Indian Journal of Animal Sciences **92** (7): 846–848, July 2022/Short Communication
https://doi.org/10.56093/ijans.v92i7.122941

Novel Cold-Inducible RNA-Binding Protein (CIRBP) in goat spermatozoa: A possible cryo-stress reliever during the process of Cryopreservation

A ARANGASAMY[1]✉, S BACKIALAKSHMI[1], J N SHARANYA[1], M VENKATA KRISHNAIAH[1], S SELVARAJU[1], K S ROY[1], I J REDDY[1] and R BHATTA[1]

ICAR-National Institute of Animal Nutrition and Physiology, Bengaluru, Karnataka 560 030 India

Received: 1 April 2022; Accepted: 11 May 2022

Keywords: Bucks, CIRBP, Copper, Cryopreservation, 2D Gel, MALDI-TOF, Spermatozoa, Western blotting, Zinc

The success of the cryopreservation depends on the revival of the spermatozoa motility after thawing process. Various *in vivo* or *in vitro* approaches have been made for the past few decades to retain spermatozoa motility at maximum level. The *in vivo* method, consists of supplementation of mineral, proteins, high energy diet to male animals and enhancement of semen characteristic prior to collection of semen samples (Arunachalam *et al.* 2018; Hemalatha *et al.* 2018; Narasimhaiah *et al.* 2018). In the *in vitro* method, various types of semen additives are included during the dilution and equilibration period and prior to cryopreservation process (Dance *et al.* 2016, Arangasamy *et al.* 2018, Mayasula *et al.* 2022). The obtained results and success of the *in vivo* and *in vitro* approaches are variable at a larger scale. The major problems in cryopreservation process are cryodamage to cells and reduction in morphological and physical properties of the spermatozoa at cryopreservation (Yash Pal *et al.* 2011). Semen additives could be cryoprotectants, antioxidants, fatty acids, antifreeze proteins (AFP), animal serum, nanoparticles, plant essential oils, amino acids, seminal plasma protein (Sangeeta *et al.* 2015, Himanshu B 2019). Inclusion of these kind of additives at the time of semen dilution in fresh or frozen semen has promised to enhance the quality of frozen spermatozoa with more intact sperm plasma/ acrosome membrane after cryopreservation (Sangeeta *et al.* 2015) as defensive strategies. In offensive approaches, researchers have experimented with application of stress pre-conditioning of spermatozoa before cryopreservation (Horváth *et al.* 2016, Hezavehei *et al.* 2016), hence the application of sub-lethal stress in spermatozoa may induce a general adaptation and increased resistance to various future stresses (Pribenszky *et al.* 2011). This study was conducted to confirm the MALDI-TOF identified CIRBP protein via Western blotting using the low abundant protein in high organic Zinc supplemented group of bucks.

Experimental plan and 2 D gel electrophoresis and Immunoblotting: This is the continuation of previously published work in cryobiology (Arunachalam *et al.* 2018, Mayasula *et al.* 2022, B. Sekar *et al.* 2021). The 2D Gel analysed differentially expressed protein localized and identified through MALDI-TOF (methodology followed as discussed by Mayasula *et al.* 2022). Briefly the 2-DE was carried out by re-suspending 60 µg protein in 125 µl rehydration buffer (0.2% biolyte ampholyte, 2 M thiourea, 50 mM dithiothreitol, 8 M urea, 2% CHAPS and added to immobilized *p*H gradient (IPG) strip (7 cm, 4–7 pI) (Biorad), followed by rehydration (19 h, 50 V at 20°C) (Protean IEF cell, Bio-Rad). After rehydration, electro focussing was carried out at 250 V for 2 h, 500 V for 1 h, 1000 V for 1 h, 2000 V for 1 h, 4000 V for 1 h, and 8000 V until it reaches 16,000 Vh. After this the IPG strips were equilibrated with equilibration buffer (57 mM Tris–HCl *p*H 8.8, 6 M urea, 2% SDS, and 29.3% glycerol) containing 30 mg/ml DTT and equilibration buffer containing 30 mg/ml iodoacetamide for 25 min each. The equilibrated strips were subjected to SDS-PAGE (12.5%) and focussed at 100 V. Silver nitrate staining was carried out (Gromova and Celis 2006), and the images were captured in the gel documentation system by gene snap image acquisition software (G-BOX, Syngene, UK). The analysis of differential spots between control and treatment groups was carried out by using the Dymension tool (Syngene, UK). The differential spots identified was excised (@ 60 mg/kg bodyweight DM basis organic Zn supplemented group bucks had poor initial semen quality as well as very low freezability along with low quantity of protein), digested, extracted, and submitted for mass spectrometric analysis for the identification of protein. The mass to charge ratio were uploaded to Mascot search engine (https://www.matrixscience.com/) and the protein was identified as cold-inducible RNA-binding protein isoform X3, NCBIprot 20191120 database, molecular weight 26.95 kDa, protein score 279. The identified cold inducible RNA binding protein was later confirmed with the Western Blotting method. Protein samples from all the 10 treatment groups (10 µg/well) were denatured at 95°C for 5 min and loaded on SDS-PAGE. The separated proteins were subjected to

Present address: [1]ICAR-National Institute of Animal Nutrition and Physiology, Bengaluru, Karnataka. ✉Corresponding author email: aarangasamy@rediffmail.com

western blotting by transferring to nitrocellulose membrane at 20V, 70 mA, and 8.01 hlm for three and half hours. After transfer, the blot was blocked using 5% Bovine serum albumin (BSA) in Tris-buffered saline-Tween 20 (TBST) for 1 h at 4°C. Blot was incubated with CIRBP primary antibody (monoclonal mouse Anti-CIRBP, Sigma-Aldrich, USA) of 1:1000 dilution at 4°C overnight. The membrane was washed thrice using TBST and HRP conjugated secondary antibody (1:5000 dilution) was added and incubated at RT for 2 h. The membranes were washed and incubated with ECL substrate (Immobilin® ECL substrate, Merck, Germany) and images were captured using Chemi-documentation system (Fig.1) (iChemi XR, Syngene, UK). The immunoblot was quantified using Image J software (Fig. 2).

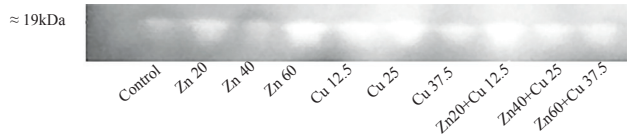

Fig. 1. Immunoblotting of CIRBP in goat spermatozoa of ten experimental groups.

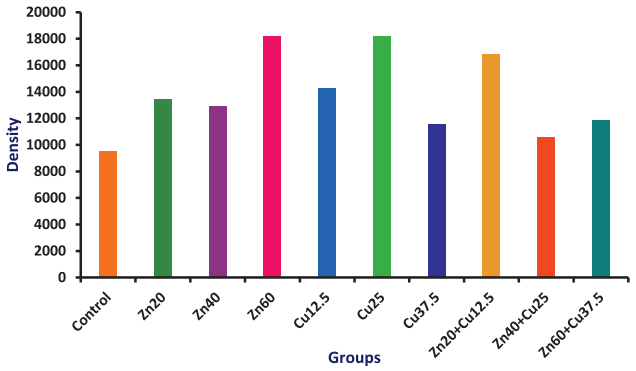

Fig. 2. Quantification of immunoblot using Image J software.

The reproductive physiology laboratory at ICAR-National Institute of Animal Nutrition and Physiology, Bengaluru, India, identified a protein with a molecular weight ≈ 19 kDa in goat spermatozoa through Mass Spectrometry (MALDI-TOF). The ≈ 19 kDa protein later confirmed (Western Blotting) as a cold-inducible RNA-binding protein (CIRBP). The CIRBP is expressed in testes and its expression level goes down or up depending on testes exposure to stress condition (Masuda *et al.* 2012, Xia *et al.* 2012). Recent studies indicate that, CIRBP protein is involved in the cellular response to oxidative stress inducer, cancer cells and in spermatogenesis (Nishiyama *et al.* 1998). In cancer patients, it was noticed that it inhibits the cell growth or involved in the cellular stress and in hypothermic conditions, it prevents the cold-induced delay of cell proliferation (Roilo *et al.* 2018). Antifreeze glycoproteins (AFGP) and Antifreeze proteins (AFP) are natural biological antifreeze molecules found in various species across the globe. It is believed that AFP and AFGP can help to preserve the integrity of cellular membranes during sperm freezing. In our previous mineral (Zn and Cu) supplementation studies related to puberty onset in bucks, we identified that the sperms of T4 (Zn 60 mg on kg- DM) group semen had lost their viability at the end of equilibration (4 hr) and after cryopreservation (Arunachalam *et al.* 2018, Arangasamy *et al.* 2018, Mayasula *et al.* 2022). Initially it was believed that this could be due to the deleterious effect of higher Zn on the spermatozoa plasma membrane or accelerated cold shock induced damage to spermatozoa. Later, confirmed that the missing/ low expressed protein in the T4 group (differential expression among various Zn, Cu and Zn and Cu combination group) was CIRBP (Western Blotting confirmed) protein. The expression level of this protein has role in spermatozoa cryopreservation failure during pre-freeze and post-thaw stage (Arunachalam *et al.* 2018). This is the first report, presence of CIRBP in spermatozoa and its importance in cryopreservation, and maintaining the viability of spermatozoa. Our finding was in similar lines with the recent work on rooster spermatozoa, that the CIRBP expression pattern varies depending upon exposure to the type of stress and subsequent upregulation and down regulations (Qi XL *et al.* 2020). This protein has the novelty towards minimizing the cryoinjury and the success of the cryopreserved semen in terms of post-thaw seminal characteristics. Similarly, as per our results, CIRBP mimics the Antifreeze glycoproteins (AFGP) and Antifreeze proteins (AFP) in terms of the possible beneficial effects towards freezing spermatozoa, however it has not been elucidated in detail. Use of CIRBP as a cryopreservation semen additive for preserving spermatozoa from different species still needs to be investigated further.

SUMMARY

The great expectation of the dairy owners from the frozen semen industry is quality semen with high fertility. Many researchers concentrate in this area to produce frozen semen of high quality and fertility. However, the problem still exists to minimize or reduce the cryoinjury related damages that occur during the process of cryopreservation with the use of all kinds of semen additives. The success rate of the additives added semen is variable at a larger scale. Therefore, any new molecules which can effectively prevent or minimize the cryoinjury are warranted and it could be low cost substances. Our incidental finding and protein identification with molecular weight ≈ 19 kDa (cold-inducible RNA-binding protein CIRBP) in goat spermatozoa seems to be effective in minimizing cryoinjury. The identified CIRBP was confirmed via Western blotting. In future, further studies are needed to confirm the molecular mechanism of the CIRBP on the cryoinjury as semen additives, and application of this protein added semen in the field to improve the level of conception rate in cattle.

ACKNOWLEDGEMENTS

The authors acknowledge Director, ICAR-NIANP, Bengaluru, India for permitting the research at the institute. We acknowledge the Director, Department of

Biotechnology, Government of India for funding the project (BT/PR10901/AAQ/1/581/2014).

REFERENCES

Arangasamy A, Venkata Krishnaiah M, Manohar N, Selvaraju S, Guvvala P R, Soren N M, Reddy I J, Roy K S and Ravindra J P. 2018. Advancement of puberty and enhancement of seminal characteristics by supplementation of trace minerals to bucks. *Theriogenology* **110**: 182–91.

Arunachalam A, Krishnaiah M V, Manohar N, Selvaraju S, Rani G P, Soren N M, Reddy I J and Ravindra J P. 2018. Cryoprotective role of organic Zn and Cu supplementation in goats (*Capra hircus*) diet. *Cryobiology* **81**: 117–24.

Dance A, Thundathil J, Blondin P and Kastelic J. 2016. Enhanced early-life nutrition of Holstein bulls increases sperm production potential without decreasing postpubertal semen quality. *Theriogenology* **86**: 687–94.e2.

Gromova I and Celis J E. 2006. Protein detection in gels by silver staining: A procedure compatible with mass spectrometry. *Cell Biology* **4**: 219–23.

Hemalatha K, Arangasamy A, Selvaraju S, Krishnaiah M V, Rani G P, Mishra A, Soren N B, Reddy I J and Ravindra J P. 2018. Effect of dietary supplementation of organic zinc and copper on *in vitro* semen fertility in goat. *Small Ruminant Research* **161**: 68–72.

Hezavehei M, Sharafi M, Kouchesfahani H M, Henkel R, Agarwal A, Esmaeili V and Shahverdi A. 2018. Sperm cryopreservation: A review on current molecular cryobiology and advanced approaches. *Reproductive Biomedicine Online* **37**: 327–39.

Himanshu B. 2019. Dietary flaxseed and coconut oil supplementation effect on semen profile and hormone level in adult ram. IVRI, Izatnagar, Bareilly, Uttar Pradesh.

Horváth A, Szenci O, Nagy K, Végh L and Pribenszky C. 2016. Stress preconditioning of semen before cryopreservation improves fertility and increases the number of offspring born: A prospective randomised study using a porcine model. *Reproduction, Fertility and Development* **28**: 475–81.

Masuda T, Itoh K, Higashitsuji H, Hisako H, Nakazawa N, Sakurai T, Liu Y, Tokuchi H, Fujta T, Zhao Y, Nishiyama H, Tanaka T, Fukumoto M, Ikawa M,Okabe M and Fujita J. 2012. Cold-inducible RNA-binding protein (Cirp) interacts with Dyrk1b/Mirk and promotes proliferation of immature male germ cells in mice. *Proceedings of the National Academy of Science* **109**: 10885–90.

Mayasula V K, Arangasamy A, Selvaraju S, Guvvala P R and Ghosh J. 2022. Organic Zinc and Copper supplementation-associated changes in gene expression and protein profiles in Buck spermatozoa. *Biological Trace Elements Research* **200**: 1626–39.

Narasimhaiah M, Arunachalam A, Sellappan S, Krishnaiah M V, Rani G P, Ghosh S, Chandra V, Ghosh J and Kumar H. 2018. Organic zinc and copper supplementation on antioxidant protective mechanism and their correlation with sperm functional characteristics in goats. *Reproduction in Domestic Animals* **53**: 644–54.

Nishiyama H, Danno S, Kaneko Y, Itoh K, Yokoi H, Fukumoto M, Mill'an J L, Matsuda T, Yoshida O and Fujita J. 1998. Decreased expression of cold-inducible RNA-binding protein (CIRP) in male germ cells at elevated temperature. *American Journal of Pathology* **152**: 289.

Pribenszky C, Horváth A, Végh L, Huang S-Y, Kuo Y-H and Szenci O. 2011. Stress preconditioning of Boar spermatozoa: A new approach to enhance semen quality. *Reproduction in Domestic Animals* **46**: 26–30.

Qi X L, Xing K, Huang Z, Chen Y, Wang L, Zhang L C, Sheng X H, Wang X G, Ni H M and Guo Y 2020. Comparative transcriptome analysis digs out genes related to antifreeze between fresh and frozen–thawed rooster sperm. *Poultry Science* **99**: 2841–51.

Roilo M, Kullmann M K and Hengst L. 2018. Cold-inducible RNA-binding protein (CIRP) induces translation of the cell-cycle inhibitor p27Kip1. *Nucleic Acids Research* **46**: 3198–210.

Sangeeta S, Arangasamy A, Kulkarni S and Selvaraju S 2015. Role of amino acids as additives on sperm motility, plasma membrane integrity and lipid peroxidation levels at pre-freeze and post-thawed ram semen. *Animal Reproduction Science* **161**: 82–8.

Sekar B, Arangasamy A, Naidu S J, Reddy I J and Bhatta R. 2021. Organic mineral supplementation on differential protein profile of Osmanabadi bucks (*Capra hircus*). *Reproductive Biology* **21**: 100533.

Xia Z, Zheng X, Zheng H, Liu X, Yang Z and Wang X. 2012. Cold-inducible RNA-binding protein (CIRP) regulates target mRNA stabilization in the mouse testis. *FEBS Lett* **586**: 3299–308.

Yash Pal, A Arangasamy, R A Legha, J Singh, R S Bansal, S K Khurana and Tandon S N. 2011. Freezability and fertility of Marwari stallion semen. *Indian Journal of Animal Science* **81**: 445–92.