BF-1470
[1-6]

RESEARCH ARTICLE Indian Journal of Animal Research

Effect of L-arginine and Eugenol on Ram Semen Kinematic Parameters and Post Thawed Fertility Rate after Trans-cervical Artificial Insemination

D. Berean[1], A. Blaga-Petrean[1], I. Bogdan[2], S. Bogdan[1], O.M. Tamas-Krumpe[1], R. Cimpean[1], E. Pall[1], M.E. Nap[3], L.M. Bogdan[1]

10.18805/IJAR.BF-1470

ABSTRACT

Backgroung: Long term storage of spermatozoa is one of the most desirable tools of assisted reproductive technologies. The purpose of this research was to evaluate the effect of two antioxidants on sperm kinematic parameters after the freezing-thawing process and the post thawed fertility rate after the trans cervical artificial insemination.

Methods: Ejaculates from Lacune rams were collected using artificial vagina, were pooled and diluted using based extender (Biladyl, Minitube) and based extender with antioxidants (Eugenol, Arginine). Trans-cervical artificial insemination was performed with frozen seminal material in straws.

Result: No significant differences were observed in samples cryopreserved in a base extender and in the base extender supplemented with Arginine. In Eugenol supplemented extender the kinematic parameters were significantly higher for p=0.5 (30.15% progressive motility after thawing) than in the others groups (23.57%, 24.38%). The fertility rate was higher for the straws conserved in extender supplemented with Eugenol (80%) compared with the base extender (55.55%) and the base extender supplemented with Arginine (60%).

Key words: Antioxidants, Cryopreservation, Ram semen.

INTRODUCTION

Long term storage of spermatozoa is one of the most desirable tools of assisted reproductive technologies. Cryopreservation of semen is a beneficial method to save sperm cells, which allows increasing the reproductive performances in herd (Toker *et al*., 2016). Different semen extenders have been used for ram semen cryopreservation in the last decades (Watson 2000).

Semen freezing procedures cause adverse changes in sperm structure and function, after thawing, the percentage of motile sperm and velocity is significantly reduced, sperm viability can be decreased by even 50% of initial (pre-freezing) values (Lemma 2011). These changes are accompanied by a reduction in motility, impaired transport and decreased viability of spermatozoa in the female genital tract and reduced fertility after artificial insemination (Stradaioli *et al*., 2007).

The storage of spermatozoa under artificial condition is stimulated by the need to fertilize large numbers of ewes with semen of outstanding rams, by the necessity to use the rams over extended periods, or at different times of the year (Salamon and Maxwell 2000).

Normally, the semen is a complex redox system, with a balance between antioxidant properties of seminal plasma/ sperm and the oxidant potential of sperm metabolites, which are particularly activated in non-physiological conditions and control sperm lipoperoxidation rate (Stradaioli *et al*., 2007, Pankaj Kumar *et al*., 2014, Kalmath and Narayana Swamy, 2019). If the oxidative balance became pro-oxidant, due to excessive ROS production or decreased antioxidant activity, the biological system is under oxidative stress, damaging sperm and reducing fertility (Câmara *et al*., 2016, Wang *et al*., 2003).

Protein oxidation, lipid peroxidation and plasma nitric oxide (NO) levels are increased by free radicals. The free

[1]Faculty of Veterinary Medicine, University of Agricultural Sciences and Veterinary Medicine from Cluj-Napoca, 400372, Mănăştur street, no. 3-5, Romania.
[2]Faculty of Agriculture, University of Agricultural Sciences and Veterinary Medicine from Cluj-Napoca, 400372, Mănăştur street, no. 3-5, Romania.
[3]Faculty of Horticulture, University of Agricultural Sciences and Veterinary Medicine from Cluj-Napoca, 400372, Mănăştur street, no. 3-5, Romania.

Corresponding Author: D. Berean, Faculty of Veterinary Medicine, University of Agricultural Sciences and Veterinary Medicine from Cluj-Napoca, 400372, Mănăştur street, no. 3-5, Romania.
Email: daniel.berean@usamvcluj.ro

How to cite this article: Berean, D., Blaga-Petrean, A., Bogdan, I., Bogdan, S., Tamas-Krumpe, O.M., Cimpean, R., Pall,E., Nap, M.E. and Bogdan, L.M. (2022). Effect of L-arginine and Eugenol on Ram Semen Kinematic Parameters and Post Thawed Fertility Rate after Trans-cervical Artificial Insemination. Indian Journal of Animal Research. DOI: 10.18805/IJAR.BF-1470.

Submitted: 23-11-2021 **Accepted:** 26-07-2022 **Online:** 04-08-2022

radicals can transfer electrons to oxidizing agents and are inplicated in cell damages (Vincent 2004, Maritim *et al.*, 2003). The principle of antioxidant activity is based on the availability of electrons to neutralize any free radicals. In addition, antioxidant activity is related to the number and the nature of the hydroxylation pattern on the aromatic ring. It is generally assumed that the ability to act as hydrogen donor and the inhibition of oxidation is enhanced by the increase in the number of hydroxyl groups in the phenyl ring (Gülçin 2011, Samir Z. 2019).

L-Arg is a semi-essential amino acid that participates in protein and creatine synthesis, anabolic hormone stimulation and nitrogen balance improvement (Lass *et al.*, 2002). Eugenol (4-allyl-2-methoxyphenyl), a methoxy-phenol with a short hydrocarbon chain, is the major component (80%-95%) of clove oil (Szabadics and Erdelyi 2000). Eugenol has induced reactive oxygen species-mediated apoptosis in HL-60 human pro-myelocytic leukemia cells. It depresses neuromuscular transmission and central nervous system function. Eugenol prevented radiation-induced chemical oxidative damage in membranes and modified the membrane-associated signalling process after radiation exposure (Pandey *et al.*, 2006). Computerized motility analysis provides a number of objective measures of sperm motion characteristics taken from tracks of large numbers of sperm, including the percentage of motile sperm, percentage of progressively motile sperm (i.e., above a preset cutoff for speed and direction of movement), the amplitude of lateral head displacement during forwarding movement, average path velocity in micrometers per second and curvilinear velocity in micrometers per second (Steven and David 2011).

Artificial insemination (AI) of sheep is an advantageous management practice aimed at the genetic improvement at farm level and a programme of genetic selection (Leethongdee 2010), this is one of the most efficient biotechnology at cows in all the world, but, in sheep, the technology is not so used because is difficult to find seminal material with high characteristics at thawing and the cervical passage is a little bit more difficult than in cows. Artificial insemination (AI) of sheep with frozen-thawed semen is a growing practice, especially in countries importing new breeds of sheep and by producers of unique genotypes within selected sheep breeds (Campbell *et al.*,1996).

There generally are 3 AI techniques 1) vaginal insemination, 2) the laparoscopic intrauterine insemination 3) cervical insemination that has been used in the sheep industry and newly developed fourth technique, trans-cervical artificial insemination (TCAI) (Leethongdee 2010). Intra- cervical insemination is performed by insemination at the cervical opening or at the deepest possible intra-cervical site that is easily accessible without attempting to force the inseminating pipette into the cervical canal (Ayad *et al.*, 2004, King *et al.*, 2004).

When the semen is frozen and stored in liquid nitrogen (-196°C), metabolic reactions of the sperm are stopped, allowing the preservation of seminal material for long periods. In this way, genetic material can be available at any time of the year. The use of frozen semen has a great impact on genetic improvement worldwide, by considerably increasing the flow of genetic material to general sheep flocks, as well as facilitating national and international transport and commercialization (Alejandro *et al.*, 2019).

The objective of our study was to evaluate the effect of two different antioxidants (arginine, eugenol) on the kinematic parameters of ram semen after freezing-thawing process and on the fertility rate after trans-cervical artificial insemination.

MATHERIALS AND METHODS

Semen samples were collected from Lacaune rams using artificial vagina mehod. The rams were raised in a semi-confinement system with natural light in Covasna county, Romania. Ejaculates collected were pooled at 37°C, afterwards, were divided and diluted in order to eliminate individual differences. The pooled semen was divided into three aliquots and diluted 1:1 at 37°C with the base extender (Biladyl) supplemented with Arginine (0.1 mm, gr Bil A), Eugenol (35 µM, gr Bil E) and control group (no antioxidant, gr Bil). After the first dilution, the samples have been preserved for 15 minutes at 15°C for equilibration. The second dilution 1/10 was performed after equilibration and, then, the aliquots were kept at 4°C for 6h while were transported at the seminal laboratory of Faculty of Veterinary Medicine Cluj Napoca. The kinematic parameters were analyzed via the CASA system before and after freezing-thawed process. The samples were filled in 0.5 ml french straws, sealed with polyvinyl alcohol powder and frozen in liquid nitrogen vapour 15 minutes and stored in liquid nitrogen container (-196°C). The experiment was repeated 3 times, the kinematic parameters were compared.

Estrous synchronisation and transcervical artificial insemination with frozen semen have been carried out to determinate the fertility rate *in vivo*. The estrous synchronisation was performed with intravaginal sponges with 60 mg progesterone (Ovigest, Hipra Spain) for 12 days and 500 I.U of PMSG (Folligon, MSD) at sponges withdrawal. At 52 h after the injection with PMSG, the transcervical artificial insemination was performed with AI gun (IMV-Casso). Ewes were alternatively inseminated with semen diluted and frozen in Bil, BilA and BilE extender. AI was performed just at the ewes where the transcervical passage of the cervix was possible. The animals where was not possible the passage of the cervix were out of the study. At 75 days after the AI, the diagnosis of gestation was performed by transabdominal ultrasonography (Easy Scan Linear, BCF Technology, Late Mode).

RESULTS AND DISCUSSION

In this work, we demonstrated the cryoprotective capacity of Eugenol added to base extender Biladyl, both in vitro and in vivo trials. Sperm motility is an indicator of male fertility because of its importance for sperm migration through the female genital tract and for gamete interaction at fertilization (Robayo *et al.* 2008). The effects of antioxidants on the kinetic parameters were evaluated in 3 different groups. All samples showed good mobility after thawing and therefore can be used for artificial insemination. The mean percentage of motility, velocity and progressivity before and after freezing thawing process are presented in Tables 1 and 2. From 34 animals synchronized, in 29 cases (85.29%) artificial insemination was performed. In 9 cases were used straws from group Bil, in 10 cases straws from group BilA and straws from group BilE for the rest of 10 cases. From 29 animals

Table 1: Motility parameters before and after freezing-thawing process.

Groups	Sample	Before freezing		After thawing	
		Total motility (%)	Progressive motility (%)	Total motility (%)	Progressive motility (%)
Group bil	1	92.02	40.26	73.54	25.8
	2	91.55	42.03	71.96	23.12
	3	91.74	42.15	74.25	24.22
	Average	91.77	41.48	73.25	24.38
	StDev	0.23	1.05	1.17	1.34
	St Err (p=0.05)	0.26	1.19	1.32	1.52
Group bil A	1	92.22	36.75	75.29	24.1
	2	92.17	37.15	75.96	23.65
	3	90.23	38.12	76.45	22.96
	Average	91.54	37.34	75.9	23.57
	StDev	1.13	0.70	0.58	0.57
	St Err (p=0.05)	1.28	0.79		0.64
Group bil E	1	92.5	36.37	78.89	29.8
	2	90.85	36.13	80.55	29.15
	3	92.35	35.95	81.25	31.5
	Average	91.9	36.15	80.23	30.15
	StDev	0.91	0.21	1.21	1.21
	St Err (p=0.05)	1.03	0.23	1.37	1.37

Table 2: Velocity and progressivity parameters before and after freezing-thawing process.

	Groups	Stastical parameters	Velocity and progressivity %			
			Rapid progressive	Medium progressive	Non progressive	Immobile
Before freezing	Bil	Media	14.41	27.44	49.91	8.23
		DS	0.61	0.51	1.68	2.09
		EroareaSt (p=0.05)	0.69	0.58	1.90	2.36
	Bil A	Media	15.48	21.16	54.56	8.1
		DS	0.60	1.016	2.09	0.92
		Eroarea St (p=0.05)	0.68	1.15	2.36	1.04
	Bil E	Media	17.25	18.91	55.39	8.3
		DS	1.26	1.17	0.92	4.21
		Eroarea St (p=0.05)	1.42	1.33	1.04	4.77
After thawing	Bil	Media	2.5	21.88	48.88	26.74
		DS	0.32	0.64	2.32	2.87
		Eroarea St (p=0.05)	0.37	0.73	2.63	3.25
	Bil A	Media	2.08	21.49	51.53	24.83
		DS	0.06	1.42	0.58	1.60
		Eroarea St (p=0.05)	0.06	1.61	0.66	1.81
	Bil E	Media	1.84	28.31	50.08	19.65
		DS	0.26	0.92	0.53	0.77
		Eroarea St (p=0.05)	0.29	1.04	0.60	0.87

which were inseminated in 19 sheep the gestation was confirmed (65.51%) (Table 3).

Alcay *et al*., 2015 reported after cryopreservation of ram semen with lyophilized egg yolk-based extendera total motility after thawing around 53% . In our study the total motility after thawing was 73.25% for group Bil, 75.09% for group BilA and 80.23% for group BilE. Bucack *et al*., 2007, reported freezing extender supplemented with 50 mM trehalose, 25 mM taurine and 5 and 10 mM cysteamine led to higher motility percentages, in comparison to control levels (P<0.01) after thawing. As set out in Table 5, progressive motility post thawing was significantly higher (30.15%), in group supplemented with 35 µM Eugenol compared to other groups (23.57%, 24.38%). Pool *et al*., 2020, reported that Melatonin-treated rams had a greater percentage of frozen-thawed spermatozoa with progressive motility (38.78%) compared with the control group (25.34%). We obtained similar values after the supplementation of the base extender with Eugenol. Freezing thawing process descrease the velocity and velocity and progressivity parameters. In case of the BilE group was obtained in our study the best percent of rapid velocity (26.04 %, Table 4) and the lowest percent of immobile spermatozoa after thawing (19.77%, Table 5). In a study of Rasa Aragonesa breed, it has been reported that high-fertility rams produced a higher proportion of fast and linear spermatozoa than did low-fertility rams (Yániz *et al*., 2015).

Mammalian spermatozoa are highly sensitive to lipid peroxidation (LPO), which occurs as a result of the oxidation of membrane lipids by partially reduced oxygen molecules, *e.g.* superoxide, hydrogen peroxide and hydroxyl radicals (Bucak *et al*., 2007). It is well known that cryopreservation causes physical and chemical stresses to sperm membranes, along with oxidative stress, which can reduce sperm viability and fertilizing capability (Watson, P.F., 2000), being the sperm damage due to the release of endogenous reactive oxygen species the major cause of the reduction in sperm motility and conception rate associated with semen cryopreservation (Bucak *et al*., 2007).

Hill *et al*.,1998, presented results from AI with frozen and fresh semen considering the factors influencing pregnancy. The study comprised a high number of animals (28,447 Australian Merino ewes), using semen from 468 rams. The overall pregnancy rate was 71.7%. Pregnancy rates varied with the type of progestogen implant, type and dosage of eCG, fresh or frozen semen, the month of the year. The pregnancy rate obtained with MAP sponges (64.6%) was significantly lower than with 30 mg FGA (74.7%) or 40 mg FGA (72.1%) or CIDR implant (71.7%; P<0.05). An eCG dose of 200 IU resulted in significantly lower pregnancy rate (62.4%) compared with 250 IU (72.9%) or 300 IU (79.1%) (P<0.05). Ewes inseminated with fresh semen were significantly more likely to become pregnant (82.2%) than those inseminated with semen frozen in pellets (69.5%) or straws (71.6%) (P<0.001). Donovan *et al*., 2004 reported a non-return rate of 58% following deep cervical insemination with Frozen-thawed semen. In our study, the pregnancy rate average was 65.51% for the ewes inseminated with frozen semen in straws. In case of straws frozen with Bil extender, the gestation was confirmed in 5 cases (55.55%), in case of the straws frozen in Bil A extender in 6 cases (60%) and in case of straws frozen in Bil E extender in 8 cases (80%).

Table 3: Results of artificial insemination with frozen semen.

	Number (%)
Synchronized animals	34 (100)
AI sheep	29 (85.29)
Out of study at AI	5 (14.7)
Pregnant sheep	19 (65.51)
Non-pregnant sheep	10 (34.09)
Pregnants bil	5/9 (55.55)
Pregnants bilA	6/10 (60%)
Pregnants bil E	8/10 (80%)

Table 4: Comparative analysis of sperm motility.

Groups	Before freezing		After thawing	
	Total motility (%)	Progressive motility (%)	Total motility (%)	Progressive motility (%)
Bil	91.77b	41.48b	73.25a	24.38a
Bil A	91.54a	37.34a	75.9b	23.57a
Bil E	91.9a	36.15a	80.23c	30.15b
St Err for p=0.05	0.51%	0.40%	0.50%	0.62%

* Different letters show statistically significant differences for p = 0.05.

Table 5: Comparative analysis of sperm velocity.

Groups	Velocity before freezing (%)			Velocity after tnawing (%)			St Err for p = 0.05
	Lot Bil	Lot Bil A	Lot Bil E	Lot Bil	Lot Bil A	Lot Bil E	
Rapid (%)	31.9f	24.75c	26.4d	21.63a	22.83b	28.64c	0.37%
Medium (%)	23.55c	22c	19.69b	11.63a	11.06a	10.89a	0.52%
Slow (%)	36.36a	45.15c	45.61c	40b	40.70b	41.28b	0.94%
Immotile (%)	8.23a	8.1a	8.3a	26.74c	19.65c	24.83b	1.44%

* Different letters show statistically significant differences for p = 0.05.

CONCLUSION

The addition of Eugenol to Byladil extender improves sperm kinematic properties following liquid storage, according to the findings of this study. Increasing the kinematic characteristics of semen liquid storage is a step toward improving the success rate of artificial insemination. In our investigation, the antioxidant Arginine had no effect on kinematic measures. Transcervical artificial insemination is a technique for artificial insemination in sheep that allows for the use of frozen-thawed sperm. The frozen-thawed semen is deposited directly intrauterine via the passage of an insemination pipette through the cervical canal. The supplementation of the base extender Biladyl with Eugenol increase both: the kinematic parameters and the fertility rate after the transcervical insemination.

Conflict of interest: None.

REFERENCES

Alcay, S., Toker, M.B., Gokce, E., Ustuner, B., Onder, N.T., Sagirkaya, H., Kemal Soylu, M. (2015). Successful ram semen cryopreservation with lyophilized egg yolk-based extender. Cryobiology. 71(2): 329-333. doi:10.1016 j.cryobiol.2015.08.008.

Alejandro, E.G., Jimena, F., María, M.B.G., María, V.S., Marcela, I.C. (2019). Technical recommendations for artificial insemination in sheep. Anim Reprod. 16: 803-809.

Ayad, V.J., Leung, S.T., Parkinson, T.J., Wathes, D.C. (2004). Coincident increases in oxytocin receptor expression and EMG responsiveness to oxytocin in the ovine cervix at oestrus. Anim. Reprod. Sci. 80(3-4): 237-258.

Bucak, M.N., Ateşşahin, A., Varışlı, Ö., Yüce, A., Tekin, N. and Akçay, A. (2007). The influence of trehalose, taurine, cysteamine and hyaluronan on ram semen. Theriogenology. 67(5): 1060-1067. doi:10.1016/j.theriogenology. 2006. 12.004.

Bucak, M.N., Atessahim, A., Yüce, A., (2008) Effect of anti-oxidants and oxidative stress parameters on ram semen after the freezing-thawing process. Small Rumin Res. 75: 128-134.

Câmara, D.R., Pinto, L.C., Pinto, M.M.C.M., Kastelic, J.P., Nunes, J.F., Barbosa, J.M.P., Guerra, M.M.P. (2016). Influence of catalase and pre-freezing equilibration on post-thaw semen quality and conception rate in ewes laparoscopically inseminated. Animal Reproduction. 13: 21-27. 10.21451/ 1984-3143-AR729.

Campbell, J.W., Harvey, T.G., McDonald, M.F., Sparksman, R.I. (1996). Transcervical insemination in sheep: An anatomical and histological evaluation. Theriogenology. 45(8): 1535-1544. doi: 10.1016/0093-691x(96)00121-5.

Donovan, A., Hanrahan, J.P., Kummen, E., Duffy, P., Boland, M.P. (2004). Fertility in the ewe following cervical insemination with fresh or frozen-thawed semen at a natural or synchronised oestrus. Anim Reprod Sci. 84(3-4): 359-368. doi:10.1016/ j.anireprosci.2003.12.014.

Gülçin, I. (2011) Antioxidant activity of eugenol: A structure-activity relationship study. Journal of Medicinal Food. 14: 975-85. 10.1089/jmf.2010.0197.

Hill, J.R., Thompson, J.A., Perkins, N.R. (1998). Factors affecting pregnancy rates following laparoscopic insemination of 28,447 merino ewes under commercial conditions: A survey. Theriogenology. 49(4): 697-709. doi: 10.1016/ S0093-691X(98)00019-3.

Kalmath, G.P. and Swamy, M.N. (2019). Effect of antioxidants supplementation on plasma hormonal profiles in Hallikar cattle during different seasons. Indian Journal of Animal Research. (53): 28-3.

King, M.E., McKelvey, W.A., Dingwall, W.S., Matthews, K.P., Gebbie, F.E., Mylne, M.J., Stewart, E., 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(7): 1236-1244.

Kumar, P., Yadav, B. and Yadav, S. (2014). Effect of zinc and selenium supplementation on semen quality of barbari bucks. Indian Journal of Animal Research. (48): 366-369.

Lass, A., Suessenbacher, A., Wolkart, G., Mayer, B., Brunner, F. (2002). Functional and analytical evidence for scavenging of oxygen radicals by L-arginine. Mol Pharmacol. 61(5): 1081-8.

Leethongdee, S. (2010). Development of trans-cervical artificial Insemination in sheep with special reference to anatomy of cervix. Suranaree J Sci Technol. 17.

Lemma, A. (2011). Effect of Cryopreservation on Sperm Quality and Fertility. In: [Manafi, M. (ed.)] Artificial insemination in farm animals. ISBN978-953-307-312-5.

Maritim, A.C., Sanders, R.A., Watkins, J.B. (2003). Diabetes, oxidative stress and antioxidants: A review. J Biochem Mol Toxicol. 17(1): 24-38.

Pandey, B.N., Lathika, K.M., Mishra, K.P. (2006) Modification of radiation-induced oxidative damage in liposomal and microsomal mem-brane by eugenol. Rad Physics Chem. 75: 384-391.

Pool, K.R., Rickard, J.P., Tumeth, E., Graaf, S.P. (2020). Treatment of rams with melatonin implants in the non-breeding season improves post-thaw sperm progressive motility and DNA integrity. Animal Reproduction Science. Volume 221.

Robayo, I.M., Valdes, V., Cristian, C., Jose, F. (2008). CASA Assessment of kinematic parameters of Ram spermatozoa and their relationship to migration efficiency in ruminant cervical mucus. Reproduction in domestic animals = Zuchthygiene. 43: 393-9. 10.1111/j.1439-0531.2007.00920.x.

Salamon, S., Maxwell, W.M. (2000). Storage of ram semen. Animal Reproduction Science. 62(1-3): 77-111. doi: 10.1016/ s0378-4320(00)00155-x.

Samir, Z., El-Zarkouny and Eman, A., Elwakeel. (2019). Biochemical changes and pregnancy toxemia in pregnant and lactating Harri ewes under arid lands conditions. Indian Journal of Animal Research. 53: 1188-1193.

Steven, P., Brinsko, Terry, L., Blanchard, Dickson, D., Varner, James Schumacher, Charles, C., Love, Katrin Hinrichs, David, L. Hartman, (2011). Manual of Equine Reproduction (Third Edition), Mosby, Chapter 18- Evaluation of Breeding Records,Pages. 288-301.

Stradaioli, G., Noro, T., Sylla, L., Monaci, M. (2007). Decrease in glutathione (GSH) content in bovine sperm after cryopreservation: Comparison between two extenders. Theriogenology. 67: 1249-1255.

Szabadics, J., Erdelyi, L. (2000). Pre-and postsynaptic effects of eugenoland related compounds on Helix pomatia L.Acta Biol Hung. 51: 265-273.

Toker, M.B., Alcay, S., Gokce, E., Ustuner, B. (2016). Cryopreservation of ram semen with antioxidant supplemented soybean lecithin-based extenders and impacts on incubation resilience. Cryobiology. 72(3): 205-209. doi: 10.1016/j.cryobiol.2016.05.001.

Vincent, A.M., Russell, J.W., Low, P., Feldman, E.L. (2004). Oxidative stress in the pathogenesis of diabetic neuropathy. Endocr Rev. 25(4): 612-28.

Wang, X., Sharma, R.K., Gupta, A., George, V., Thomas, A.J., Falcone, T., Agarwal, A. (2003). Alterations in mitochondria membrane potential and oxidative stress in infertile men: A prospective observational study. Fertil Steril. 80: 844-850.

Watson, P.F. (2000). The causes of reduced fertility with cryopreserved semen. Anim Reprod Sci. 61: 481-492.

Yániz, J.L., Palacín, I., Vicente-Fiel, S., Sánchez-Nadal, J.A., Santolaria, P. (2015) Sperm population structure in high and low field fertility rams. Anim. Reprod. Sci. 156: 128-134.