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Research Article

Effect of the Ethylene Glycol Tetra acetic Acid Supplementation in the Freezing Extender on Quality of Cryopreserved Boar Sperm

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Abstract

The aim of this study was to evaluate the effect of the inclusion of divalent ion chelating agent ethylene glycol tetra acetic acid (EGTA) in the freezing extender on quality of cryopreserved boar sperm. For boar sperm the freezing extender (lactose egg yolk) was supplemented with 0, 1, 2, 4 and 8 mM EGTA.

The percentages of sperm with normal acrosomal ridge (%NAR), sperm with intact plasmatic membrane (%SIPM), total motile sperm (%TMS), progressive motility sperm (%PMS), and kinematic parameters, were evaluated in samples incubated at 37ºC during 30 and 150 min after thawing.

The supplementation with 2 mM EGTA showed a higher significant sperm viability (SIPM) and normal acrosome ridge (NAR), than non-supplemented sample. In sperm motility no significant differences were observed between treatments in all parameters studied. In addition, greater concentrations than 2 mM EGTA did not show a detrimental effect on quality of cryopreserved boar sperm. Our results suggest the EGTA supplementation in the freezing extender of boar sperm might improve the post-thawing sperm quality. But it would be necessary to carry out further experiments to determine how exactly the chelating agents protect the sperm during cryopreservation process.

Keywords: *Freezing extenders; Cryopreservation; ethylene glycol tetra acetic acid; Boar sperm; sperm quality*

Abbreviations: %NAR: percentages of sperm with normal acrosomal ridge; %PMS: percentages of progressive motility sperm; %SIPM: percentages sperm with intact plasmatic membrane; %TMS: percentages of total motile sperm; ALH: (μm) mean amplitude of lateral head displacement; BCF: (Hz) means of the beat cross frequency; EGTA: ethylene glycol tetra acetic acid; LIN: the percentage of linearity (%; ratio between VSL and VCL); LN2: into liquid nitrogen; PI: propidium iodide; STR: the percentage of straightness (%; ratio between VSL and VAP); VAP: (μm/s) the average path velocity; VCL: (μm/s) the curvilinear velocity; VSL: (μm/s) the straight-line velocity; WOB: oscillation index (ratio between VAP and VCL); LEY: Lactose egg yolk freezing extender; BTS: Beltsville Thawing Solution

Introduction

The cryopreservation process causes severe damage to sperm. After insemination, the sperm have to survive for long time in the female reproductive tract and colonize the oviduct in order to fertilize an oocyte. But about 50% of the population of the sperm does not survive the cryopreservation process and the survivors remain with sublethal dysfunctions [1] which reduce the longevity of sperm and their fertilizing ability.

This means that there are still many weaknesses in the sperm cryopreservation process that should be improved, and the composition of the freezing extenders is one of the most important. The basic ingredients of current sperm freezing extenders are the same as those used 35 years ago, although in recent years several authors have investigated the incorporation of new compounds to the freezing extender, like low-density lipoproteins (LDL) [2,3], different non-permeating sugars [4,5], seminal plasma [6], antioxidants [7,8] showing that it is possible to improve the post-thaw sperm quality modifying the actual cryopreservation extenders. Among one of these new compounds that could improve the quality of frozen semen is the ethylene glycol tetra acetic acid (EGTA). This compound is a chelating agent which is able to capture divalent metal ions as Mg^{2+}, Cu^{2+}, and Ca^{2+} among others, and in addition may limit their movement across the plasma membrane [9]. The chelating agents are routinely added to conservation extender solutions of boar semen such as Modena or BTS solution [10,11] and recently in the thawing extenders [12], to block the action of divalent metal ions, like calcium ion which is a mediator of sperm capacitation and the acrosome reaction (reviewed by Gadea, [13]). But little information about the effects of the chelating agents on the cryopreservation process is available. Few studies have investigated the supplementation of the freezing media with chelating agents and they showed that these may improve the post-thaw sperm quality in some species like human, ram or bear [14-17]. Although the fact of how many of them may preserve semen quality is not completely established. Moreover, the inclusion of EGTA as chelating agent in the boar sperm freezing extender has never been tested. Therefore, the aim of this study was to evaluate the effect of the EGTA (ethylene glycol tetra acetic acid) supplementation in the boar freezing extender, determining their effect on post-thaw sperm quality.

Materials and Methods

Reagents and media

Unless otherwise stated, all media components were purchased from Sigma Chemical Co. (St. Louis, MO, USA), with purified water (18 MΩ cm; Automatic GR Wasserlab, Spain). Orvus ES Paste is marketed as Equex STM, Nova Chemical Sales Inc., Scituate, MA, USA. The basic medium used for sperm extension was Beltsville Thawing Solution (BTS, composed of 205 mM glucose, 20.39 mM NaCl, 5.4 mM KCl, 15.01 mM NaHCO3, and 3.35 mM EDTA; [18]), containing kanamycin sulfate (50 μg/mL). The extender used for boar sperm freezing was Lactose egg yolk (LEY) (80% [v/v] 310 mM lactose, 20% [v/v] egg yolk, 100 μg/mL kanamycin sulfate; 330 ± 5m Osm kg^{-1}, and pH 6.2).

Boar semen collection and freezing protocol

Ejaculate sperm samples were collected from three sexually mature pietrain boars once a week for five consecutive weeks. Boar ejaculates were obtained using the gloved-hand method and extended in BTS (1:1 [v/v]). The ejaculates were pooled prior to centrifugation and cryopreservation (the number of boars and ejaculates, and the performance of a pool is suitable to investigate improvements in sperm freezing [4,19,20]). Boars were housed at a commercial semen collection center, fed a diet providing 100% of their nutritional needs, and provided water ad libitum.

Sperm were cryopreserved using the straw freezing procedure described by Westendorf., *et al*. [21] and modified by Thurston., et al. [22] and Carvajal., *et al*. [23]. Diluted semen samples were pooled and centrifuged at 2400 × g for 3 min at 15ºC. The pellet was diluted in LEY extender to a concentration of 1.5 × 109 cells/mL. After further cooling to 5ºC within 120 min, diluted sperm samples were re-suspended in LEY-glycerol-Orvus ES Paste (LEYGO) extender (92.5% LEY, 1.5% Equex STM [Nova Chemical Sales Inc, Scituate, Mass] and 6% glycerol [v/v]; 1650 ± 15 mOsm kg^{-1}, pH was adjusted for each experiment) to yield a final concentration of 1 × 109 cells/mL. The re-suspended and cooled sperm were packed into 0.5 mL PVC-French straws (Minitüb, Tiefenbach, Germany), which were frozen using a controlled-rate freezer (IceCube 14 S; Minitüb) as follows: from 5 to -5ºC at a rate of 6ºC/min, from -5 to -80ºC at 40ºC/min, held for 30 s at -80ºC, then cooled at 70ºC/min to -150ºC and plunged into liquid nitrogen (LN2). The straws remained in the LN2 tank for at least two weeks before thawing. Thawing of straws was done in a circulating water bath at 37ºC for 20 seconds. Thawed sperm samples were extended in BTS (1:1 [v/v]; 37ºC, and incubated in a water bath at 37ºC to 150 minutes).

Sperm assessments

Sperm quality was determined by assessing for motility, acrosomal status, and viability (plasma membrane integrity) at 30 and 150 minutes.

Sperm motility was objectively evaluated using a computer-assisted sperm analysis system (Integrated Semen Analysis System, ISAS® Prosier, Valencia, and Spain) following the procedure described by Cremades., *et al*. [24].

Diluted-thawed boar sperm were rediluted in BTS to a concentration of 30 × 106/mL. For each evaluation, a 2 μL aliquot of sperm sample was placed in a pre-warmed (37ºC) Makler counting chamber (Sefi Medical Instruments, Haifa, Israel) and three fields were analyzed at 37 ºC, assessing a minimum of 100 sperm per sample. The proportions of total motile sperm (% TMS) and progressive motility sperm (%PMS) were determined. The kinematic parameters measured for each spermatozoon included the curvilinear velocity (VCL, μm/s), the straight-line velocity (VSL, μm/s), the average path velocity (VAP, μm/s), the percentage of linearity (LIN, %) (Ratio between VSL and VCL), the percentage of straightness (STR, %) (Ratio between VSL and VAP), oscillation index (WOB, ratio between VAP and VCL), mean amplitude of lateral head displacement (ALH, μm) and means of the beat cross frequency (BCF, Hz). For more detailed descriptions of these parameters, see Mortimer [25].

The acrosome morphology was evaluated by phase contrast microscopy at 1000x. Samples were fixed in 2% glutaraldehyde and a minimum of 200 acrosomes per sample were examined. The damage to the acrosome cap for boar sperm was classified by the scoring system reported by Pursel., *et al*. [26]. Only the percentage of sperm with normal acrosomal ridge (NAR) was considered in the results.

Sperm plasma membrane integrity (viability) was assessed by dual fluorescent probes, SYBR-14 and propidium iodide, PI (L-7011, Live/Dead Sperm Viability Kit; Molecular Probes Europe, Leiden, the Netherlands), as was described by Garner and Johnson [27]. The samples were evaluated by means of fluorescence microscope (Nikon Eclipse E400, Tokyo, Japan) equipped with Nikon G-2A filter with excitation/barrier filter of 510/590, which allowed dual excitation of blue and green for SYBR-14 and PI, respectively. The sperm cells with undamaged membrane were stained for SYBR-14 resulting in a bright green fluorescence, while PI only stained red the nuclei of the damaged membrane sperm. A minimum of 300 cells per slide were examined in random fields of each sample. Only the percentage of sperm with intact plasmatic membrane (SIPM) was considered in the results (SYBR-14 positive and PI negative).

Experimental design

To determine the effect of the EGTA supplementation on the quality of frozen boar sperm, different concentrations of this compound were supplemented in the freezing extender. Pools were divided into five aliquots, and diluted with LEY freezing extender supplemented with 0 (non-supplemented), 1, 2, 4 and 8 mM EGTA.

Statistical analysis

Statistical analyses were performed using SAS system version 9.1 (SAS Institute Inc., Cary, North Caroline, USA 2004). The MIXED model analysis of variance (ANOVA) procedure was used to evaluate the effects of the EGTA inclusion in the freezing extender and their effect on sperm motility, plasma membrane integrity, and acrosomal morphology. The statistical model included the fixed effects of the recollection week. When ANOVA revealed a significant effect, values were compared using the Tukey-Kramer test and were considered to be significant when P was less than 0.05. Results are presented as least-squares means ± SEM.

Results and Discussion

Our study investigated the effect of EGTA supplementation in the boar sperm freezing extender on the post-thawing sperm quality. The supplementation with 2 mM EGTA showed a higher significant sperm viability (SIPM) and normal acrosome ridge (NAR), than non-supplemented sample, at 30 and 150 min of post-thawing incubation (Table 1). In addition, greater concentrations than 2 mM EGTA did not show a detrimental effect on quality of cryopreserved boar sperm.

	EGTA Concentrations (mM EGTA)					
Variables 30 and 150 min	0 mM (non-supplemented)	1 mM	2 mM	4 mM	8 mM	SEM
% SIPM-30	50.9[a]	56.9[ab]	62.7[b]	57.2[ab]	51.9[ab]	2.5
% SIPM-150	43.5[a]	51.7[ab]	56.9[b]	51.6[ab]	51.7[ab]	2.7
% NAR-30	52.6[a]	53.2[a]	61.6[b]	53.8[a]	51.3[a]	1.9
% NAR-150	37.8[a]	43.5[ab]	49.6[b]	43.5[ab]	42[ab]	2.4
% TMS-30	51.8	54.9	59.6	53.7	52.5	4.2
% TMS-150	39.6	43	49.1	42.3	49.1	3.4
% PMS-30	45.8	48.4	52.1	47.3	45.3	3.9
% PMS-150	35.2	36.9	43.1	36.5	43.9	3.4

Table 1: *Effect of the EGTA supplementation at different concentrations in the freezing media (lactose egg yolk) on boar sperm quality after cryopreservation at two incubation times (30 and 150 minutes).*

§Data are from five replicates of pooled sperm from three boars. Values are expressed as mean ± S.E.M. Values within the same row with different letters (a, b) differed significantly ($P < 0.05$). SIPM: spermatozoa with intact plasmatic membrane; NAR: normal acrosomal ridge; TMS: total motile spermatozoa; PMS: progressive motility spermatozoa.

The improvement of post-thaw sperm quality has also been observed by other authors in different species when the freezing extender was supplemented with a chelating agent [14-17]. Determining that, the chelating agent incorporation in the freezing extender has a beneficial effect on post-thaw sperm quality. But how the chelating agents are able to protect during the cryopreservation process is not fully established.

It is known the main changes suffered during cooling and freezing of sperm are changes in membrane fluidity [28], readily the sperm undergo an acrosome reaction [29-31] and an increase in the intracellular ion concentrations is observed [32]. This ion concentration increase is due to membrane rearrangements and consequent lipid packing faults [33], which can cause a loss of the effectiveness of the plasma membrane as a barrier, resulting in an increase in membrane permeability and thus influx of extracellular ions such as Ca^{2+} and Mg^{2+}. This redistribution causes some cations to be unbalanced, increasing the amount of some of them in the intracellular sperm and others in the external environment [34-36]. These unbalanced cations were negatively correlated with fertility, contributing to high percentage of the variation in the fertility of cryopreserved sperm [37,38]. Moreover, some authors have shown that high concentrations of cations such as calcium or magnesium in the environment provided significantly less protection to the cells during freezing and thawing [39], and even can affect the activity of the acrosin [40] and consequently the acrosome reaction. Also some authors have documented for the sperm of some species that during the freezing process there is an increase of intracellular calcium concentration [34,41-43]. This increase may cause perturbations of the plasma membrane [44], because they may have a detrimental effect in the acrosome state [45,46], and a higher concentration in the external medium may decrease the motility and viability of sperm [46,47]. Therefore, the beneficial effect of inclusion of EGTA during freezing may be determined by their ability to chelate divalent ions such as Ca^{2+} and Mg^{2+}, especially the calcium ion [48], preventing or diminishing the unbalanced cations that occur during freezing, and especially in the acrosome protection, where it is known that the calcium increase triggered acrosome reaction [49-51]. All of this corresponds with the best results of viability and acrosome status of treatment with 2 mM EGTA respect of the control.

With respect to the quality of sperm motility, no significant differences were observed in all parameters (Tables 1 and 2), except for the kinetic parameters VSL and WOP at 150 min of incubation, but this could not determine a clear pattern of differences. Although extracellular calcium is required for sperm motility and is known to regulate both activated and hyper activated motility [52, 53], our results show that there are no differences in the treatments with EGTA than the control. That EGTA does not affect sperm motility may

be due to motility (especially hyper activated motility), and may be regulated by intracellular calcium stored [54]. Therefore EGTA, by not penetrate cells, only affect the extracellular cations, improving the viability and acrosome status as stated before, without affecting sperm motility.

	EGTA concentrations (mM)					
Variables 30 and 150 min	**0 mM (non-supplemented)**	**1 mM**	**2 mM**	**4 mM**	**8 mM**	**SEM**
VCL-30	76.9	74.2	74.7	75.6	72.4	3.2
VCL-150	75.2[a]	68.7[ab]	72.7[ab]	66.1[b]	71.6[ab]	3.5
VSL-30	55.4	52.9	52.6	54.9	52	2.9
VSL-150	55	49.6	54.9	50	53.3	3
VAP-30	65.6	63	64.1	65.7	62.4	3.2
VAP-150	63.7	57.6	63.1	57.4	62	3.3
LIN-30	71.9	71.1	70.3	72.6	71.9	1.9
LIN-150	72.9	72.1	75.4	75.5	71.4	1
STR-30	84.3	83.8	82	83.6	83.3	1.2
STR-150	86.2	86.1	87	87	85.9	0.8
WOB-30	85.2	84.8	85.8	86.8	86.3	1.3
WOB-150	84.5[ab]	83.8[b]	86.7[ab]	86.8[a]	86.6[ab]	0.7
ALH-30	2.4	2.3	2.3	2.2	2.3	0.1
ALH-150	2.3	2.2	2.2	2.1	2.1	0.07
BCF-30	8.2	8.2	8.2	8.3	8	0.2
BCF-150	8.6	8.4	8.7	8.3	8.5	0.2

Table2: *Effect of the EGTA supplementation at different concentrations in the freezing media (lactose egg yolk) on boar sperm kinematics parameters after cryopreservation at two incubation times (30 and 150 minutes).*

§Data are from five replicates of pooled sperm from three boars. Values are expressed as mean ± S.E.M. Values within the same row with different letters (a, b) differed significantly ($P < 0.05$). VCL indicates curvilinear velocity; VSL, straight-line velocity; VAP, average path velocity; LIN, linearity; STR, straightness; WOB, oscillation index; ALH, mean amplitude of lateral head displacement; and BCF, beat cross frequency.

Conclusion

In conclusion, the EGTA supplementation in the freezing extender improves the quality of cryopreserved boar sperm, improving sperm viability and the percentage of sperm with an intact acrosome, without detriment of motility. However, it would be necessary to carry out further experiments to determine the evolution of divalent cations, especially calcium, when EGTA are present or not, during the cryopreservation process.

Bibliography

1. Watson PF. "The causes of reduced fertility with cryopreserved semen". *Animal Reproduction Science* 60-61 (2000): 481-492.
2. Wang P., *et al*. "Effects of low-density lipoproteins extracted from different avian yolks on boar spermatozoa quality following freezing-thawing". *Zygote* 22.2 (2014): 175-181.
3. Yamauchi S., *et al.* "Characteristics of Okinawan native agu pig spermatozoa after addition of low-density lipoprotein to freezing extender". *Journal of Reproduction and Development* 55.5 (2009): 558-565.

4. Gómez-Fernández, J., *et al*. "Effect of different monosaccharides and disaccharides on boar sperm quality after cryopreservation". *Animal Reproduction Science* 133.2 (2012): 109-116.
5. Malo C., *et al*. "Comparing sugar type supplementation for cryopreservation of boar semen in egg yolk based extender". *Cryobiology* 61.1 (2010): 17-21.
6. Gómez-Fernández, J., *et al*. "Inclusion of seminal plasma in sperm cryopreservation of Iberian pig". *Animal Reproduction Science* 130.2 (2012), 82-90.
7. Malo C., *et al*. "Antioxidant effect of rosemary (Rosmarinus officinalis) on boar epididymal spermatozoa during cryopreservation". *Theriogenology* 75 (2011): 1735-1741.
8. Varo-Ghiuru F., *et al*. "Lutein, Trolox, ascorbic acid and combination of Trolox with ascorbic acid can improve boar semen quality during cryopreservation". *Cryo Letters* 36.1 (2015): 1-7.
9. Watson PF. "Artificial insemination and the preservation of semen. In: Lamming", G. (Ed), Marshall's Physiology of Reproduction 4th edn. vol. 2 (1990). Churchill Livingstone, Edinburgh, pp. 747-869.
10. Pursel VG and Johnson LA. "Freezing of boar spermatozoa: Fertilizing capacity with concentrated semen and a new thawing procedure". *Journal of Animal Science* 40.1 (1975), 99-102.
11. Funahashi H and Sano T. "Select antioxidants improve the function of extended boar semen stored at 10 degrees C". *Theriogenology* 63.6 (2005): 1605-1616.
12. Okazaki T., *et al*. "The addition of calcium ion chelator, EGTA to thawing solution improves fertilizing ability in frozen-thawed boar sperm". *Animal Science Journal* 82.3 (2011): 412-419.
13. Gadea J. "Review: Semen extenders used in the artificial insemination of swine". *Spanish Journal of Agricultural Research* 1.2 (2003): 17-27.
14. Hong CY., *et al*. "Calcium antagonists stimulate sperm motility in ejaculated human semen". *British Journal of Clinical Pharmacology* 19.1 (1985): 45-49.
15. Fong JC., *et al*. "Calcium buffering capacity of human seminal plasma: the role of EGTA in stimulating sperm motility". *Chinese Journal of Physiology* 29.1 (1986): 7-12.
16. Aisen EG., *et al*. "Effect of trehalose and EDTA on cryoprotective action of ram semen diluents". *Theriogenology* 53.5 (2000): 1053-1061.
17. Anel L., *et al*. "Effect of basic factors of extender composition on post-thawing quality of brown bear electroejaculated spermatozoa". *Theriogenology* 74.4 (2010): 643-651.
18. Johnson LA., *et al*. "Artificial insemination of swine: fecundity of boar stored in Beltsville TS (BTS), modified Modena (MM) or MR-A and inseminated on one, three and four days after collection". *Zuchthygyene* 23.2 (1988): 49-55.
19. Gil MA., *et al*. "Pentoxifylline added to freezing or post-thaw extenders does not improve the survival or in vitro fertilising capacity of boar spermatozoa". *Reproduction* 139.3 (2010): 557–564.
20. Juarez JD., *et al*. "Boar semen can tolerate rapid cooling rates prior to freezing". *Reproduction Fertility and Development* 23.5 (2011): 681–690.
21. Westendorf P., *et al*. "Deep freezing of boar sperma. Laboratory and insemination results using the Hulsenberger paillete method". *Deutsche Tierärztliche Wochenschrift* 82.7 (1975): 261-267.
22. Thurston LM., *et al*. "Morphologically distinct sperm subpopulations defined by Fourier shape descriptors in fresh ejaculate correlate with variation in boar semen quality following cryopreservation". *Journal of Andrology* 22.3 (2001): 382-394.
23. Carvajal G., *et al*. "Effects of centifugation before freezing on boar sperm cryosurvival". *Journal of Andrology* 25.3 (2004): 389-396.
24. Cremades T., *et al*. "Kinematic changes during the cryopreservation of boar spermatozoa". *Journal of Andrology* 26.5 (2005): 610-618.

25. Mortimer ST. "A critical review of the physiological importance and analysis of sperm movement in mammals". *Human Reproduction Update* 3.5 (1997): 403-439.
26. Pursel VG., *et al*. "Interaction of extender composition and incubation period on cold shock susceptibility of boar spermatozoa". *Journal of Animal Science* 35.3 (1972): 580-584.
27. Garner DL and Johnson LA. "Viability assessment of mammalian sperm using SYBR-14 and propidium iodide". *Biology of Reproduction* 53.2 (1995): 276-284.
28. Harrison RAP., *et al*. "Bicarbonate/CO2, an effectors of capacitation, induces a rapid and reversible change in the lipid architecture of boar sperm plasma membranes". *Molecular Reproduction and Development* 45.3 (1996): 378-391.
29. Clarke RN and Johnson LA. "Effect of liquid nitrogen storage and cryopreservation of boar spermatozoa on acrosomal integrity and the penetration of zona-free hamster ova *in vitro*". *Gamete Research* 16.3 (1987): 193-204.
30. Gillan L., *et al*. "Fate and functional integrity of fresh and frozen–thawed ram spermatozoa following intrauterine insemination". *Reproduction Fertility and Development* 11.6 (1999): 309-315.
31. Dubé C., *et al*. "The importance of calcium in the appearance of p32, a boar sperm tyrosine phosphoprotein, during *in vitro* capacitation". *Journal of Andrology* 24.5 (2003): 727-733.
32. Adeoya-Osiguwa SA and Fraser LR. "A biphasic pattern of 45 Ca^{2+} uptake by mouse spermatozoa in vitro correlates with changing functional potential". *Journal of Reproduction and Fertility* 99.1 (1993): 187-194.
33. Drobnis EZ., *et al*. "Cold shock damage is due to lipid phase transitions in cell membranes: a demonstration using sperm as a model". *Journal of Experimental Zoology* 265.4 (1993): 432-437.
34. Quinn PJ and White IG. "The effect of cold shock and deep-freezing on the concentration of major cations in spermatozoa". *Journal of Reproduction and Fertility* 12.2 (1996): 263-270.
35. Profirov I., *et al*. "Role of the plasma membrane in the distribution of potassium, sodium and calcium in ram sperm during deep freezing". *Veterinarno-meditsinski nauki* 23.3 (1986): 73-77.
36. Courtens JL., *et al*. "Preliminary study of water and some element contents in boar spermatozoa, before, during and after freezing". *Journal of Reproduction and Fertility* 87.2 (1989): 613-626.
37. Mahadevan MM and Trounson AO. "Biochemical factors affecting the fertility of cryopreserved human semen". *Clinical reproduction and fertility* 2.3 (1983): 217-227.
38. Kim JC., *et al*. "Effects of cryopreservation on Ca^{2+} signals induced by membrane depolarization, caffeine, thapsigargin and progesterone in boar spermatozoa". *Molecules and Cells* 26.6 (2008): 558-565.
39. García MA and Graham EF. "Development of a buffer system for dialysis of bovine spermatozoa before freezing. III. Effect of different inorganic and organic salts on fresh and frozen-thawed semen". *Theriogenology* 31.5 (1989): 1039-1048.
40. Brown CR., *et al*. "Studies on ram acrosin. Isolation from spermatozoa, activation by cations and organic solvents, and influence of cations on its reaction with inhibitors". *Biochemical Journal* 149.1 (1975): 133-146.
41. Karagiannidis A. "The distribution of calcium in bovine spermatozoa and seminal plasma in relation to cold shock". *Journal of Reproduction and Fertility* 46.1 (1976): 83-90.
42. Simpson AM and White IG. "Measurement and manipulation of cytoplasmic free calcium of ram and boar spermatozoa using quin 2". *Cell Calcium* 9.1 (1988): 45-56.
43. Bailey JL and Buhr MM. "Ca^{2+} regulation by cryopreserved bull spermatozoa in response to A23187". *Cryobiology* 30.5 (1993): 470-481.
44. Harrison RAP., *et al*. "Flow cytometric studies of bicarbonate-mediated Ca^{2+} influx in boar sperm populations". *Molecular Reproduction and Development* 35.2 (1993): 197-208.
45. Dragileva E., *et al*. "Intracellular Ca(2+)-Mg(2+)-ATPase regulates calcium influx and acrosomal exocytosis in bull and ram spermatozoa". *Biology of Reproduction* 61.5 (1999): 1226-1234.

46. Yamaguchi S., *et al*. "Improved fertility in gilts and sows after artificial insemination of frozen-thawed boar semen by supplementation of semen extender with caffeine and $CaCl_2$". *Journal of Reproduction and Development* 55.6 (2009): 645-649.
47. Tash JS and Means AR. "Cyclic adenosine 3',5' monophosphate, calcium and protein phosphorylation in flagellar motility". *Biology of Reproduction*. 28.1 (1983): 75-104.
48. Bett GCL and Rasmusson RL. "Computer Models of Ion Channels". *In: Cabo, Candido*. Rosenbaum, David, S. Quantitative Cardiac Electrophysiology (2002). Marcel Dekker. pp. 48.
49. Aguas AP and Pinto Da Silva P. "Bimodal redistribution of surface transmembrane glycoproteins during Ca^{2+} -dependent secretion (acrosome reaction) in boar spermatozoa". *Journal of Cell Science* 93 (1989): 467-479.
50. Birck A., *et al*. "Dynamics of the induced acrosome reaction in boar". *Animal Reproduction Science* 115.4 (2009): 124-136.
51. Bragado MJ., *et al*. "Platelet-activating factor in Iberian pig spermatozoa: receptor expression and role as enhancer of the calcium-induced acrosome reaction". *Reproduction in Domestic Animals* 46.6 (2011): 943-949.
52. Suarez S., *et al*. "Hyperactivated motility induced in mouse sperm by calcium ionophore A23187 is reversible". *Journal of Experimental Zoology* 244.2 (1987): 331-336.
53. Ho HC., *et al*. "Hyperactivated motility of bull sperm is triggered at axoneme by Ca^{2+} and not cAMP". *Developmental Biology* 250.1 (2002): 208-217.
54. Ho H and Suarez S. "Characterization of the intracellular calcium store at the base of sperm flagellum that regulates hyperactivated motility". *Biology of Reproduction* 68.5 (2003): 1590-1596.

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