad "Koroneiki" en lactosa yema. Pajas con $5.10^7$ espermatozoides / ml, fueron congeladas y almacenadas en nitrógeno líquido. La descongelación se produjo a 37°C durante 30 segundos y se evaluó: la motilidad, la funcionalidad de la mitocondria, la integridad del ADN, la tasa de penetración de ovocitos y el número de espermatozoides por ovocito (en el ensayo de penetración *in vitro*). Las variables se compararon mediante la prueba de Kruskal-Wallis. Aunque no hubo diferencia estadística entre el control y los tratamientos

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que contienen aceite de oliva (p> 0,05), la concentración de 0,25% de este aceite mostró tendencias de protección de esperma, superando el control en la funcionalidad de las mitocondrias y en la preservación de la capacidad fertilizante en el ensayo de penetración *in vitro*. Por lo tanto, el aceite de oliva puede representar una alternativa para ayudar en la criopreservación de semen porcino.

**Palabras clave**

*Antioxidantes, ensayo de penetración in vitro, funcionalidad de la mitocondria.*

**Resumo**

O objetivo de este estudo foi avaliar diferentes concentrações de azeite de oliva para a criopreservação de sêmen suíno. Utilizaram-se 21 ejaculados de 18 machos com motilidade igual ou superior ao 70%. Para o congelamento, o sêmen se dilui-o nos tratamentos: controle (Cont) (lactose-gema), 0,25% (A025); 0,50% (A050); 0,75% (A075) e 1,0% (A10) de azeite da variedade "Koroneiki" em lactose-gema. Palhetas com uma dose de $5.10^7$ espermatozoides/ml foram congeladas e armazenadas em nitrogênio liquido. O descongelamento se produz a 37°C durante 30 segundos e se avaliaram: motilidade, funcionalidade da mitocôndria, integridade do DNA, taxa de penetração de ovócitos e o número de espermatozoides por ovócito (no teste de penetração *in vitro*). As variáveis se compararam mediante o teste de Kruskal-Wallis. Ainda que não houve diferença estatística entre o controle e os tratamentos que continham azeite de oliva (p> 0,05), a concentração de 0,25% de azeite de oliva teve tendências de proteção do esperma, superando o controle na funcionalidade das mitocôndrias e na preservação da capacidade fertilizante no teste de penetração *in vitro*. Assim sendo, o azeite de oliva pode representar uma alternativa para ajudar na criopreservação do sêmen suíno.

**Palavras chave**

*Antioxidantes, funcionalidade da mitocôndria, teste de penetração in vitro.*

# Introduction

The frozen boar semen does not present results as efficient as those of cooled semen, due to the high sensitivity of the sperm to damages during cryopreservation and thaw[1]. Thus, 99% of the total dose is preserved by cooling, method that ensures a period of storage for up to seven days[1]. However, only the cryopreservation technology enables the prolonged storage of semen for international exchange of genetic material and generation of gene banks[2]. In this context, it is justified to research new strategies for decrease the sperm post-thaw damages for the use of cryopreservation on routine of farms for breeding intensification[3].

The process of freezing and thawing, provide an increase in generation of reactive oxygen species (ROS) and consequently the oxidative stress[4]. The ROS are involved in important processes of sperm physiology, such as hyperactivation, capacitation, acrosome reaction and events of binding to the zona pellucida[5,7]. However, the ROS excess causes oxidative stress which generates detrimental effects on sperm such as lipid peroxidation[8], DNA damage[9], reduction of the capacity for fusion with the oocyte[10] and reduction of the motility and viability of sperm[8].

The boar semen is particularly sensitive to oxidative stress due to high content of polyunsaturated fatty acids (PUFAs) in phospholipids of membrane and the low level in proportion cholesterol: phospholipid membrane[11], besides the low antioxidant activity in seminal plasma that can facilitate oxidative damage also *in vivo*[12].

Thus, research on antioxidants has been intense in recent years. Großfeld *et al*. (2008)[2] found that the addition of butylated hydroxytoluene, catalase, reduced glutathione, superoxide dismutase, and vitamin E (analogue Trolox) on extenders for freezing boar semen can improve sperm survival after thawing. However, there are few reports on the effects of natural antioxidants for sperm, despite the growing interest in research on natural substances due to security problems and toxicity of some synthetic antioxidants such as butylated hydroxyanisole (BHA) and propyl gallate (PG)[13].

In research of natural antioxidants, an attractive alternative is olive oil that is extracted from fruit of the olive tree (*Olea europaea*) through mechanical processes[14]. Olive oil contains a large amount of natural

antioxidants which provide oxidative stability during storage[15]. Among the antioxidants present in this oil can mention the tocopherols, sterols, carotenoids and phenolic compounds, being the o-dihydroxy-phenolic a potent antioxidant[16]. It is instigating the fact of this compound have not yet been added to the freezing extender because its antioxidant effect is widely reported in the Mediterranean diet, with protective effects against oxidative stress-related diseases such as cancer and neurodegenerative diseases[17, 18].

The viability of using antioxidants in oils has been demonstrated by Kaeoket *et al.*, (2012)[19] that used rice bran oil containing the antioxidant gamma-oryzanol for the boar semen cryopreservation and obtained a significantly higher percentage of progressive motility, viability and acrosomal integrity on the supplemented groups compared to control (lactose-yolk).

Thus, the addition of olive oil to freezing extender could neutralize the production of ROS to contain a mixture of antioxidants. The objective of this study was to evaluate the antioxidant activity of olive oil as an additive to the extender for cryopreservation of boar semen.

## Materials and methods

The animals used were boars of Large White and Landrace breed, sexually mature and healthy, housed in a commercial farm in Estrela/RS (longitude 51°57' 59 "and latitude 29° 30' 07"). A total of 21 ejaculated from 18 different males are used. The collection occurred by the gloved hand technique and the semen was deposited on tubes with a filter to separate the gelatinous fraction at 38 °C[21]. Immediately after collection, the semen was diluted at 38 °C, 1: 1 (v / v) in the Beltsville Thawing Solution extender (BTS). After dilution, the semen was placed at 17 °C and sent to Pelotas / Rio Grande do Sul not exceeding 4 h between collection and processing. Cryopreservation occurred in the Veterinary School of the Federal University of Pelotas / Rio Grande do Sul. Only samples that had at least 70% motility in optical microscopy[22, 23] upon arrival in the laboratory were used for cryopreservation.

For freezing sperm a volume of 15 ml of diluted semen in BTS was centrifuged for 10 minutes at 800*g*. The supernatant was discarded and the pellet resuspended with 400 µL cooling extender (CE, lactose-yolk) with osmolarity of 355 mOsm and pH of 7.2, containing 80% (v / v) lactose solution at 11 and 20% (v / v) egg yolk. After, the 400 µL above mentioned (CE plus cooling extender) were distributed and diluted on the following treatments: Cont (CE only); A0,25 (0.25% olive oil in CE); A0,50 (0.5% olive oil in CE); A0,75 (0.75% olive oil in CE) and A1,0 (1.0% olive oil in CE).

The olive oil used was the variety "Koroneiki" got picked fruit grown olive trees in the city of Bage/RS mechanically cold processed with extractor Spremoliva (Oliomio/ Italy). The determination of antioxidant compounds was performed by spectrophotometry to determine the phenols and carotenoids and liquid chromatography (HPLC) for total tocopherols, these compounds being shown in Table 1. After dilution in treatments semen was cooled in the refrigerator at 5 °C and remained at that temperature for 90 minutes.

**Table 1.** Antioxidant compounds present in olive oil variety "*Koroneiki*" values expressed as mean and standard deviation.

| *Total Phenols* | *Total Carotenoids* | *Tocopherols* | | |
|---|---|---|---|---|
| (mg/kg) | (mg/kg) | Alfa-α | Gama β+γ | Delta-δ |
| 112.2±1.3 | 6.53± 0.05 | 39.5±1.6 | 1.1±0.01 | - |

After this period at 5 °C, semen was diluted in freezing extender (FE) with 83.5% of CE; 1.5% of *Orvus Ex Paste* and 15% of cryoprotectant N, N-dimethylacetamide (DMA) ($C_4H_9NO$) (v/v). The addition of FE occurred in 1:2 ratio, i.e. for each 2 ml of CE was adding 1 mL of FE to present a DMA final concentration of 5%[24]. At the end of this dilution in DC, each treatment had a concentration of 5 x$10^7$ sperm / mL per 0.25 mL straws. After filling, the straws were stored at 5 cm from nitrogen vapor for 10 minutes. Then these straws were immersed and stored in liquid nitrogen at -196 °C.

After 7 days of storage in liquid nitrogen, the straws were thawed in a water bath at 37 °C for 30 seconds and its contents stored in conical tubes containing 2.5 mL of BTS, preheated to 37 °C[25, 26]. The thawed semen was incubated for 10 minutes and analysis of sperm performed.

Motility was assessed by Bearden & Fuquay (1997) and CBRA, (1998)[22,23] by viewing through optical microscopy in 200x magnification, an aliquot of 20 µL in slide under coverslip, both previously heated to 37 °C.

For evaluation of the DNA integrity in a 20 µL semen aliquot added 10 µL of buffer TNE (0.01 M Tris-HCl, 0.15 M NaCl, 0.001 M EDTA, pH 7.2) after 30 seconds, 20 µL of detergent Triton 1X and after another 30 seconds added 10 µL of acridine orange (2 mg/mL in deionized $H_2O$). The evaluation of DNA integrity occurred 5 minutes after addition of reagents to the semen in epifluorescence microscope, by counting 100 cells, considering cells with damaged DNA those presenting red or orange fluorescence and integrity those presenting green fluorescence as described by Varela *et al*. (2012)[27].

Functionality of mitochondria was assessed using a probe specific, Rhodamine 123 (Rh123) (R8004-5 mg) together with propidium iodide (PI) second modified protocol of Garner *et al*. (1997)[28]. At a 20 µL of semen aliquot was added 30 µL of a working solution containing: 960 µL of sodium citrate solution (2.94%), 10 µL of formaldehyde (1.7 mm), 10 µL of PI (7.3 mm ) and 20 µL of Rh123 (0.2 mM). A total of 100 cells were evaluated at 400x magnification on epifluorescence microscope. Cells that had intermediate piece with an intense green fluorescence were classified like intact mitochondria (functionally active), whereas cells with low green fluorescence (matte) in the intermediate piece were deemed functional.

To evaluate the rate penetration of oocyte and number of spermatozoa per oocyte (on *in vitro* penetration test), ovaries of prepubertal gilts were collected at a slaughterhouse and transported to the laboratory within 60 min, in saline solution (0.90%) containing gentamicin (40 mg/ml) at 30 °C. In the laboratory, follicles (3-6 mm) are were aspirated with the aid of a vacuum pump and oocytes with intact zona pellucida were frozen (-18 °C) for later use. After thawing, the *cumulus* cells were mechanically removed with the aid of a micropipette of 200 µL and processing of oocytes was performed as described by Macedo *et al*. (2006) and Macedo *et al*. (2010)[29,30]. The *in vitro* rate penetration test occurred at 30 oocytes per sample according Maleszewski et al. (1995)[31] with modifications: the fertilization medium was the modified Tris (mTBM) comprising: 113.1 mM NaCl, 3.0 mM KCl, 10.0 mM $CaCl_2$, 20.0 mM Tris, 11.0 mM glucose, 5.0 mM sodium pyruvate, 0.4% of bovine serum albumin and 5 mM caffeine. The co-incubation of gametes was carried out in microcentrifuge tubes with 1 mL of mTBM in a water bath at 37 °C for 2 hours. The sperm concentration of $4.10^6$/mL. After co-incubation, oocytes were recovered, washed, stained with Hoechst 33342 (10 mg / ml) and evaluated in epifluorescence microscope at 400x. Finally, oocytes were considered penetrated when their zona pellucida contained at least one sperm in perivitelline or cytoplasm space [29, 30].

For each male, the number of oocytes penetrated were counted and the rate of *in vitro* penetration calculated by the division of the number of oocytes penetrated on total oocytes (not penetrated and penetrated).

The variables of motility, DNA integrity, mitochondrial functionality, number of spermatozoa per oocyte and rate penetration were subjected to the Shapiro-Wilk normality test, which indicated the absence of normality. Thus, the averages for these variables were compared by Kruskal-Wallis test, and all statistical analyzes the software Statistix 9.0 (2008)[32]. The confidence interval for all evaluations was 5%.

## Results

There was no statistical difference between the different treatments with olive oil and control (lactose-yolk only) for motility, DNA integrity and functionality of mitochondria (Table 2).

**Table 2.** Thawed boar semen: Mean and standard error of the mean for motility (MOT), functionality of mitochondria (FM) and DNA integrity (IDNA).

| *Treatment* | *MOT (%)* | *IDNA (%)* | *FM (%)* |
|---|---|---|---|
| Cont | 21.1±3.1 | 87.7±5.4 | 47.7±5.1 |
| A025 | 14.0±2.1 | 89.9±5.8 | 50.5±5.4 |
| A050 | 14.7±2.2 | 91.7±4.7 | 40.7±6.2 |
| A075 | 13.5±2.0 | 82.6±7.2 | 40.8±5.8 |
| A10 | 14.0±2.3 | 88.4±4.9 | 40.2±4.8 |

Cont (control, lactose-yolk); A025 (lactose-yolk more 0.25% olive oil); A050 (lactose-yolk more 0.50% olive oil); A075 (lactose-yolk more 0.75% olive oil); A10 (lactose-yolk more 1.0% olive oil), means were compared by Kruskal-Wallis.

There was no statistical difference between treatments with olive oil and control (lactose-yolk only) for the number of spermatozoa per oocyte and penetration rate (Table 3).

The variables analyzed showed no statistical difference compared to control. However, treatments with olive oil showed a tendency to protect against damage from freezing compared to control (lactose-yolk) (Table 2 and Table 3).

**Table 3.** Thawed boar semen: Mean and Standard Error for the average sperm number per oocyte (SN) and penetration rate (PR).

| *Treatment* | *SN* | *PR* |
|---|---|---|
| Cont | 2.6 ± 0.4 | 76.9 ± 5.2 |
| A025 | 4.4 ± 0.1 | 83.9 ± 4.6 |
| A050 | 2.6 ± 0.3 | 73.2 ± 5.2 |
| A075 | 3.2 ± 0.5 | 74.6 ± 3.8 |
| A10 | 2.9 ± 0.5 | 73.1 ± 4.1 |

Cont (control, lactose-yolk); A025 (lactose-yolk more 0.25% olive oil); A050 (lactose-yolk more 0.50% olive oil); A075 (lactose-yolk more 0.75% olive oil); A10 (lactose-yolk more 1.0% olive oil), means were compared by Kruskal-Wallis.

## Discussion

This is the first report of the use of olive oil for freezing boar semen and demonstrates an interesting field of research, especially due to be a natural antioxidant with superior safety and potential toxicity lower than synthetic antioxidants[13].

Among the trends of protection (Tab. 2 and Tab. 3) has verified that sperm motility showed levels below 20% in all treatments and 21% for the control, similar to the averages reported by Jiang *et al.*, (2007)[33]; Bianchi *et al.*, (2008)[24]; Gutiérrez-Pérez *et al.*, (2009)[34]; Zeng *et al.*, (2014)[35]. The reduced motility in treatment may be justified due to the viscosity of olive oil[36], does not represent a loss for this variable. Moreover, a higher viscosity may reduce sperm metabolic demand prolonging the viability of gametes, as demonstrated by Nagy *et al*. (2002)[37] and López-Gatius *et al*. (2005)[38] for rabbit spermatozoa.

The trends of semen protection are shown in other results helps to prove the fact that reduced motility is not due to a toxic effect but to a higher viscosity of olive oil. Thus, the protection trend against cryopreservation damages is remarkable to the variables of DNA integrity and functionality of mitochondria, being the highest averages for DNA integrity at concentrations of 0.25% and 0.50% olive oil and for mitochondria functionality the highest average at concentrations 0.25% olive oil ($p > 0.05$) exceeding the control for both. In this context, this trend of protection is very promising, since mitochondrial function is critical for ATP production, which sustains the flagellar beats for the motility[39], and DNA integrity is essential for the correct embryonic development[40] and birth of healthy offspring.

The 0.25% concentration had already demonstrated beneficial effects for the variables of DNA integrity and function of mitochondria, also demonstrated trend of protection by penetration testing. Thus, olive oil generated the greatest number of sperm per oocyte as well as the higher rate of penetration. Penetration testing conducted by co-incubation of gametes is more predictive of fertility compared to tests that evaluate the sperm alone[41, 42], in addition to this test have shown sensitivity in detecting fertilizing ability of boar sperm[29].

Semen is a biological sample with large variation, since hardly showed normal distribution in various evaluations of seminal quality [43,44,45,46] and that increased sampling contributes to decrease variability[47]. Thus, it is believed that increasing the sampling demonstrate the differences statistically between treatments containing olive oil and control because many experiments assessing sperm quality with larger samples obtained superior results in the treated groups[24, 33, 48].

The results in this paper are promising due to trends protection has presented considering the great sensitivity of boar semen to freezing. Thus, is there the perspective to evaluate other varieties of olive oil, beyond the "Koroneiki", because according Minioti and Georgiou (2008)[15] the composition of the most potent antioxidants (phenols), is variable between different olive oils.

## Conclusion

Olive oil at a concentration of 0.25% was found for the protection of boar sperm against damage from freezing.

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