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SHORT COMMUNICATIONS

Recombinant TrxAFNIIx4His$_6$ improves post-thaw motility of ram sperm measured by a sperm motility tracker software

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Abstract

The aim of the present study was to evaluate a freezing extender supplemented with recombinant TrxAFNIIx4His$_6$, a reported decapacitating factor. Semen samples were diluted in tris-egg yolk medium with 0, 1.5 µM and 3.0 µM of TrxAFNIIx4His$_6$. Computer-assisted sperm motility tracking and subpopulations evaluation showed that addition of TrxAFNIIx4His$_6$ improved post-thaw total and progressive motility at both concentrations evaluated. TrxAFNIIx4His$_6$ increased the sperm subpopulation with the highest progressiveness and great velocity and decreased the subpopulation of poorly motile and almost non-progressive sperm. Incorporation of TrxAFNIIx4His$_6$ to freezing extender shows potential for the development of cryoprotection media which may lead to improved fertility after artificial insemination.

Keywords Sperm subpopulations · Recombinant proteins · Semen extender · Ram

Introduction

Sperm cryopreservation is a very useful tool to disseminate superior germplasm and maintain genetic diversity. However, the cryopreservation process negatively affects sperm cells (Yeste 2016). Incubation of frozen/thawed ram sperm with seminal plasma (SP) improved sperm functionality (Muiño-Blanco et al. 2008). Ram SP is enriched in two proteins, named RSVP14 and RSVP20, characterized by the presence of two fibronectin type II (FNII) tandem domains. The FNII domain interact with choline-phospholipids of the sperm plasma membrane preventing the free movement of phospholipids and stabilizing the membrane structure (Manjunath and Therien 2002). Since the concentration of RSVP14 and RSVP20 in SP is highly variable (Ledesma et al. 2015), much interest has been focused on their in vitro production (Serrano et al. 2013, 2015). Given the binding properties of FNII domain, we cloned and expressed a recombinant peptide composed of four FNII tandem repeats, named TrxAFNIIx4His$_6$. The protein demonstrated to be a decapacitation factor (DF) over ram sperm, evidenced by attachment to the sperm surface and reduction in cryocapacitation signals without inteferring with the in vitro fertilization rate (Ledesma et al. 2019). However, in this previous study, the addition of the protein was performed after thawing.

Thus, in the present study, we analyzed the effect of a novel extender formulation based on the addition of the recombinant TrxAFNIIx4His$_6$ over the post-thawing motility. One of the sperm traits believed to play an important role exclusively in the context of sperm competition is sperm velocity (Donnelly et al. 1998; Gage et al. 2004; Gomendio and Roldan 2004). Moreover, mammalian ejaculates contain sperm subpopulations with differences in their kinematic characteristics, and it has been suggested that the presence of these subpopulations might be related to sperm functionality or fertilizing ability (Quintero-Moreno et al. 2007). Knowing that semen handling and the application of a given treatment can differently affect subpopulations within a sample (Ledesma et al. 2017), we considered both average CASA parameters and supopulations defined on the basis of kinetic variables.

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Materials and methods

Five fertile mature Texel rams were used for semen collection and cryopreservation. Rams were kept under semi-extensive conditions at the Experimental Station of the Instituto Nacional de Tecnología Agropecuaria, INTA Argentina (37° 450 S, 58° 180 W). Ejaculates were obtained during the natural breeding season with an artificial vagina, and nine semen collections were made. Ejaculates with sperm mass motility ≥ 4 and concentration $\geq 3 \times 10^9$ cells/mL were pooled, obtaining one pool of semen per session. Pooled ejaculates were divided into three fractions and diluted with a tris-egg yolk-based extender (TRIS-glucose-citric acid extender, 10% v/v egg yolk, 7% v/v glycerol, Osm 300 mOsm/Kg) supplemented with 0 (control), 1.5 µM or 3.0 µM of TrxAFNIIx4His$_6$. The concentration used in the present work were tenfold higher compared to our previous study considering that the protein components of the semen extender reduce sperm binding proteins accessibility to the sperm surface (Ramírez-Vásquez et al. 2019). Toxicity of the protein was discarded by motility analysis of fresh samples upon dilution. Samples were cryopreserved as previously reported (Ledesma et al. 2019), and ten straws per treatment were cryopreserved. To study the effect of TrxAFNIIx4His$_6$ in the cryopreservation extender, three straws per treatment and control were randomly selected, pooled, and thawed by immersion in a water bath (37 °C, 1 min), layered over 1 mL of Ovipure colloid and processed according to the developers instructions (Morrell and Rodríguez-Martínez 2008). Subsequently, sperm cells were washed with PBS (800 × g, 5 min at 37 °C) and evaluated. Three thawing sessions and motility evaluation were made per treatment.

Sperm motility was analyzed by the sperm motility tracker software (Buchelly Imbachí et al. 2018). A warmed Cell-Vu chamber (20 µm depth, Millennium Sciences) was filled with 7 µL of sample and examined with a Nikon Eclipse E 200 microscope (10×, negative phase contrast field). Images were captured with a Coolpix S10, Nikon digital camera at 30 frames per second, and four fields were analyzed for each treatment and replicate. The kinematic variables analyzed were total motility (TM, %); progressive motility (PM, %); curvilinear velocity (VCL, µm/s); straight-line velocity (VSL, µm/s); average path velocity (VAP, µm/s); linearity (LIN, %); straightness (STR; %); wobble (WOB; %); lateral head displacement (ALH; µm); and beat-cross frequency (BCF; Hz). Sperm cells with VCL ≥ 10 µm/seg were considered as motile and STR ≥ 80% as sperm with progressive motility. Immotile sperms were excluded to calculate the averages.

Subpopulation distribution analysis was conducted in a two-step procedure considering the total sperm from each sample, followed by partition of the samples by a hierarchical method selecting all kinematic parameters as classifiers (total motility, progressive motility, curvilinear velocity, straight-line velocity, average path velocity, linearity, straightness, wobble, lateral head displacement, and beat-cross frequency). The number of clusters chosen was validated according to the best silhouette average index, $S = 0.88$, a measure of the average clustering quality of the individual elements (Rousseeuw 1987), corresponding to four clusters that combined the 8 kinematic parameters. Cells from each treatment and replicate were assigned to the previous clusters defined by a k-nearest neighbor supervised classification procedure (Weinberger and Saul 2009).

Statistical analysis

To evaluate the effect of recombinant protein on kinematic parameters and to determine statistical significance between the relative abundances of subpopulations, data were analyzed by generalized linear mixed effect models (GLMM), considering the "pooled ejaculates" as the aleatory variable. Normality of residuals was assessed by plotting theoretical quantiles versus standardized residuals (Q–Q plots). Homogeneity of variance was evaluated by plotting residuals versus fitted values. All analyses were performed using R software version 3.3.3, and significant differences were determined at $p < 0.05$.

Results and discussion

The effects of the addition of 1.5 or 3 µM TrxAFNIIx4His$_6$ to the semen extender are shown in Figs. 1, 2 and 3. Sperm cryopreserved in extender with 1.5 µM TrxAFNIIx4His$_6$

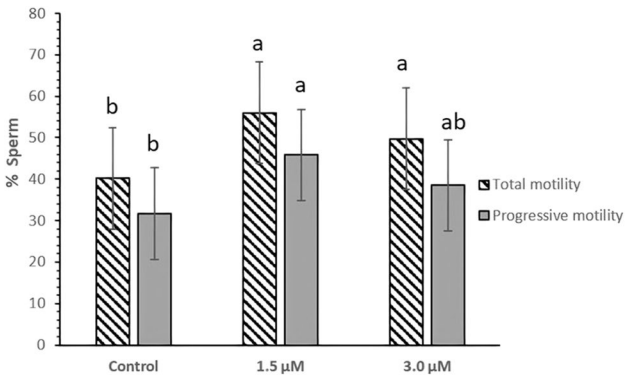

Fig. 1 Total and progressive motility of frozen-thawed ram sperm cryopreserved with or without TrxAFNIIx4His$_6$ (1.5 µM or 3.0 µM). Data represent mean ± SEM of three experiments. Columns of the same color and different letters are statistically different ($p < 0.05$)

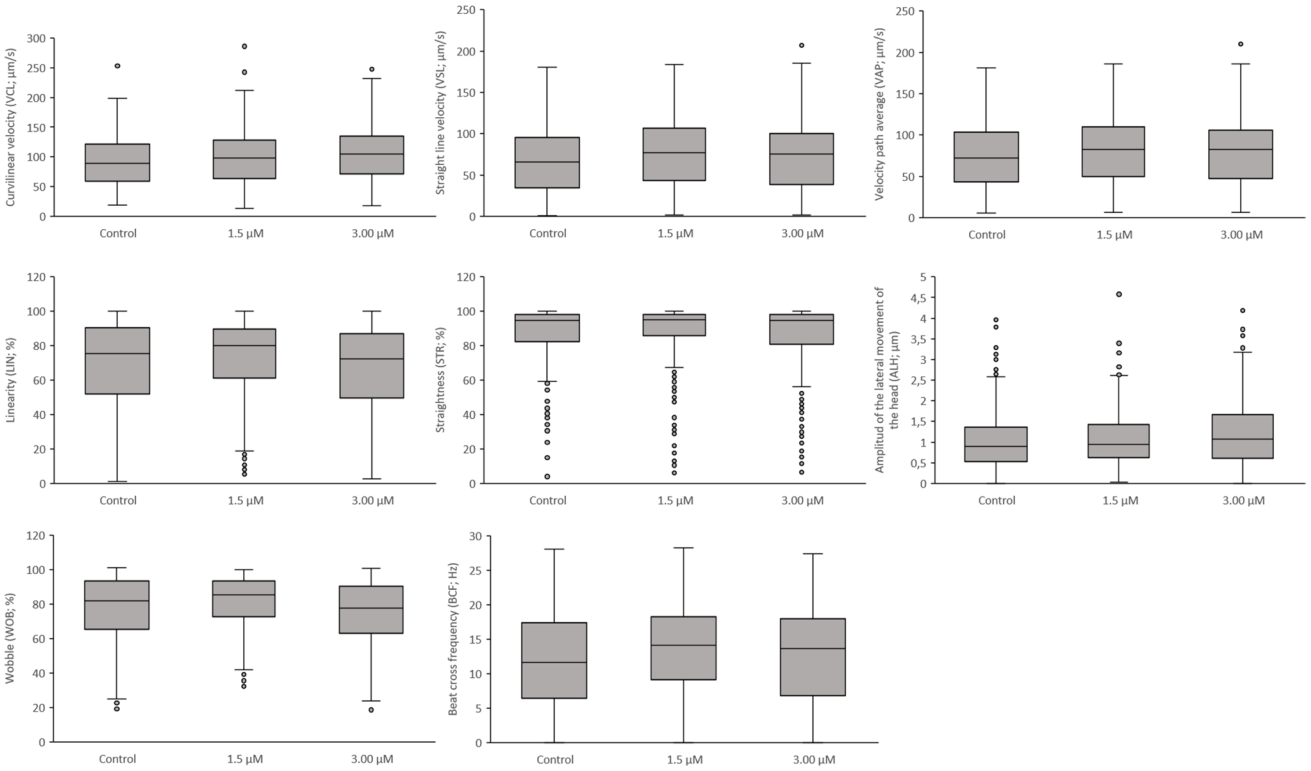

Fig. 2 Effect of TrxAFNIIx4His$_6$ addition on kinematic parameters (VCL, VSL, VAP, LIN, STR, WOB, ALH, and BCF) on the overall population. Spermatozoa were cryopreserved in an extender with or without TrxAFNIIx4His$_6$ (1.5 µM or 3.0 µM). Data represent mean ± SEM of three experiments

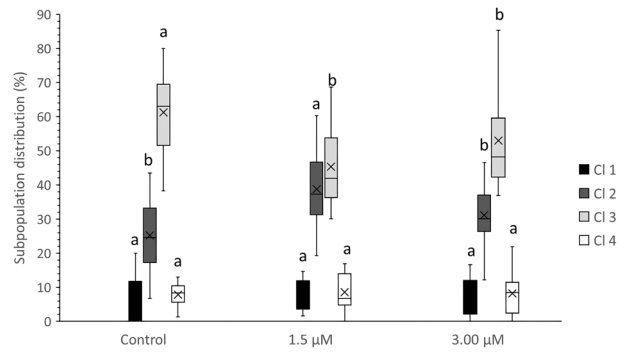

Fig. 3 Distribution of subpopulations in sperm cryopreserved with or without TrxAFNIIx4His$_6$ (1.5 µM or 3.0 µM). subpopulation 1, black bars; subpopulation 2, dark grey bars; subpopulation 3, light grey bars; subpopulation 4, white bars. Different letters indicate significant differences in subpopulations distribution between treatments ($p < 0.05$)

had higher total and progressive motility than sperm cryopreserved with control extender ($p = 0.0004$ and $p = 0.0016$, respectively). Sperm cryopreserved with 3.0 µM TrxAFNIIx4His$_6$ also had statistically greater motility than control sperm ($p = 0.028$), and, on the other side, progressive motility, although greater, was not statistically different from control ($p = 0.135$). Meanwhile, no differences were found between the two concentrations (1.5 and 3.0 µM) of TrxAFNIIx4His$_6$ evaluated (Fig. 1). Previously, we showed that same recombinant protein added at thawing increased ram sperm in vitro fertilization potential and decreased cryocapacitation signals without effects over motility (Ledesma et al. 2019).

TrxAFNIIx4His$_6$ is composed by a double FN type II domain, based on the structure of seminal plasma (SP) proteins RSVP14 and RSVP20. Barrios et al. (2005) reported that RSVP14 and RSVP20 increase the resistance of ram spermatozoa to cooling and cold-shock, preserving membrane integrity. It has been reported that a SP fraction that improves cryopreserved sperm quality is enriched in FNII containing proteins (Barrios et al. 2005; Muiño-Blanco et al. 2008). However, the protective effect of SP against the damage caused by exposure to low temperatures has shown great variability (Muiño-Blanco et al. 2008; Bernardini et al. 2011). As the benefits of any additive for freezing must be consistent, we and others have been working on the cloning and expression of recombinant components based on SP proteins with cryoprotective capacity. Serrano et al. cloned and expressed RSVP14 (2013) and RSVP20 (2015). However, the effects of the inclusion of these recombinant proteins in the freezing/thawing media have not been reported. We previously demonstrated that SPINK3, a mouse recombinant

decapacitation protein, prevented and reverted freezing damage and improved ram sperm motility (Zalazar et al. 2016, 2020). Variability in the results might be explained by the fact that the effect of the additives depends on the moment of incorporation (before or after freezing), concentration, and presence of egg yolk in the diluent (Ramírez-Vásquez et al. 2019).

The use of standard approaches for CASA data analysis might led to disappointing results, as the evaluation of mean values ± standard deviations hidden the behavior of subgroups of cells within the sample. That is to say that sometimes, differences produced by some treatments are hidden within average data. In the present study, no effect was observed over the average sperm kinematic parameters ($p > 0.05$) (Fig. 2). Thus, while some subpopulations might be affected by a treatment, other non-responding co-exist in the sample (Martínez-Pastor et al. 2011; Ledesma et al. 2016). Therefore, we used a sperm motility tracking software to detect the presence of subpopulations according to sperm motility characteristics and to evaluate the effect of TrxAFNIIx4His$_6$ on the distribution of the subpopulations. According to the motility characteristics of our sperm samples ($n = 2285$) and the best silhouette index found after the clustering procedure, four sperm subpopulations or clusters (CL) could be defined (Table 1). Subpopulation 1 (CL 1) was characterized by highly motile sperm with the highest velocities (VCL, VSL, and VAP), a bit undulatory and vigorous beating as indicated by the highest BCF value; subpopulation 2 (CL2) was characterized by the highest degree of progressiveness, inferred by LIN value and great velocity, however, less than CL1. ALH value was low, indicating movement with few undulatory characteristics; subpopulation 3 (CL3) contained poorly motile, almost non-active and non-progressive sperm, as indicated by the least values of VCL, VSL, VAP, ALH, and BCF, together with the least LIN, STR, and WOB values; subpopulation 4 (CL4) was represented by sperm with moderate velocities (medium VCL and VAP) and low progressiveness (low LIN, STR, and WOB). Sperm trajectories were less straight than CL1 and CL2. We also observed that addition of 1.5 μM of TrxAFNIIx4His$_6$ increased the proportion of motile and progressive sperm (CL2) and decreased the proportion of immotile sperm (CL3) in comparison to control ($p = 0.0017$ and $p = 0.0003$, respectively). Addition of 3.0 μM of TrxAFNIIx4His$_6$ decreased the proportion of sperm in CL3 ($p = 0.023$) and increased the proportion of CL2 compared with control, although statistically insignificant ($p = 0.219$) (Fig. 3). Curiously, unlike previous studies carried out in rams, we did not detect a subpopulation with hyperactive motility, characterized by active but non-progressive sperm. According to Mortimer and Maxwell (1999) hyperactive ram sperm are defined by VCL > 250.0 μm/s, VSL ≤ 100.0 μm/s, LIN ≤ 30%, and ALH > 9.0 μm. It is worth remembering that the term "hyperactivation" refers to a characteristic motility pattern that sperm acquire during the capacitation process (Ho and Suarez 2001). Therefore, we can infer that under our experimental conditions sperm are yet non-capacitated consistent with the fact that cryopreserved ram sperm capacitation requires at least 15 min incubation under capacitating conditions (Peris-Frau et al. 2020). TrxAFNIIx4His$_6$ caused an increase in the subpopulation composed by rapid and progressive sperm and a decrease in the subpopulation composed of almost non-active sperm. These findings corroborate our previous reports suggesting the protective effect of SP proteins is related to their ability to bind to the sperm membrane exerting a defense against the harmful cryopreservation process (Ledesma et al. 2016). Since motile and progressive sperm has more possibilities to reach the insemination site and fertilize an ovum (Li et al. 2016), this might result in higher fertile cryopreserved semen doses.

Fibronectin type II domains have the capacity to stabilize membranes through their interaction with cholinephospholipids (Manjunath and Therien 2002), and this stabilization, in turn, would increase the resistance to cryopreservation. In rams, the plasma membrane contains high levels of unsaturated phospholipids and low levels

Table 1 Descriptive parameters of the subpopulations (CL) identified in frozen-thawed ram sperm (mean ± SD; $n = 2285$)

Variable	CL 1	CL 2	CL 3	CL 4
VCL (μm/s)	158.77 ± 3.94	101.63 ± 17.43	1.46 ± 0.70	92.43 ± 9.71
VSL (μm/s)	115.70 ± 8.70	82.99 ± 10.52	0.19 ± 0.08	41.52 ± 5.97
VAP (μm/s)	124.65 ± 8.54	87.01 ± 12.01	0.56 ± 0.30	59.50 ± 6.72
LIN (%)	73.05 ± 3.95	80.18 ± 1.93	0.61 ± 0.22	40.37 ± 2.65
STR (%)	92.40 ± 1.06	94.03 ± 0.55	1.68 ± 0.54	63.40 ± 5.52
ALH (μm)	2.06 ± 0.11	1.01 ± 0.33	0.03 ± 0,01	1.44 ± 0.13
WOB (%)	78.73 ± 3.61	84.71 ± 1.65	1.46 ± 0.61	63.14 ± 6.98
BCF (Hz)	17.48 ± 0.91	13.36 ± 0.84	0.16 ± 0.07	14.39 ± 0.93

CL1 very fast/undulatory/linear, *CL2* fast/progressive/linear, *CL3* poorly motile, *CL4* slow/non-linear, *VCL* curvilinear velocity, *VSL* straight-line velocity, *VAP* average path velocity, *LIN* linearity, *STR* straightness, *WOB* wobble, *ALH* lateral head displacement, *BCF* beat cross frequency

of cholesterol, which declines the resistance to the freezing–thawing process (Darin-Bennett and White 1975). During freezing, phospholipids undergo a redistribution, and some of them change from a liquid-state to a gel-state earlier than others, resulting in a lipid-phase separation. In consequence, the lipid-protein interactions are disturbed, and some surface proteins are lost or translocated causing loss of function (Amann and Pickett 1987). Another possible mechanism is through antioxidant properties of the FNII domains, since Marti et al. (2007) reported an antioxidant potential of RSVP14 and RSVP20. Free radicals produced in excess during the freezing/thawing cause structural damage in sperm membranes and decrease motility (Aitken et al. 1993). Accordingly, supplementation of extenders with antioxidants such as vitamin E, cysteine, and carotenoids produced great improvements in sperm motility (Silva et al. 2013; Büyükleblebici et al. 2014; Zalazar et al. 2019).

In conclusion, addition of TrxAFNIIx4His$_6$ to egg yolk-based freezing extender increased ram sperm motility and shows potential for the development of cryoprotection techniques which may lead to improved fertility after artificial insemination. Future studies are needed to understand the effect of molecular and cellular mechanisms involved in sperm-TrxAFNIIx4His$_6$ interaction and the impact over in vivo fertility.

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Author contribution Conception and design of the work: Alba Ledesma, Andreina Cesari. Data collection: Alba Ledesma, Lucía Zalazar, Micaela Greco. Data analysis and interpretation: Alba Ledesma, Lucía Zalazar, Andreina Cesari. Drafting the article: Alba Ledesma. Critical revision of the article: Andreina Cesari, Federico Hozbor.

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Data availability Not applicable

Code availability Not applicable

Declarations

Ethics approval All procedures involving animals were in accordance with the good animal practice and conditions reviewed and approved by the Animal Ethics Committee of the Instituto Nacional de Tecnología Agropecuaria, Argentina (Protocol ID 156/2018).

Conflict of interest The authors declare no competing interests.

References

Aitken, R., Buckingham, D., Harkiss, D., 1993. Use of a xanthine oxidase free radical generating system to investigate the cytotoxic effects of reactive oxygen species on human spermatozoa. Reproduction, 97, 441-50.

Amann, R., Pickett, B., 1987. Principles of cryopreservation and a review of cryopreservation of stallion spermatozoa. Journal of Equine Veterinary Sciences, 7, 145-173.

Barrios, B., Fernández-Juan, M., Muiño-Blanco, T., Cebrián-Pérez, J. A., 2005. Immunocytochemical localization and biochemical characterization of two seminal plasma proteins that protect ram spermatozoa against cold shock. Journal of Andrology, 26, 539-549.

Bernardini, A., Hozbor, F., Sanchez, E., Fornes, M., Alberio, R., Cesari, A., 2011. Conserved ram seminal plasma proteins bind to the sperm membrane and repair cryopreservation damage. Theriogenology, 76, 436-447.

Buchelly-Imbachí, F., Zalazar, L., Pastore, J., Greco, M., Iniesta-Cuerda, M., Garde, J., Soler, A., Ballarin, V., Cesari, A., 2018. Objective evaluation of ram and buck sperm motility by using a novel sperm tracker software. Reproduction, 156, 11-21.

Büyükleblebici, S., Tuncer, P., Bucak, M., Eken, A., Sarıözkan, S., Taşdemir, U., Endirlik, B., 2014. Cryopreservation of bull sperm: Effects of extender supplemented with different cryoprotectants and antioxidants on sperm motility, antioxidant capacity and fertility results. Animal Reproduction Science, 150, 77-83.

Darin-Bennett, A., White, I., 1975. Cholesterol and phospholipid content of mammalian spermatozoa and its relation to membrane structure and cold shock. Journal of Reproduction and Fertility, 2, 383-384.

Donnelly, E. T., Lewis, S. E., McNally, J. A., Thompson, W. 1998. In vitro fertilization and pregnancy rates: the influence of sperm motility and morphology on IVF outcome. Fertility and sterility, 70, 305-314.

Gage, M. J., Macfarlane, C. P., Yeates, S., Ward, R. G., Searle, J. B., Parker, G. A. 2004. Spermatozoal traits and sperm competition in Atlantic salmon: relative sperm velocity is the primary determinant of fertilization success. Current biology, 14, 44-47.

Gomendio, M., Roldan, E. 2004. Implications of diversity in sperm size and function for sperm competition and fertility. International Journal of Developmental Biology, 5,6, 439-447.

Ho, H., Suarez, S., 2001. Hyperactivation of mammalian spermatozoa: function and regulation. Reproduction, 122, 519-526.

Ledesma, A., Manes, J., Ríos, G., Aller, J., Cesari, A., Alberio, R., Hozbor, F., 2015. Effect of Seminal Plasma on Post-Thaw Quality and Functionality of Corriedale Ram Sperm Obtained by Electroejaculation and Artificial Vagina. Reproduction in Domestic Animals, 50, 386-392.

Ledesma, A., Fernández-Alegre, E., Cano, A., Hozbor, F., Martínez-Pastor, F., Cesari A. 2016. Seminal plasma proteins interacting with sperm surface revert capacitation indicators in frozen-thawed ram sperm. Animal reproduction science, 173, 35-41.

Ledesma, A., Zalazar, L., Fernández-Alegre, E., Hozbor, F., Cesari, A., Martínez-Pastor, F. 2017. Seminal plasma proteins modify the distribution of sperm subpopulations in cryopreserved semen of rams with lesser fertility. Animal Reproduction Science, 184, 44-50.

Ledesma, A., Zalazar, L., Buchelly-Imbachi, F., Pastore, J. I., Brown, P., Eddy, E. M., Hozbor F, Cesari, A., 2019. Recombinant peptide reverses cryo-capacitation in ram sperm and improves in vitro fertilization. Animal Reproduction Science, 207, 61-72.

Li Y., Kalo D., Zeron Y., Roth, Z., 2016. Progressive motility a potential predictive parameter for semen fertilization capacity in bovines. Zygote, 24, 70-82.

Manjunath, P., Therien, I., 2002. Role of seminal plasma phospholipid-binding proteins in sperm membrane lipid modification that occurs during capacitation. Journal of Reproductive Immunology, 53, 109-119.

Marti, E., Mara, L., Marti, J., Muiño-Blanco, T., Cebrián-Pérez, J., 2007. Seasonal variations in antioxidant enzyme activity in ram seminal plasma. Theriogenology, 67, 1446-1454.

Martínez-Pastor, F., Tizado, E., Garde, J., Anel, L., de Paz, P., 2011. Statistical series: opportunities and challenges of sperm motility subpopulation analysis. Theriogenology, 75, 783-795.

Morrell, J., Rodríguez-Martínez, H., 2008. Biomimetic techniques for improving sperm quality in animal breeding: a review. The Open Andrology Journal, 1, 1-9.

Mortimer, S.T., Maxwell, W.M., 1999. Kinematic definition of ram sperm hyperactivation. Reproduction Fertility and Development, 11, 25-30.

Muiño-Blanco, T., Pérez-Pé, R., Cebrián-Pérez, J., 2008. Seminal plasma proteins and sperm resistance to stress. Reproduction in Domestic Animals, 43, 18-31.

Peris-Frau, P., Martín-Maestro, A., Iniesta-Cuerda, M., Sánchez-Ajofrín, I., Cesari, A., Garde, J., Villar, M., Soler, A., 2020. Cryopreservation of ram sperm alters the dynamic changes associated with in vitro capacitation. Theriogenology, 145, 100-108.

Quintero-Moreno, A., Rigau, T., Rodríguez-Gil, J., 2007. Multivariate cluster analysis regression procedures as tools to identify motile sperm subpopulations in rabbit semen and to predict semen fertility and litter size. Reproduction in Domestic Animals, 42, 312-319.

Ramírez-Vásquez, R., Cano, A., Hozbor, F., Cesari, A., 2019. Cryopreservation and egg yolk extender components modify the interaction between seminal plasma proteins and the sperm surface. Theriogenology, 140, 153-163.

Rousseeuw P., 1987. Silhouettes: A graphical aid to the interpretation and validation of cluster analysis. Journal of Computational and Applied Mathematics, 20, 53-65.

Serrano, E., Pérez-Pé, R., Calleja L., Guillén, N., Casao, A., Hurtado-Guerrero, R., Muiño-Blanco, T., Cebrián-Pérez, J., 2013. Characterization of the cDNA and in vitro expression of the ram seminal plasma protein RSVP14. Gene, 519, 271-278.

Serrano, E., Martínez, A., Arruga, D., Pérez-Pé, R., Sánchez-Ferrer, A., Muiño-Blanco, T., Pérez-Pé, J., 2015. New insights into the phylogeny and gene context analysis of binder of sperm proteins (BSPs). Plos One. 10, e0137008.

Silva, S., Soares, A., Batista, A., Almeida, F., Nunes, J., Peixoto, C., Guerra, M., 2013. Vitamin E (Trolox) addition to Tris-egg yolk extender preserves ram spermatozoon structure and kinematics after cryopreservation. Animal Reproduction Science, 137, 37-44.

Weinberger, K., Saul, L.K., 2009. Distance metric learning for large margin nearest neighbor classification. Journal of Machine Learning Research, 10, 207–244.

Yeste, M. 2016. Sperm cryopreservation update: Cryodamage, markers, and factors affecting the sperm freezability in pigs. Theriogenology, 85, 47-64.

Zalazar, L., Ledesma, A., Hozbor, F., Cesari, A., 2016. Heterologous recombinant protein with decapacitating activity prevents and reverts cryodamage in ram sperm: An emerging biotechnological tool for cryobiology. Animal Reproduction Science, 164, 31-39.

Zalazar, L., Pagola, P., Miró, M., Churio, M., Carleti, M., Martínez, C., Iniesta-Cuerda, M., Soler, A., Cesari, A., De castro, R., 2019. Bacterioruberin extracts from a genetically modified hyperpigmented Haloferax volcanii strain: antioxidant activity and bioactive properties on sperm cells. Journal of Applied Microbiology, 126, 796-810.

Zalazar, L., Iniesta-Cuerda, M., Sánchez-Ajofrín, I., Garde, J. J., Valls, A. J. S., Cesari, A., 2020. Recombinant SPINK3 improves ram sperm quality and in vitro fertility after cryopreservation. Theriogenology, 144, 45-55.