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Effect of olive-derived antioxidants (3,4-dihydroxyphenylethanol and 3,4-dihydroxyphenylglycol) on sperm motility and fertility in liquid ram sperm stored at 15°C or 5°C

Running title: Effect of olive-derived antioxidants in liquid ram sperm

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SUMMARY

The aim of the present study was to evaluate the effect of the addition of two olive oil-derived antioxidants, hydroxytyrosol (3,4-dihydroxyphenylethanol, HT) and 3,4-dihydroxyphenylglycol (DHPG), on ovine semen during liquid storage at 5°C and 15°C.

Semen was collected, pooled, diluted and then divided into aliquots supplemented with different concentrations (5 µg/ml, 10 µg/ml, 50µg/ml and 100 µg/ml) of HT, DHPG and a mixture (MIX) of both antioxidants. Sperm motility characteristics were assessed in the different samples at 0, 6, 24, 48, 72 and 96 h after cooling and a fertility trial was also conducted. The results showed that the antioxidant addition did not significantly improve total and progressive motility in ovine cooled sperm maintained at 15° or 5°C. However, in samples stored at 5°C, LIN (48, 72, 96 h), STR (0h) and WOB (0, 48, 72, 96h) values significantly decreased in comparison with control treatment when high antioxidant concentrations were added (MIX100 or HT100). When samples were maintained at 15°C, MIX50 showed significantly higher VCL values than the control treatment after 6h cooling, and MIX100 showed significantly lower VCL values at 96h after cooling. According to the artificial insemination trial, no significant differences were observed when antioxidants were added. In conclusion, the use of HT and DHPG showed small impact in sperm motility and fertility was not affected (nor detrimentally nor positively) when insemination was carried out using antioxidant-supplemented liquid sperm.

Keywords: Artificial insemination; Olive Oil; Ovine; Phenolic antioxidants.

1. Introduction

Artificial insemination (AI) is an important tool for the dissemination of the genetic of high merit individuals, offering advantages against natural service (O'Hara et al., 2010). However, the implementation of this technique in ovine is far away that which occurs in bovine or porcine. In order to explain the slow development of this technique in ovine it can be argued the low economical profitability of these farms, the high variability of the fertility rates, or the loss of sperm quality associated with cryopreservation or cooling process (Mata-Campuzano et al., 2014).

Different strategies for extending the fertile life of spermatozoa induce a reversible reduction of sperm metabolism and motility through the refrigeration of sperm at low temperatures (5° or 15°C), using diluents containing different components to reduce the production of reactive oxygen species (ROS), among other actions (Allai et al., 2015).

Mammalian sperm cells are highly sensitive to ROS due to a high presence of polyunsaturated fatty acid (PUFA), a low cholesterol:phospholipids ratio and also to the extrusion of its antioxidant-rich cytoplasm during maturation stages (Holt & North, 1985; Maxwell & Watson, 1996; Motlagh et al., 2014).

Under physiological conditions, spermatozoa have endogenous antioxidant defence mechanisms that help them to counteract ROS proliferation and to avoid oxidative stress. This is composed by enzymatic antioxidants, such as superoxide dismutase, catalase and glutathione peroxidase, and other non-enzymatic antioxidants, such as vitamin C, vitamin E (α -tocopherol), pyruvate, glutathione and carnitine, which can be found in the seminal plasma (Agarwal & Saleh 2002).

However, under stressful conditions, as could be semen handling or cooling process, the high generation of ROS cannot be counteracted by antioxidant defence mechanism. This situation induces lipid, protein and DNA damages in the spermatozoa, as well as

motility impairment and alteration in acrosomal functions (Saraswat, Kharche & Jindal, 2014).

In recent years, many studies have been focused in the use of exogenous antioxidants to ram semen extenders to diminish the negative effect induced by ROS (Çoyan et al., 2010; Mata-Campuzano et al., 2014; Moradi, Malekinejad, Farrokhi-Ardabili & Bernousi, 2013; Allai et al., 2015; Fang et al., 2015, Rather, Islam, Malik & Lone, 2016; Allai, Benmoula, da Silva, Nasser & El Amiri, 2018). The main antioxidants are extracts from the leaves, seeds and roots of plants due to their high content of polyphenols, flavonoids, carotenes, gallic acid, tannins and essential oils (Zhong & Zhou, 2013).

A large amount of phenolic component could be extracted of olive fruit, as occur with hydroxytyrosol (3,4-dihydroxyphenylethanol, HT) and 3,4-dihydroxyphenylglycol (DHPG). HT is a simple phenol obtained from the alperujo (the waste of olive oil) which has a high antioxidant ability (Fernández-Bolaños, López, Fernández-Bolaños, Rodríguez, 2008), reduces the oxidation of low-density lipoproteins, protects against H₂O₂ cytotoxicity and minimises lactate dehydrogenase activity (Hashimoto et al., 2004; Roche et al., 2009; Choucair, Saliba, Jaoude & Hazzouri, 2018). In relation to DHPG, it is also an alperujo-derived phenolic component with high antioxidant and anti-inflammatory potential, similar to HT but with an additional hydroxyl group in the β position (Bermúdez-Oria, Rodríguez-Gutiérrez, Rodríguez-Juan, González & Fernández-Bolaños, 2018). DHPG is compared with vitamin E due to its antioxidant and antiradical ability (Al-Daraji, 2012), summed to its anti-inflammatory properties (De Roos et al., 2011).

In our knowledge, few studies evaluate the effect of olive oil on the sperm quality. Al-Daraji et al. (2012) studied the effect of the direct addition of olive oil into the extender

media in rooster sperm, while others evaluated the effect of HT-based extender on rat (Hamden et al., 2010), human (Kedechi et al., 2017) and ram sperm (Balaganur et al., 2018, Arando et al., 2019). However, the use of DHPG and the mixture of HT and DHPG as a coadjuvant of semen extender has been recently reported by Arando et al. (2019) in frozen-thawed ram sperm samples, observing that **lipid peroxidation** values significantly decreased, leading us to hypothesise that the addition of these antioxidants in ram sperm preservation media could reduce the detrimental effects associated with liquid sperm storage. The aim of the present study was to evaluate the effect of HT, DHPG or the combination of both substances on the quality of ovine sperm motility maintained under refrigeration to 15° and 5°C during 96 hours.

2. Materials and methods

2.1. Isolation of bio-active compounds (HT and DHPG) from olive oil waste

In order to obtain HT and DHPG, alperujo olive pulp (semi-solid waste generated during the olive oil extraction) was submitted to thermal extraction and chromatographic purification. Samples of alperujo (between 30 and 50 kg) were inserted in a reactor chamber at an average temperature of 160-170°C for 60 minutes at 9 Kg/cm². The sample was partially hydrolysed to facilitate solid-liquid separation. The wet material was centrifuged after cooling at 4700 g for 10 min (Comteifa, S.L., Barcelona, Spain) to obtain a liquid fraction as a source rich in antioxidants. The liquid was extracted by chromatography fractionation in two steps, with a final yield of 99.6% and 95% purity relative to dry matter for HT and DHPG, respectively . Fernández-Bolaños et al. (2002, 2014). A volume of 1 litter of stock solution was obtained for each antioxidant and 500µl aliquots were stored at -21°C until their use. Final antioxidant solutions were prepared immediately prior to use.

2.2. *Animals and semen collection*

Six Merino Fleischschaf and nine Segureña breed rams (3-5 years of age) of proven fertility from the Diputacion de Cordoba and Granada (Spain), respectively, were involved in the present study. Animals were individually housed and they were fed with a commercial concentrate (0.5 kg), and ad libitum access to alfalfa hay, water and mineral supplementation blocks. Semen was collected by artificial vagina once a week during breeding season (October-February). These ejaculates were assessed to determine the volume (ml) using graduated tubes, masal motility (0-5, 40 x magnification; Olympus, Tokyo, Japan) and sperm concentration by photometer (Accuread, IMV technologies, France). The criteria for inclusion of sperm samples were volume ≥ 0.5 , concentration $\geq 3000 \times 10^6$ sperm/ml and masal motility ≥ 4 . Animal management were carried out according to the European Union Regulations (2010/63/UE), transposed to the Spanish law (RD 53/2013). In addition, the experiment was authorized by the Bioethics Committee of the University of Cordoba (N. 2018PI/29).

2.3. *Experimental design*

After collection, the ejaculates were placed at 37°C in a water bath and diluted 1:2 with INRA 96® (INRA, IMV Technologies, France) for the initial sperm assessment. Immediately, they were pooled to avoid the individual rams variability and then diluted to a final concentration of 600×10^6 sperm/ml. After that, in order to evaluate sperm motility at different temperatures (15°C and 5°C), pooled samples were split into 26 aliquots containing 150×10^6 sperm/ml. One tube per temperature served as a control (without antioxidant) and the remaining (12 aliquots per each temperature) were supplemented with HT, DHPG and the combination of both (MIX, maintaining 1:10 relation between HT and DHPG) for a final concentration as following described: HT5

(5µg/ml), HT10 (10µg/ml), HT50 (50µg/ml) and HT100 (100µg/ml); DHPG5 (5µg/ml); DHPG10 (10µg/ml); DHPG50 (50µg/ml); DHPG100 (100µg/ml); MIX5 (5µg/ml HT+0.55 µg/ml DHPG), MIX10 (10µg/ml HT+1.11 µg/ml DHPG), MIX50 (50µg/ml HT + 5.55 µg/ml DHPG) and MIX100 (100µg/ml+11.11 µg/ml DHPG). Subsequently, 13 aliquots were stored at 5°C (during two hours, decreasing temperature at 0.3 °C/min) while the others were stored at 15°C. Sperm motility was assessed at 0, 6, 24, 48, 72 and 96 h after sperm dilution. This experiment was repeated 6 times in Merino Fleischschaf rams, using a total of 36 ejaculates.

2.4. Sperm motility assessment

Motility analysis was performed using software ISAS v.1.2 (Integrated Semen Analyser System, Proiser Valencia, Spain). Sperm aliquot was diluted with INRA to a final concentration of 20×10^6 spz/ml. After dilution, sperm samples were warmed at 37°C for 10 min. Five µl were placed on a slide, covered with a coverslip (22 x 22mm) and put on a microscope plate at 37°C. Four fields were randomly captured. It was determined the total motility (TM, %), progressive motility (PM, %), curvilinear velocity (VLC, µm/sec), straight-line velocity (VSL, µm/sec), average path velocity (VAP, µm/sec), straightness (STR, %), linearity (LIN, %), wobble (WOB, %), amplitude of lateral head displacement (ALH, µm), beat cross frequency (BCF, Hz). Head area of spermatozoa ranged between 10 µm and 70 µm. They were considered as motile when VAP >10 µm/sec and linearly motile when they were deviated <75% from a straight-line.

2.5. Fertility trial

Two Segureña breed farms reared under extensive production systems and located in Huescar (Granada, Spain) were choose for this trial. Semen from nine tested rams (3-4 years old) was used for this trial. As previously described, semen was diluted with

INRA extender and four different groups were tested: Control (without antioxidants), HT10, DHPG50, and MIX50. The antioxidant concentration used for this experimental trial was choose based on the best VCL value obtained at 6h of storage. A total of 220 ewes ranged between 2 and 5 years-old and with a lambing-insemination interval extended from 64 to 98 days were randomly assigned to each experimental group (55 ewes in each group). Ewes were synchronized using intravaginal devices impregnated with 60 mg medroxyprogesterone acetate (Esponjavet®, HIPRA) and 400 I.U. eCG (Oviser®, HIPRA) were i.m. administered at the sponge withdrawal. Time-fixed AI was realized by vaginal procedure, using cooled (15°C) sperm. Sperm were used around 6 hours after collection and females were inseminated 55 ± 1 h after the sponge withdrawal, with 400×10^6 sperm per ewe, using a 0.25 ml straw. Fertility (i.e. ewes lambing per ewe inseminated) was determined at parturition.

2.6. Statistical analysis

For the statistical analysis of data, SPSS 22.0 software (Chicago, IL, USA) was used. Normality was tested using the Kolmogorov-Smirnov test. As the data exhibited a non-normal distribution, arcsin and ln transformation was carried out for percentages and continuous data, respectively. The effect of storage time (0, 6, 24, 48, 72, and 96 hours), antioxidant concentration and their interactions on the motility and kinematic parameters were tested using repeated measure analysis. Fertility was analysed using logistic regression, and farm and antioxidant concentration factors were included in the model. When significant differences ($p < 0.05$) were detected, a LSD post-hoc test was carried out. The results are shown as mean $\pm$ SEM.

3. Results

3.1. Effect of extender and storage time at 5°C

The effect of the antioxidant concentration, storage time and their interactions were analysed. Antioxidant concentration*storage time interactions were statistically significant ($p<0.05$) for LIN (Table 1). By contrast, no interactions were observed for TM, PM, VCL, VSL, VAP, STR, WOB, ALH and BCF. Therefore, these factors were studied as the main effect.

As seems in table 1, all variables were negatively diminished throughout the storage time, and the antioxidant concentration only significantly affected on LIN, STR and WOB parameters. The supplementary table S1 shows results obtained in stored samples throughout 96 h.

In reference to LIN, a significant interaction was observed between antioxidant and the storage time. After 48h, it was observed that high concentrations of the combination of both antioxidants (MIX100) significantly decreased LIN values in comparison with control group. HT100 showed worse values than control at 48 h and 72 h of storage. When STR was evaluated, MIX100 showed significantly poorer results than the others treatments at 0h. In addition, at 24 and 48 h, control group showed significantly higher values than HT100. The WOB values were also affected by different antioxidant concentrations. MIX100 was significantly lower than the control group at 0, 48, 72 and 96 h of storage.

3.2. Effect of extender and storage time at 15°C

Results showed a similar trend that observed at 5°C, but no interactions were observed for any of the studied variables (Table 1). Therefore, these factors were studied as the main effect. All variables showed lower values throughout the storage time.

Supplementary table S2 shows the results obtained over 96 h of storage time.

According to the antioxidant concentration effects, significant differences were only observed for VCL. At 0 h of storage, MIX100 and HT10 showed significantly higher

values than the control group. In addition, the control group showed significantly lower values than MIX50 and MIX100 at 6 h and 24 h of storage, respectively. By contrast, control group presented significantly higher values than MIX100 at 96 h of storage.

3.3. Fertility trial

Low fertility results were obtained in all the groups. After cervical artificial insemination using sperm at 15°C diluted in commercial extender or containing different olive-derived antioxidants, no differences were detected. Fertility was 15.09% in the control group, while in ewes inseminated using sperm supplemented with HT10, DHPG50 and MIX50, the results were 23.63, 13.46 and 15.38%, respectively.

4. Discussion

In recent years, several studies have been carried out to evaluate the effect of the addition of exogenous antioxidants in ram semen extenders to reduce the negative effect induced by ROS (Budai, Egerszegi, Olah, Javor & Kovacs, 2014; Allai, Benmoula, da Silva, Nasser & El Amiri, 2018). However, to our knowledge, this is the first attempt to evaluate the effect of derived-olive oil antioxidants (HT, DHPG and the combination of both) on the ram sperm motility characteristics during liquid storage at 15° and 5°C.

In comparison with freezing, the liquid storage of spermatozoa reduces injuries linked to cryopreservation and allows extending its useful life, although it does not avoid the decrease of semen quality over time (Mata-Campuzano et al., 2014). In fact, the maintenance of sperm at cooling conditions produces oxidative stress, as a consequence of free radical production, accumulation of waste substances and by the decrease of the antioxidant capacity of the sample (Donnelly, McClure, & Lewis, 2000).

The present study evaluates the motility characteristics of sperm maintained at 15° and 5°C for 96 h, using different concentrations of HT, DHPG and mixture of both antioxidants, and also determined the fertility obtained after cervical insemination with

sperm samples maintained at 15°C. As previously described by Palacin et al. (2013), sperm motility is one of the most important parameters to determine quality and energy status of sperm cells. Furthermore, the quantitative assessment of sperm motility by CASA is correlated with the fertilization capacity of spermatozoa. Kasimanickam et al. (2011) suggests that motility is the most meaningful and useful sperm quality indicator, beyond DNA and mitochondria activity assessment.

In the present study, the addition of different antioxidant types and concentrations did not significantly improve sperm TM and PM for both studied temperatures, according with our previous results obtained in cryopreserved samples, where no significant effects were observed for TM and PM when HT, DHPG and the combination of both were used (Arando et al., 2019). By contrast, the addition of 80 µM HT has recently demonstrated a positive effect on the post-thawed sperm motility in ram sperm (Balaganur et al. 2018). Hamden et al. (2010) observed that rat sperm samples previously incubated with high concentrations of glucose (that is toxic for spermatozoa) showed lower damage when they were protected with 50 µg/ml HT for 1h at 32°C. In roosters, Al-Daraji (2012) added olive oil to the diluents and observed higher values for massal and individual motility in sperm samples maintained during 72h at 5°C.

However, human sperm samples supplemented with HT did not show any variation in motility (Kedechi et al., 2017), in consonance with the results here obtained.

When other antioxidants have been tested in liquid ram sperm, contradictory results have been obtained, suggesting that results could be influenced by other factors such as the male, extender, antioxidant or concentration (Silva, Cajueiro, Silva, Soares & Guerra, 2012). Assorted results are found in literature showing positive (using catalase, methionine, astaxanthin or vitamine C) (Câmara et al., 2010; Bucak, Cayan, Oztürk, Güngör, & Omür, 2012; Fang et al., 2015) or not positive effects (such as glutathione,

ergothioneine, reduced glutathione or trolox) (Bucak & Tekin, 2007; Mata-Campuzano et al., 2014) of antioxidants on the sperm motility. In this sense, some authors suggested that the addition of natural substances having antioxidant effect acts as double-edged swords, since high exogenous antioxidants may disrupt redox balance and acts like a pro-oxidant by activating pathways such as increasing pro-inflammatory mediators production, nitrosylation of proteins, glycation effect and endocrine disrupting activities (Bouayed & Bohn, 2010).

Several studies report the correlation between the kinematic sperm parameters and the spermatozoa fertilization ability. As described by Larsen et al. (2000), VCL was the most significant parameter correlated with the fertilization rate in human sperm. In ovine, Sanchez-Partida et al. (1999) described VCL, VSL, VAP and ALH as positively correlated parameters with fertility after intrauterine insemination. In the same way, VAP and VCL values are good predictors of the ability of spermatozoa to migrate in ewe cervical mucus (Robayo, Montenegro, Valdés & Cox, 2008).

In the present study, VCL at 0 h of storage at 15°C were significantly higher in MIX100 and HT10 groups than the control group, same as MIX50 at 6 h and MIX100 at 24 h. By contrast, control group significantly improved VCL in comparison with MIX100 at 96h of storage. Nevertheless, in samples maintained at 5°C, MIX100 significantly decreased LIN values up to 48 h of storage, STR values during 24 and 48 h of storage and WOB values at 0, 48, 72 and 96 h of storage in comparison with control group, suggesting that high concentration of both antioxidants provides negative effect in sperm quality. However, Maia et al. (2009) observed that high trolox concentrations induce significantly higher values for LIN and STR during ram sperm cryopreservation, affirming the existence of dose–response relationship in which antioxidant absence or low concentration may not be sufficient to provide sperm cell protection. Similarly,

Moradi et al. (2013) reported VLC and VSL enhancement when samples were supplemented with royal jelly, which contains natural antioxidants.

In a recent study, it has been reported that the addition of HT, DHPG and the combination of both in criopreserved ram sperm did not offer neither better nor detrimental effects on the kinetic and velocity parameters in comparison with the control group (Arando et al., 2019), probably because lower antioxidant concentrations were added to the sperm samples than in the present study. In the same way, when antioxidants such as catalase, glutathione or astaxanthin, were added to the sperm dilution media during liquid storage, no significant differences were observed for kinematic parameters (Câmara et al., 2010; Fang et al., 2015) suggesting that factors aside from oxidative effects, such as reduced energy production or metabolism, may contribute to their decline during storage at 4°C.

The results show that the duration of storage is significantly associated with the deterioration of sperm motility for both studied storage temperatures, in agreement with others (Gundogan, Yeni, Avdatek & Fidan, 2010; Allai et al., 2017). A sperm motility reduction during liquid storage has been related with the action of the cellular components of semen (the superoxide anion radical, the hydrogen peroxide, nitric oxide and peroxynitrite anion) and the lipid hydroperoxides formed via lipid peroxidation of the spermatozoa membranes (Allai et al., 2017). However, lower sperm motility and metabolism could be associated with pH reduction due to bacterial contamination and metabolite production (Yániz, Mateos & Santolaria, 2011). Previous studies reported that 15°C is the optimal temperature to maintain a good percentage of motile cells and a high motility score during short-term storage (Upreti, Oliver, Munday & Smith, 1992), while the storage of semen at 5°C is clearly advantageous for long-term storage (Mata-Campuzano et al., 2014).

Although there are many reports describing in vitro quality of ram sperm after antioxidant supplementation, only few studies report the fertility rates. In the present study, fertility was not significantly associated with the supplementation of antioxidants to cooled semen at 15°C. By contrast, Mara et al. (2005) observed higher fertility and blastocyst rates when semen was supplemented with Tempol and stored at 15°C. Similarly, Casao et al. (2009) reported significantly greater cleavage rates when low amounts of melatonin were added. In the same way, in ewes inseminated with semen treated with GSH and stored at 15°C the lambing rates were significantly improved (Mata-Campuzano et al., 2014), and pregnancy rates significantly increased in samples supplemented with superoxide dismutase and catalase and stored at 5°C (Maxwell & Stojanov, 1996). In addition, Kubovičová et al. (2010) also reported a positive effect on the fertilization and pregnancy rate in ewes after cervical insemination with semen stored at 5°C and supplemented with glutathione. As highlighted by Mata-Campuzano et al. (2015), the effects of antioxidants could be modulated by other extender components and possibly by other factors such as cooling rate, equilibration time or sample source. This could partially explain the results obtained in the present study on sperm motility parameters.

5. Conclusions

In conclusion, it is the first study evaluating the effect of olive oil-derived antioxidants on the liquid storage ram sperm. The use of HT, DHPG and the combination of both antioxidants showed slight impact on the sperm motility and did not show significant effects on the fertility. Further studies are required to evaluate interactions between these antioxidants and other factors, in order to obtain protective and enhanced properties on this kind of sperm conservation.

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Declaration of interest

None.

Data Availability Statement

The data will be made available upon reasonable request.

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544

545 **TABLES**

546 **Table 1.** P value results in motility and kinematic parameters in samples maintained at 5 and
547 15°C. Significant effects ($p < 0.05$) are highlighted in bold.

548

549 **Supplementary table S1.** Mean values ($\pm$ SEM) for motility and kinematic parameters from
550 samples maintained at 5°C throughout 96 h of storage time.

551 Uppercase letters (A, B, C) show significant differences between antioxidants within each time / Lowercase
552 letters (a, b, c) show the effect of time for each antioxidant ($p < 0.05$).

553 **Supplementary table S2.** Mean values ($\pm$ SEM) for motility and kinematic parameters
554 from samples maintained at 15°C throughout 96 h of storage time.

555 Uppercase letters (A, B, C) show significant differences between antioxidants within each time /
556 Lowercase letters (a, b, c) show the effect of time for each antioxidant ($p < 0.05$).

557 **Table 1.** P value results in motility and kinematic parameters in samples maintained at 5
558 and 15°C. Significant effects (p<0.05) are highlighted in bold.

	15°C			5°C		
	Concentration	Time	Concentration* Time	Concentration	Time	Concentration * Time
TM (%)	0.485	0.0001	0.714	0.958	0.0001	0.918
PM (%)	0.253	0.0001	0.851	0.869	0.0001	0.367
VCL (μ/s)	0.043	0.0001	0.104	0.955	0.0001	0.977
VSL (μ/s)	0.424	0.0001	0.821	0.916	0.0001	0.318
VAP (μ/s)	0.084	0.0001	0.256	0.915	0.0001	0.499
LIN (%)	0.455	0.0001	0.279	0.0001	0.0001	0.026
STR (%)	0.737	0.0001	0.59	0.0001	0.0001	0.34
WOB (%)	0.369	0.0001	0.221	0.03	0.0001	0.214
ALH (μ)	0.686	0.0001	0.234	0.119	0.003	0.818
BCF(Hz)	0.951	0.0001	0.12	0.139	0.0001	0.993

559