Pakistan J. Zool., pp 1-3, 2021. **DOI**: https://dx.doi.org/10.17582/journal.pjz/20190422070439

Short Communication

Effect of Glutathione and L-Cysteine Supplementation on the Quality of Frozen Beetal Buck Spermatozoa

Muhammad Hammad Fayyaz*, S. Murtaza Hassan Andrabi and Muhammad Shafiq Haider

Animal Reproduction and Genetics Program, Animal Sciences Institute, National Agricultural Research Centre, PARC, Islamabad 44000

Article Information
Received 22 April 2019
Revised 30 May 2020
Accepted 22 April 2021
Available online 13 December 2021 (early access)

Authors' Contributions
MHF preformed the research, analyzed the data and prepared the manuscript. SMHA edited the manuscript and supervised the overall research. MSH designed the research and assisted in analyzing the data.

Key words
Buck semen, Cryopreservation, Cryo-injuries, Oxidative stress.

ABSTRACT

This study was designed to assess the cryosurvival of beetal buck spermatozoa with the supplementation of Glutathione (GLU) and L-cysteine (CYS) in the extender. In the first experiment, semen samples from each of the five mature bucks were pooled and diluted in Tris-citric acid (TCA) extender containing Glutathione (0.5mM, 1mM), L-cysteine (5mM CYS, 10mM CYS), and control. In experiment 2, semen samples (n=5) were diluted in TCA extender containing differential doses of combined antioxidants (0.5 mM GLU+ 5mM CYS, 0.5mM + 10mM CYS, 1mM GLU + 5mM CYS, 1mM GLU + 10mM CYS and Control). At post-thawing, progressive motility, plasma membrane integrity (PMI), and livability of buck spermatozoa were higher ($P< 0.05$) with 1mM GLU and 10mM CYS as compared to control. In the second experiment, post-thaw progressive motility, PMI, and livability were higher ($P< 0.05$) with 1mM GLU + 10mM CYS and 1mM GLU + 5mM CYS compared to other doses of GLU+CYS and control. We concluded that optimized supplementation of Glutathione and L-cysteine in TCA extender ameliorated frozen-thawed quality of beetal buck spermatozoa.

Artificial insemination (AI) is one of the major assisted reproductive techniques used to impregnate the female without direct involvement of the male. It offers a low cost and easy way to disseminate desired valuable genetic factors (Leboeuf *et al*., 2000). Breeding programs of goat majorly depend upon AI of frozen-thawed spermatozoa (Andrabi *et al*., 2016). Success in fertilization is the main goal of artificially incorporated cryopreserved spermatozoa in the female reproductive tract (Purdy, 2006). Although, cryopreservation process is an efficient technique to store spermatozoa for long periods but its initiated injuries are also harmful (Andrabi, 2009). Oxidative stress is one of the major injuries faced by spermatozoa during the freeze-thawing process (Fayyaz *et al*., 2017). Reactive oxygen species (ROS) are highly reactive molecules that are normally produced during cellular respiration (Aitken *et al*., 2016). The rate of ROS generation is high during the freeze-thaw process (Shah *et al*., 2017) which compromises fertilization potential spermatozoa by affecting its various qualities *i.e.* motility, acrosomal integrity, plasma membrane integrity, mitochondrial membrane potential, and DNA integrity (Baumber *et al*., 2000). Buck sperm also constitutes a high amount of polyunsaturated fatty acids (PUFA) (Dolatpanah *et al*., 2008) which are more susceptible to lipid peroxidation (Shah *et al*., 2017) leading to ROS generation (Alvarez and Storey, 1995). Therefore, a powerful exogenous antioxidant system should be required to avoid cryoinjuries and lipid peroxidation (Irvine, 1996). In this regard, the addition of antioxidants in cryodiluent is considered helpful to improve the frozen-thawed quality of spermatozoa (Rao *et al*., 2013).

Glutathione is the biological antioxidant that normally exists in semen. Moreover, exogenous supplementation of glutathione has also been reported to provide intracellular defense against oxidative stress (Salmani *et al*., 2013). Interestingly, cysteine is the main component of glutathione, which has also been reported to prevent lipid peroxidation in spermatozoa (Topraggaleh *et al*., 2014). Cysteine also consists of thiol groups, which individually act as a non-enzymatic antioxidant and easily penetrates the sperm (Çoyan *et al*., 2011).

There is no comprehensive data available on the combined useful effects of glutathione and L-cysteine on post-thaw sperm quality of buck. Therefore, the present study was designed to determine the optimal combination of glutathione and L-cysteine in extender, to improve the post-thaw quality of beetal buck spermatozoa.

* Corresponding author: hammad.fayyax@gmail.com
0030-9923/2021/0001-0001 $ 9.00/0

Table I.- Motility, plasma membrane integrity (PMI) and liveability of frozen thawed beetal buck spermatozoa supplemented with different concentrations of glutathione (GLU) and L-cysteine (CYS). Values are the mean±S.E.M. of five treatment groups. Means with different superscripts are significantly different (P<0.05).

	Motility	PMI	Livability
Experiment 1			
Control	35.00±2.00^{A}	44.73±1.42^{A}	49.37±1.55^{A}
0.5mM GLU	40.00±2.00^{B}	46.93±1.32^{AB}	52.82±1.07^{AB}
1mM GLU	45.00±1.00^{C}	49.80±0.879^{B}	52.73±1.55^{AB}
5mM CYS	45.00±1.20^{C}	53.18±0.871^{C}	55.19±0.93^{B}
10mM CYS	48.00±1.58^{C}	55.46±1.11^{C}	55.18±1.40^{B}
Experiment 2			
Control	40.50 ± 1.48^{A}	51.65±1.90^{A}	55.45± 1.40^{A}
0.5mM GLU + 5mM CYS	55.00 ± 1.48^{B}	57.11±1.76^{B}	62.12± 1.55^{B}
0.5mM GLU + 10mM CYS	55.50 ± 1.48^{B}	58.61± 1.72^{B}	64.50± 1.53^{C}
1mM GLU + 5mM CYS	58.50 ± 1.40^{BC}	63.90± 1.70^{C}	67.50± 1.60^{D}
1mM GLU + 10mM CYS	60.00 ± 1.40^{BC}	65.00± 1.70^{C}	68.30± 1.60^{D}

Materials and methods

Semen from Five healthy beetal bucks each buck was collected twice a week (for 6 weeks) using an artificial vagina at 42°C. Collected samples with more than 60% motility and 1.5 × 109 sperm concentration per mL were pooled and subdivided into five aliquots. Each aliquot was diluted with tris-citric extender [Tris 3.07 g/100mL, citric acid 1.64 g/100mL, fructose 1.26 g/100mL, 1000 IU/mL streptomycin sulfate, 5 mL of glycerol (v/v); 15mL of egg yolk (v/v)] containing different concentrations of glutathione (0.5mM GLU, 1mM GLU) and L-cysteine (5mM CYS, 10mM CYS) alone and in combination *viz*. 0.5mM GLU+5mM CYS, 0.5mM GLU+10mM CYS, 1mM GLU+5mM CYS, 1mM GLU+10mM CYS.

For freezing, extended sperm suspensions were cooled from 37°C gradually to 4°C in 90 min and equilibrated at 4°C for 2 h in the cold cabinet and packaged in 0.54 mL French straws (50 million sperm/straw). Packaged straws were placed horizontally on racks in cold cabinet then held at liquid nitrogen vapors 4 cm higher than the level of liquid nitrogen for 10 min followed by immersing in liquid nitrogen at -196°C for storage.

For post-thaw sperm evaluation assays, motility, plasma membrane integrity (PMI), and livability were considered as criteria. Motility analysis was performed according to Gillan *et al*. (2008). PMI of spermatozoa was analyzed by hypo-osmotic swelling (HOS) assay as described by Correa and Zavos (1994). Post-thaw livability was assessed by eosin–nigrosine staining. A total of 200 spermatozoa were counted in different fields of the phase-contrast microscope. Spermatozoa gaining partial or complete stain were recorded non-viable, and spermatozoa with unstained heads were considered as viable.

All data were expressed as mean with the standard error of the mean (SEM) among different treatment groups. One-way analysis of variance (ANOVA) was performed for data analysis and the Tukey test as post-hoc were performed on SPSS (version 20.0). The probability level of $P < 0.05$ was considered significant.

Results

Table I shows the effects of differential concentrations of GLU and CYS (combined or separate) on post-thaw progressive motility, PMI, and livability. All sperm parameters were higher (P<0.05) in 1mM GLU, 5mM CYS and 10mM CYS groups than control. The highest sperm parameters were observed in the 10mM CYS group. The highest motility was observed in 1mM GLU + 10mM CYS and 1mM GLU + 5mM CYS groups. A similar trend was seen in the case of livability and PMI, in which high concentration groups exhibited significantly higher results as compared to control and other groups.

Discussion

The plethora of ROS during the freeze-thaw process affects sperm quality parameters, compromising its survival, declining its internal antioxidant defense system, and fertilization potential. Frozen sperm is more prone to lipid peroxidation as compared to fresh sperm.

This is the very first study to evaluate the combined effect of GLU and CYS on the post-thaw quality of buck spermatozoa. In this study, supplementation of two antioxidants in different combinations significantly improved the sperm quality in terms of motility, PMI, and livability. Our results are in agreement with the study conducted in bovine semen (Sattar *et al*., 2016). However, these findings contradicted the studies conducted in ram (Zhandi and Sharafi, 2015) and buck (Salmani *et al*., 2013)

in which inclusion ratios of GLU were high (5mM and 10mM). This may be due to mitochondrial DNA degradation is associated with high GLU concentration (Abdi-Benemar *et al*., 2015). Also, it has been studied that the high levels of antioxidants negatively affect plasma membrane functionality (Salmani *et al*., 2013). Therefore, improving the effects of GLU on PM and other parameters may be due to its low inclusions which affected the functionality of the plasma membrane. Results of our study agreed with the findings of Zhandi *et al*. (2015) who reported that low concentrations of GLU in freezing extender improved the frozen-thawed sperm qualities of buck.

Cysteine is a well-known ROS scavenger. In this study addition of CYS in extender also positively affected motility, plasma membrane integrity, and livability agreeing with the studies performed in bull (Topraggaleh *et al*., 2014) ram (Çoyan *et al*., 2011), and buck (Memon *et al*., 2011).

However, this is the first study on the combined effect of two related antioxidants tested on frozen-thawed buck sperm quality. Our findings were consistent with the same study in ram. This discrepancy may be due to the difference in antioxidant concentrations used and semen characteristics of the two species. Our results indicated that the optimum combination of GLU and CYS in freezing extender can protect buck sperm from cryoinjuries.

In conclusion, this study exhibited that glutathione and L-cysteine at the concentration of 1mM and 10mM may be the optimum combination in extender to ameliorate cryopreservation of Beetal buck spermatozoa.

Statement of conflict of interest

The authors have declared no conflict of interests.

References

Abdi-Benemar, H., Jafaroghli, M., Khalili, B., Zamiri, M.J., Ezazi, H. and Shadparvar, A.A., 2015. *Small Rumin. Res*., **130**: 166-170. https://doi.org/10.1016/j.smallrumres.2015.06.013

Aitken, R.J., Gibb, Z., Baker, M.A., Drevet, J. and Gharagozloo, P., 2016. *Reprod. Fertil. Dev*., **28**: 1-10. https://doi.org/10.1071/RD15325

Alvarez, J.G. and Storey, B.T., 1995. *Mol. Reprod. Dev*., **42**: 334-346. https://doi.org/10.1002/mrd.1080420311

Andrabi, S.H., Mehmood, A., Anwar, M. and Khan, M.F., 2016. *Small Rumin. Res*., **143**: 61-66. https://doi.org/10.1016/j.smallrumres.2016.09.002

Andrabi, S., 2009. *Reprod. Domestic Anim*., **44**: 552-569. https://doi.org/10.1111/j.1439-0531.2008.01240.x

Baumber, J., Ball, B.A., Gravance, C.G., Medina, V. and Davies-Morel, M.C., 2000. *J. Androl*., **21**: 895-902.

Correa, J.R. and Zavos, P.M., 1994. *Theriogenology*, **42**: 351-360. https://doi.org/10.1016/0093-691X(94)90280-1

Çoyan, K., Başpınar, N., Bucak, M.N. and Akalın, P.P., 2011. *Cryobiology*, **63**: 1-6. https://doi.org/10.1016/j.cryobiol.2011.04.001

Dolatpanah, M.B., Towhidi, A., Farshad, A., Rashidi, A. and Rezayazdi, A., 2008. *J. Anim. Sci*., **21**: 29. https://doi.org/10.5713/ajas.2008.70035

Fayyaz, M.H., Ahmad, M. and Ahmad, N., 2017. *Andrologia*, **49**: e12652. https://doi.org/10.1111/and.12652

Gillan, L., Kroetsch, T., Maxwell, W.C. and Evans, G., 2008. *Anim. Reprod. Sci*., **103**: 201-214. https://doi.org/10.1016/j.anireprosci.2006.12.010

Irvine, D.S., 1996. *Rev. Reprod*., **1**: 6-12. https://doi.org/10.1530/revreprod/1.1.6

Leboeuf, B., Restall, B. and Salamon, S., 2000. *Anim. Reprod. Sci.,* **62**: 113-141. https://doi.org/10.1016/S0378-4320(00)00156-1

Memon, A.A., Wahid, H., Rosnina, Y., Gohb, Y.M., Ebrahimi, M., Nadiac, F.M. and Audrey, G., 2011. *Elixir Int. J*., **38**: 4100-4104.

Purdy, P.H., 2006. *Small Rumin. Res.*, **63**: 215-225. https://doi.org/10.1016/j.smallrumres.2005.02.015

Rao, T.K.S., Kumar, N., Patel, N.B., Chauhan, I. and Chaurasia, S., 2013. *Vet. World*, **6**: 579. https://doi.org/10.5455/vetworld.2013.579-585

Salmani, H., Nabi, M.M., Vaseghi-Dodaran, H., Rahman, M.B., Mohammadi-Sangcheshmeh, A., Shakeri, M. and Zhandi, M., 2013. *Small Rumin. Res*., **112**: 123-127. https://doi.org/10.1016/j.smallrumres.2012.12.015

Sattar, A., Younas, F., Andrabi, S.M.H., Ahmad, N., Mehmood, M.U. and Durrani, A.Z., 2016. *Anim. Reprod. Sci*., **169**: 99-135. https://doi.org/10.1016/j.anireprosci.2016.03.029

Shah, S., Andrabi, S. and Qureshi, I., 2017. *Andrologia*, **49**: e12713. https://doi.org/10.1111/and.12713

Topraggaleh, T.R., Shahverdi, A., Rastegarnia, A., Ebrahimi, B., Shafiepour, V., Sharbatoghli, M. and Janzamin, E., 2014. *Andrologia*, **46**: 777-783. https://doi.org/10.1111/and.12148

Zhandi, M., Shakeri, M., Towhidi, A., Sharafi, M. and Emamverdi, M., 2015. Protective role of glutathione in buck semen cryopreservation. *Iran. J. Vet. Res*., **16**: 298-300.

Zhandi, M. and Sharafi, M., 2015. *Cell Tissue Bank*, **16**: 443-448. https://doi.org/10.1007/s10561-014-9488-z