Indian Journal of Animal Sciences **89**(9): 932–937, September 2019/Article
https://doi.org/10.56093/ijans.v89i9.93752

Effect of resveratrol supplementation to Triladyl® on the quality of ram chilled semen

MOHSEN AL-MUTARY[1]

Imam Abdulrahman Bin Faisal University, P.O. Box 2375, Dammam 31451, Saudi Arabia

Received: 9 February 2019; Accepted: 5 March 2019

ABSTRACT

The present investigation aimed to estimate the *in vitro* effects of resveratrol (RES) on the quality of chilled ram semen. Resveratrol was added to Triladyl® in three different concentration as 0 (control), 200 or 400 μM. Semen was collected from three Najdi rams, pooled and extended with one of three previous extenders for a final concentration of 600×10^6 sperm/ml. Then, the diluted semen was cooled gradually, and stored at 4°C. The total motility, viability, plasma membrane integrity and acrosome integrity were assessed at 0, 24, 72 and 120 h of storage. The thermal incubation test was evaluated at day 7. The survival time, total motility and the progressive motility were registered at day 15 of storage. After 4 h of incubation, the percentage of live sperm was significantly lower in the control group than in other groups. Results of survival time, total motility and progressive motility at day 15 of liquid storage were significantly higher in RES groups than that of the control. Based on the results of incubation resistance, survival time, total and progressive motility, it could be concluded that adding 200 and 400 μM of RES to Triladyl extender can improve the quality of ram semen during chilled storage at 4°C.

Key words: Najdi ram, Resistance, Resveratrol, Spermatozoa

Najdi sheep is a locally-important breed that lives under extreme conditions in hot environment of the Arabian Peninsula. Using liquid storage semen in sheep offers high fertility rate than frozen semen (Schuh 1992). Furthermore, the lethal harm caused by semen freezing/thawing processes influences the structure and function of spermatozoa (King *et al.* 2004, Kasimanickam *et al.* 2011). Few studies were focused on the liquid storage of ram semen compared to cryopreservation. The composition of semen extender plays an essential role in maintaining pH, osmolality and energy source that support the sperm survival. Additionally, Kasimanickam *et al.* (2011) recorded good results of ram semen liquid storage in an extender with egg yolk for eight days at 4°C. The combination of egg yolk and glycerol in extender protects ram sperm during chilled storage (Gil *et al.* 2011). Triladyl® (Minitube, Germany), is a commercial extender, commonly used for artificial insemination in ruminant animals. Triladyl® contains Tris, citric acid, sugar, glycerol, ultra-pure water and antibiotics. About 20% of fresh egg yolk should be added to Triladyl before usage according to the manufacturer's instructions.

The storage of spermatozoa at low temperature allows reducing catabolism and movement of spermatozoa and thus extends their lifetime. Also, low temperature during refrigeration may impair the quality and survival time of the spermatozoa (Kasimanickam *et al.* 2007). Reactive oxygen species (ROS), which increase during cold semen storage, cause oxidative stress (OS) leading to low antioxidant activity and damage in spermatozoa (Giaretta *et al.* 2014, Tvrda *et al.* 2015). To solve this issue, many antioxidants were added to various extenders of ram semen, and some anti-oxidative properties were shown to minimize ROS impacts and sperm injuries (Mata-Campuzano *et al.* 2014, Amidi *et al.* 2016, Toker *et al.* 2016, Vichas *et al.* 2017). Resveratrol (3,5,4'-trihydroxy-trans-stilbene) is an antioxidant, and a natural phenolic compound produced in more than 70 species of plants including grapes, blueberries, pistachios, plums and peanuts. It is responsible for dealing with injury or microbial infections (Fremont 2000, Baur and Sinclair 2006). Resveratrol have been used in numerous studies as an antioxidant agent during freezing and thawing or liquid storage of sperm. Many studies were carried out on the use of natural antioxidants as resveratrol confirmed its ability to improve health and immune responses of livestock besides reducing the risks of various animal diseases like cancer and other degenerative diseases. Such activities may be attributed to its powerful antioxidant, anti-inflammatory and immunomodulatory impacts by preventing the interaction between free radicals with cellular DNA as well as its ability to activate the intestinal microbiota and to increase nutrient digestibility and absorbance (Alagawany *et al.* 2015, Abd El-Hack *et al.* 2016). Resveratrol has the ability to scavenge the ROS in

Present address: [1](mgalmutary@iau.edu.sa), Department of Basic Sciences, College of Education.

human sperm, act against lipid peroxidation and prevent DNA fragmentation (Branco *et al.* 2010, Collodel *et al.* 2011). However, the potential effects of RES supplementation to commercial Triladyl extender on the sperm parameters during a long liquid storage of ram semen have not been studied before. Therefore, the present study was designed to explore the protective effects of Triladyl extender with RES on Najdi ram sperm storage at 4°C.

MATERIALS AND METHODS

Chemicals: Resveratrol was purchased from Sigma Co (St. Louis, MO, USA) and Triladyl® was purchased from Minitube, Germany. All other chemicals were obtained from LOBA Chemie (Mumbai, India), Scharlab S.L. (Barcelona, Spain) and UFC Biotech (Riyadh, Saudi Arabia).

Animals and their management: The present study was performed at Basic Sciences Department, College of Education, Imam Abdulrahman Bin Faisal University, Dammam, Saudi Arabia. Three healthy proven fertile Najdi rams (45–55 kg weight and 2–3.5 years old) were selected for this study. All rams were kept in barns under normal conditions. Rams were fed hay three times a day and had free access to clean drinking water.This study was approved by the Bioethics Committee of the University.

Egg yolk preparation: Before the day of semen collection, fresh hen eggs were used to prepare clarified egg yolk (CEY). Egg yolk (EY) was separated from albumin, and rolled on sterilized filter paper to remove remnants. Then yolk membranes were removed and EY collected in 50 ml conical tube. Equal volume of distilled water was added to EY, well-mixed and centrifuged at 4000 rpm at 4°C for 30 min. The lipid layer floated at the surface and the debris at the bottom was discarded and the clear water-soluble fraction (CEY) was recovered. Then the CEY was inactivated at 56°C for 30 min and kept at 4°C until use.

Semen collection and dilution: The libido of males was stimulated by the teaser female before semen collection. Thirty ejaculated samples (10 ejaculates for each ram) were collected using an artificial vagina once a week. Semen samples were collected during winter season. Ejaculates with volume of 1–1.5 ml, concentration $>3.5\times10^9$ sperm/ml, thick consistency, morphology of <10% sperm abnormalities and semen motility (score 4 from 5) were pooled to eliminate the individual variations between ram samples. Extenders were prepared by mixing one volume of Triladyl® with two volumes of distilled water and two volumes of CEY, then supplemented with (200 or 400 µM) or without (control) RES according to the experimental design. After semen collection, all accepted samples were pooled and immediately diluted in water bath by one of pervious extenders (600×10^6 sperm/ml). The diluted semen was gradually cooled to 4°C within 2 h and stored at 4°C.

Semen evaluation: The motility, viability, acrosome and membrane integrity of sperm were evaluated at various times (0, 24, 72 and 120 h). At the day seven (168 h) of chilled storage; motility, viability and membrane integrity were studied before and after incubation at 37°C for 4 h. The survival time of spermatozoa was estimated from day 10 up to the day before sperm motility became below 50%. Mass and progressive motility were evaluated at day 15. Day 0 is the time after semen sample dilution and before cooling. All the semen evaluations were assessed by the same technician (Fig. 1).

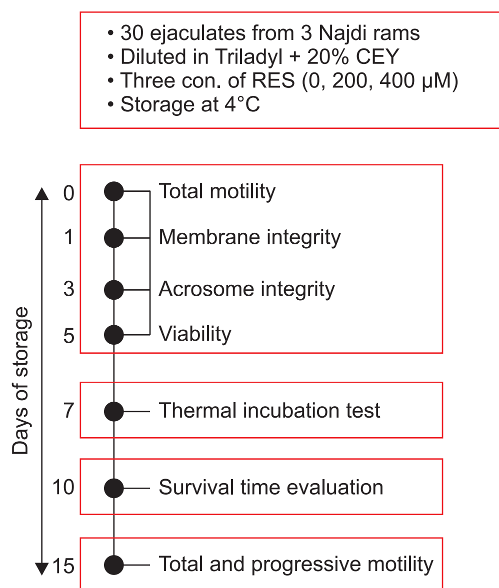

Fig. 1. Experimental design

Sperm motility: To evaluate sperm motility, samples were washed in warm phosphate buffer solution (PBS) and incubated at 37°C for 15 min before the evaluation of motility. Extended semen (20 µl) was placed on warm slides to evaluate mass motility by the assessment of wave motion of semen at 10× magnification under phase-contrast microscopy (Labomed US). The motility was recorded in scores from 0 to 5 according to the method described by Shipley *et al.* (2007). Briefly, in score 5, the moving waves were very rapid and 90% of sperm were motile. In score 4, the sperm movement was strong but less than that in the score 5 and the motile sperm are from 70 to 90%. In score 3, the low moving waves of 40–65% of sperm was observed. In score 2, up to 40% of sperm were active and no wave motion. Motility less than 10% was given score 1. And in score 0, there was no movement. The progressive motility of spermatozoa was evaluated by placing 10 µl of semen on a pre-warmed slide, and covered with a coverslip. The progressive motility was determined using a 40× objective lens in average of five different fields of the slide. Spermatozoa moving in a straight line were counted as progressively motile, while other sperm were considered as a non-progressive motile (Ahmad *et al.* 2015).

Effective survival time: The effective survival time was estimated as described by Li *et al.* (2017) with some modifications. At day 10, the sperm motility was above 50% for all groups. Thus, the mass motility was daily assessed until the day before sperm motility became below 50% for each group. The effective survival time was

calculated as: $d= h/24+10$; and was given parameter (d) while, the number of hours over 10 days was given parameter (h).

Membrane integrity: The hypo-osmotic swelling test (HOST) was used to examine the integrity of the sperm plasma membrane according to Ramu and Jeyendran (2013). The HOST solution was prepared from sodium citrate (0.367 g) and fructose (0.675 g) in 50 ml of distilled water. Ten microliters of semen and 150 microliters of HOST solution were incubated for 40 min at 37°C. Five microliters samples were examined at 40× under phase contrast microscope. The total of 200 sperm cells were observed and classified as the following: no visible coiling of spermatozoa tail (non-react spermatozoa), visible coiling of spermatozoa tail (reacted spermatozoa). The percent of reacted spermatozoa was calculated (Fig. 2A).

Sperm viability: The eosin-nigrosin stain was used to detect the viability of sperm (Evans *et al.* 1987). Eosin-nigrosin solution was prepared by dissolving 1.40 g eosin-Y, 8.72 g nigrosin and 2.53 g sodium citrate in 100 ml distilled water. Ten microliters of diluted semen were mixed with three drops of stain; smears were made on warmed slides and observed under 400× objective lens of phase contrast microscope. The unstained spermatozoon was counted as alive. A fully or partially stained spermatozoon was counted as a dead. Live-dead sperm was counted from at least six fields and 200 sperm per reading (Fig. 2B).

Acrosome integrity: The acrosome integrity of sperm samples was evaluated by Giemsa stain according to Watson (1975) and Kovács and Foote (1992) with some modification. Briefly, diluted semen was washed with PBS three times to remove the egg yolk-extender, then one drop from washed semen was smeared on slide and left to dry at room temperature. The slides were fixed in a fixation solution (86 ml of 1 N HCl, 14 ml 37% formaldehyde and 0.2 g neutral red) for 2 min. After fixation, the slides were washed under slow-running tape water followed by washing with distilled water. Finally, the slides were placed in 7.5% (v/v) Giemsa stain/distilled water (it was prepared from Giemsa stock solution: 3.8 g (w) Giemsa powder in 375 ml (v) methanol and 125 ml (v) glycerol), then left overnight. Next day, slides were washed with tap water, differentiated in distilled water for 2 min; air dried, mounted with coverslip and observed using 60× dry or oil immersion at 100×. At least, a total of 200 sperm were evaluated in each group and storage time. The sperm stained with purple color was counted as a sperm with intact acrosome while the sperm stained with pale lavender color was counted as a sperm with loose or damaged acrosome (Fig. 2C).

Sperm thermal evaluation test: At the day 7 from liquid storage, samples were incubated at 37°C for 4 h. The motility, viability and membrane integrity of sperm were evaluated at 0 and 4 h of incubation.

Statistical analysis: All calculations were done using

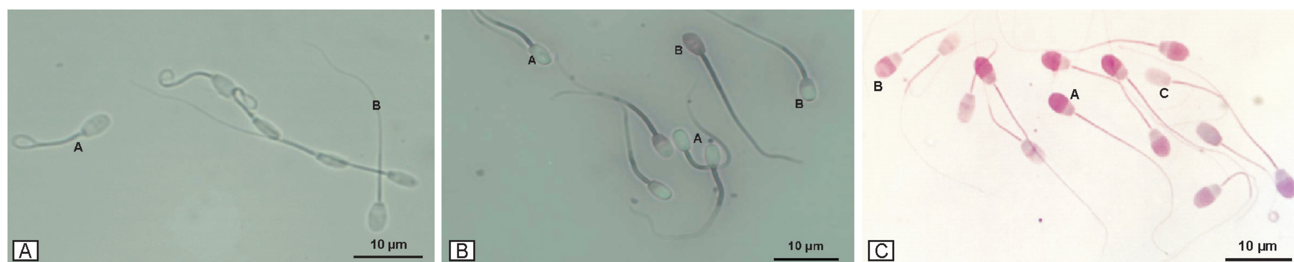

Fig. 2. **A**. Membrane integrity of Najdi ram sperm evaluated by hypo-osmotic swelling test (HOST) (A, swollen sperm; B, no swollen sperm). **B**. Viability of Najdi ram sperm evaluated by Eosin-Nigrosin stain (A, live sperm; B, dead sperm). **C**. Different acrosome states of Najdi ram sperm evaluated by Giemsa stain (A, intact acrosome; B, damaged acrosome; C, no acrosome).

Table 1. Effect of resveratrol on motility, membrane integrity, viability and acrosome integrity of Najdi ram sperm during liquid storage at 4°C

Parameter	Resveratrol Con. (µM)	Storage time (h)			
		0	24	72	120
Total motility (%)	Control (0)	86.66±1.66^{A}	84.66±0.33^{A}	79.66±3.84^{B}	68.00±1.52^{C}
	200	85.33±2.33^{A}	83.00±0.57^{A}	78.00±2.51^{B}	67.66±2.33^{C}
	400	84.66±1.66^{A}	83.33±0.88^{A}	78.00±2.00^{B}	67.66±2.33^{C}
Live sperm (%)	Control (0)	82.30±2.75^{A}	78.93±4.81^{A}	76.60±2.70^{A}	69.00±5.57^{B}
	200	76.80±1.30^{A}	78.22±1.36^{A}	75.94±2.79^{A}	71.33±3.83^{B}
	400	77.80±1.37^{A}	75.92±2.53^{A}	72.22±4.65^{A}	69.83±4.47^{B}
Membrane integrity (%)	Control (0)	78.83±4.40^{A}	78.70±3.36^{A}	76.33±0.61^{A}	62.35±3.38^{B}
	200	75.33±2.16^{A}	78.31±3.46^{A}	74.14±2.12^{A}	70.40±1.07^{B}
	400	78.33±2.20^{A}	77.21±2.67^{A}	76.67±1.21^{A}	67.63±0.00^{B}
Acrosome integrity (%)	Control (0)	96.35±0.70^{A}	88.01±1.28^{B}	87.22±1.86^{B}	87.52±2.91^{B}
	200	96.42±0.69^{A}	89.35±3.94^{B}	91.69±4.10^{B}	85.74±2.42^{B}
	400	97.13±0.18^{A}	91.36±3.22^{B}	90.87±3.94^{B}	87.04±3.57^{B}

Different superscripts in the same parameter refer to significant differences ($P<0.05$) between storage times.

SPSS 20 software (Chicago, USA). First, the Shapiro-Wilk test was used to detect the normal distribution of our results. Two way analysis of variance (ANOVA) was performed to explore the effects of resveratrol concentration and storage or incubation time on the *in vitro* parameters of Najdi ram sperm during liquid storage. Tukey post-hoc test was carried out in all parameters and between groups when the significance was P<0.05. The results were expressed as the mean±SEM.

RESULTS AND DISCUSSION

The effects of RES on the quality of Najdi ram spermatozoa after 0, 24, 72 and 120 h of chilled storage: The means of motility, membrane integrity, viability and acrosome integrity in three groups after 0, 24, 72 and 120 h from liquid storage are shown in Table 1. No significant effects of RES supplementation were recorded on the aforementioned parameters compared to the control at different times of liquid storage.

Table 2. Effect of 4 h thermal evaluation test on motility, viability and membrane integrity of Najdi ram sperm after cold storage at 4°C for 168 hours in the presence or absence of resveratrol

Time	RES Con. (µM)	Total motility (%)	Viability (%)	HOST (%)
0 h	Control (0)	67.66±1.20	66.66±4.25	69.83±1.66
	200	66.83±1.09	64.16±0.92	70.36±0.81
	400	67.83±1.09	67.50±5.76	67.50±3.81
4 h	Control (0)	61.16±0.72	53.58±1.83^{a}	53.83±1.76
	200	61.66±0.88	61.25±1.77^{b}	51.00±2.75
	400	62.16±0.72	64.08±2.16^{b}	51.50±2.29

Different superscripts in the same parameter refer to significant differences (P<0.05).

The sperm motility significantly decreased in the three groups after 72 and 120 h of storage time compared to 0 and 24 h, and significantly (P<0.05) decreased at 120 h compared to 72 h of storage. The percentage of sperm viability and membrane integrity were statistically lower in all groups after 120 h than the other storage times. The percentage of acrosome integrity significantly declined after 24, 72 and 120 h compared today 0.

The refrigerated storage of ram semen is important in artificial insemination of sheep, but it impairs the quality and survival time of the spermatozoa because of the ROS production (Kasimanickam *et al.* 2007, 2011, Gil *et al.* 2011). Therefore, the use of resveratrol as an antioxidant could be useful in reducing the negative effects of oxidative stress on sperm (Tvrda *et al.* 2015). In the current study, 20% CEY Triladyl extender was used. It already has glycerol among its components. Gil *et al.* (2011) reported that egg yolk–glycerol protected semen from a low temperature below 5°C. Similar result was observed in our study, 20% CEY Triladyl extender was able to maintain the viability and membrane integrity of ram sperm above 70% after 3 days of cold storage with and without resveratrol.

Incubation resistance after liquid storage for 7 days: The mean values of motility, viability and membrane integrity of sperm after liquid storage for 7 days and thermal incubation test are summarized in Table 2. The motility and membrane integrity of sperm of three groups was significantly lower after 4 h of incubation at 37°C than before. While, no significant difference among groups was recorded at each time the percentage of live sperm was statistically lower after 4 h of incubation in the control and 200 µM RES groups compared to before incubation. After incubation, the percentage of live sperm was significantly lower in the control group than in the other groups.

In the present study, resveratrol supplementation to 20% CEY Triladyl extender did not have any advantage at 0, 24, 72 and 120 h of liquid storage, regarding the motility, membrane integrity, viability and acrosome integrity of sperm. These findings agree with Giaretta *et al.* (2014) and Ball *et al.* (2001) who demonstrated that adding antioxidants to extender during liquid storage did not improve sperm parameters. On the other hand, our results disagree with those reported by Giaretta *et al.* (2014) who used a low concentration of resveratrol (80 µM), different extender (Kenney extender) and species (stallion) in comparison to our study. Furthermore, Longobardi *et al.* (2017) pointed out that resveratrol improved membrane stability of buffalo semen. It seems that the natural antioxidants of seminal plasma in extender during liquid storage prevents ROS production, and inhibits lipid peroxidation which damages the sperm (Kankofer *et al.* 2005, Pagl *et al.* 2006, Giaretta *et al.* 2014). However, Matheny *et al.* (2015) indicated that resveratrol had a cytotoxic effect at 1 mM and 10 mM of concentration on mobility and viability of stallion sperm. The variation among results might be due to the difference between semen extenders used, animals, and the time or type of storage (liquid storage or cryopreservation).

Survival time, total and progressive motility at day 15 of liquid storage: Results of survival time, total and progressive motility at day 15 of liquid storage demonstrated

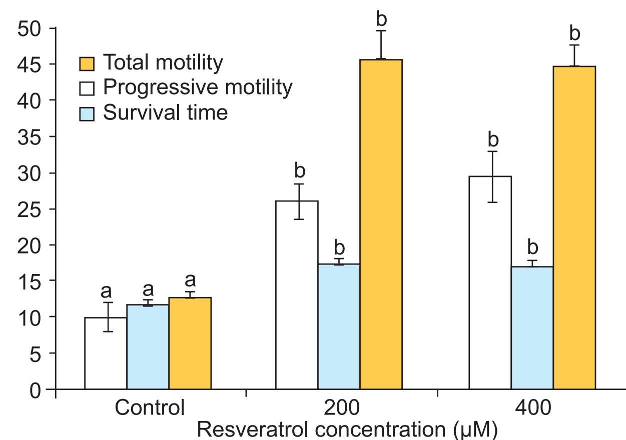

Fig. 3. Total motility (%), progressive motility (%) and survival time (days) of Najdi ram sperm at day 15 of liquid storage in different concentration of resveratrol. Different letters refer to a significant difference (P<0.05) between groups in each parameter.

that the treatment groups were significantly better (P<0.05) than that of the control group (Fig. 3). A of sperms (50%) survived in 200 and 400 µM groups until 17.33 days of refrigerated storage vs. 11.33 days in the control group.

The thermal incubation test may be a good choice to find out the ability of sperm to survive under *in vitro* conditions similar to the female reproductive tract. The following 4 h of incubation after ram sperm storage for 168 h, RES addition to Triladyl extender was effective in maintaining the viability compared to the control. A low viability of sperm might indicate the dysfunction in osmotic pressure regulation and membrane permeability alteration (Benhenia *et al.* 2018). Furthermore, due to the high levels of polyunsaturated fatty acids in plasma membrane and low natural antioxidants, mammalian sperm is very sensitive to oxidative stress (Sarlos *et al.* 2002, Naijian *et al.* 2013, Tasdemir *et al.* 2013). Our results showed that adding RES to 20% CEY Triladyl extender during refrigerated storage of Najdi ram semen provided an extra protection and helped the sperm to survive more than 2 weeks compared to the control. Interestingly, the same trend was observed in total and progressive motility in RES groups at day 15. Conversely, Giaretta *et al.* (2014) found negative impacts on total and progressive motility after 24 h of storage. However, the activity of RES may appear after longer storage with the increased oxidative stress and ROS production during liquid storage. Ortega Ferrusola *et al.* (2009) indicated that lipid peroxidation levels increase after the cold shock during sperm storage than freshly ejaculated. Moreover, the low motility, longevity and viability of spermatozoa had been correlated with the increase of ROS which damages the plasma membrane (Ball *et al.* 2001, Peris *et al.* 2007).

In conclusion, results of incubation resistance at day 7, survival time, total and progressive motility at day 15 showed the ability of RES (200 and 400 µM) to protect ram semen during liquid storage at 4°C. However, adding RES to Triladyl had no effect on the quality of chilled ram semen up to 120 h of storage time. More studies are needed to evaluate the oxidative state, DNA integrity and fertilizing capability of chilled ram semen with or without resveratrol supplementation.

ACKNOWLEDGEMENTS

The author thanks Mr. Mohammad Arafat for technical assistance.

REFERENCES

Abd El-Hack M E, Alagawany M, Ragab M F, Tiwari R, Karthik K, Dhama K and Adel M. 2016. Beneficial impacts of thymol essential oil on health and production of animals, fish and poultry: a review. *Journal of Essential Oil Research* **28**(5): 365–82.

Ahmad M, Nasrullah R and Ahmad N. 2015. Effect of cooling rate and equilibration time on pre-freeze and post-thaw survival of buck sperm. *Cryobiology* **70**: 233–38.

Alagawany M M, Farag M R, Dhama K, Abd El-Hack M E, Tiwari R and Alam G M. 2015. Mechanisms and beneficial applications of resveratrol as feed additive in animal and poultry nutrition: A review. *International Journal of Pharmacology* **11**(3): 213–21.

Amidi F, Pazhohan A, Shabani Nashtaei M, Khodarahmian M and Nekoonam S. 2016. The role of antioxidants in sperm freezing: a review. *Cell Tissue Bank* **17**: 745–56.

Ball B A, Medina V, Gravance C G and Baumbe J. 2001. Effect of antioxidants on preservation of motility,viability and acrosomal integrity of equine spermatozoa during storage at 5 degrees C. *Theriogenology* **56**: 577–89.

Baur J A and Sinclair D A. 2006. Therapeutic potential of resveratrol: the *in vivo* evidence. *Nature Reviews Drug Discovery* **5**: 493–506.

Benhenia K, Rahab H, Smadi M A, Benmakhlouf H, Lamara A, Idres T and Iguer-Ouada M. 2018. Beneficial and harmful effects of cyclodextrin-vitamin E complex on cryopreserved ram sperm. *Animal Reproduction Science (in Press)*.

Branco C S, Garcez M E, Pasqualotto F F, Erdtman B and Salvador M. 2010. Resveratrol and ascorbic acid prevent DNA damage induced by cryopreservation in human semen. *Cryobiology* **60**: 235–37.

Collodel G, Federico M G, Geminiani M, Martini S, Bonechi C, Rossi C, Figura N and Moretti E. 2011. Effect of trans-resveratrol on induced oxidative stress in human sperm and in rat germinal cells. *Reproduction Toxicology* **31**: 239–46.

Fremont L. 2000. Biological effects of resveratrol. *Life Sciences* **66**: 663–73.

Giaretta E, Bucci D, Mari G, Galeati G, Love C C, Tamanini C and Spinaci M. 2014. Is resveratrol effective in protecting stallion cooled semen? *Journal of Equine Veterinary Science* **34**: 1307–12.

Gil J, Fierro S, Bentancur O and Olivera-Muzante J. 2011. Chilled storage of ram semen improves with the addition of egg yolk and glycerol to milk-basedextenders. *Reproduction of Domestic Animals* **46**: 503–07.

Kankofer M, Kolm G, Aurich J and Aurich C. 2005. Activity of glutathione peroxidase, superoxide dismutase and catalase and lipid peroxidation intensity in stallion semen during storage at 5°C. *Theriogenology* **63**: 1354–65.

Kasimanickam R, Kasimanickam V, Pelzer K D and Dascanio J J. 2007. Effect of breed and sperm concentration on the changes in structural, functional and motility parameters of ram-lamb spermatozoa during storage at 4°C. *Animal Reproduction Sciece* **101**: 60–73.

Kasimanickam R, Kasimanickam V, Tibary A and Pelzer K. 2011. Effect of semen extenders on sperm parameters of ram semen during liquid storage at 4°C. *Small Ruminant Research* **99**: 208–13.

King M E, Mckelvey W A, Dingwall W S, Matthews K P, Gebbie F E, Mylne M J, Stewart E and Robinson J J. 2004. Lambing rates and litter sizes following intrauterine or cervical insemination of frozen/thawed semen with or without oxytocin administration. *Theriogenology* **62**: 1236–44.

Kovács A and Foote R H. 1992. Viability and acrosome staining of bull, boar and rabbit spermatozoa. *Biotechnic and Histochemistry* **67**: 119–24.

Li H, Zhang X G, Fang Q, Liu Q, Du R R, Yang G S, Wang L Q and Hu J H. 2017. Supplemental effect of different levels of taurine in Modena on boar semen quality during liquid preservation at 17°C. *Animal Science Journal* **88**: 1692–99.

Longobardi V, Zullo G, Salzano A, De Canditiis C, Cammarano A, De Luise L, Puzio M V, Neglia G and Gasparrini B. 2017. Resveratrol prevents capacitation-like changes and improves

in vitro fertilizing capability of buffalo frozen-thawed sperm. *Theriogenology* **88**: 1–8.

Mata-Campuzano M, Alvarez-Rodriguez M, Tamayo-Canul J, Lopez-Uruena E, De Paz P, Anel L, Martinez-Pastor F and Alvarez M. 2014. Refrigerated storage of ram sperm in presence of Trolox and GSH antioxidants: Effect of temperature, extender and storage time. *Animal Reproduction Science* **151**: 137–47.

Matheny K, Hopper R, King H, Memili E and Nicodemus M. 2015. Effects of resveratrol on post-thaw quality of stallion sperm. *Journal of Equine Veterinary Science* **35**: 443.

Naijian H R, Kohram H, Shahneh A Z, Sharafi M and Bucak M N. 2013. Effects of different concentrations of BHT on microscopic and oxidative parameters of Mahabadi goat semen following the freeze-thaw process. *Cryobiology* **66**: 151–55.

Ortega Ferrusola C, Gonzalez Fernandez L, Morrell J M, Salazar Sandoval C, Macias Garcia B, Rodriguez-Martinez H, Tapia J A and Pena F J. 2009. Lipid peroxidation, assessed with BODIPY-C11, increases after cryopreservation of stallion spermatozoa, is stallion-dependent and is related to apoptotic-like changes. *Reproduction* **138**: 55–63.

Pagl R, Aurich C and Kankofer M. 2006. Anti-oxidative status and semen quality during cooled storage in stallions. *Journal of Veterinary Medicine. A, Physiology, Pathology, Clinical Medicine* **53**: 486–89.

Peris S I, Bilodeau J F, Dufour M and Bailey J L. 2007. Impact of cryopreservation and reactive oxygen species on DNA integrity, lipid peroxidation, and functional parameters in ram sperm. *Molecular Reproduction and Development* **74**: 878–92.

Ramu S and Jeyendran R S. 2013. The hypo-osmotic swelling test for evaluation of sperm membrane integrity. *Methods in Molecular Biology* **927**: 21–25.

Sarlos P, Molnar A, Kokai M, Gabor G and Ratky J. 2002. Comparative evaluation of the effect of antioxidants in the conservation of ram semen. *Acta Veterinaria Hungarica* **50**: 235–45.

Schuh H. 1992. Comparison between liquid and deep-frozen semen for artificial insemination in developing and developed countries. *World Animal Review* **70–71**: 44–53.

Shipley C F B, Buckrell B C, Mylne M J A, Pollard J and Hunton J R. 2007. Chapter 86 - Artificial insemination and embryo transfer in sheep, pp. 629–41. *Current Therapy in large animal theriogenology (Second edition)*. (Eds.) Youngquist R S and Threlfall W R. Saint Louis: W.B. Saunders.

Tasdemir U, Buyukleblebici S, Tuncer P B, Coskun E, Ozgurtas T, Aydin F N, Buyukleblebici O and Gurcan I S. 2013. Effects of various cryoprotectants on bull sperm quality, DNA integrity and oxidative stress parameters. *Cryobiology* **66**: 38–42.

Toker M B, Alcay S, Gokce E and Ustuner B. 2016. Cryopreservation of ram semen with antioxidant supplemented soybean lecithin-based extenders and impacts on incubation resilience. *Cryobiology* **72**: 205–09.

Tvrda E, Kovacik A, Tusimova E, Massanyi P and Lukac N. 2015. Resveratrol offers protection to oxidative stress induced by ferrous ascorbate in bovine spermatozoa. *Journal of Environmental Science* and *Health*. *Part A, Toxic/hazardous Substances and Environmental Engineering* **50**: 1440–51.

Vichas L, Tsakmakidis I A, Vafiadis D, Tsousis G, Malama E and Boscos C M. 2017. The effect of antioxidant agents' addition and freezing method on quality parameters of frozen thawed ram semen. *Cell and Tissue Banking* **19**: 113–21.

Watson P F. 1975. Use of a Giemsa stain to detect changes in acrosomes of frozen ram spermatozoa. *Veterinary Record* **97**: 12–15.