j.Egypt.net.med.Assoc 80, no 2. 323 – 329 (2020)

EFFECT OF ADDITION OF ANTIOXIDANTS TO IN VITRO FERTILIZATION MEDIUM ON SPERM MOTILITY AND VIABILITY IN BUFFALOES

By

Hodallah H. Ahmed[1]*, Amro. M. El-Sanea A. [2], Omaima M. Kandil[2], Nahed E. El-Toukhy[1], Amal M. Aboelmaaty[2], and Ahmed S. Abdoon[2]

[1]Department of Animal Physiology, Faculty of Veterinary Medicine, Cairo University, Giza Square, 12211, Cairo, Egypt.

[2]Department of Animal Reproduction and Artificial Insemination, Veterinary Research Division, National Research Centre, Tahrir St. Dokki 12622, Cairo, Egypt.

Corresponding author: Professor Hoda A. Hatem

Email: hodaahatem@yahoo.com

ABSTRACT

The present study was designed to evaluate the effect of addition of different concentrations of ascorbic acid, glutathione and melatonin on motility and viability of frozen-thawed buffalo spermatozoa. In this experiment, frozen-thawed buffalo spermatozoa were in vitro cultured for 3 h in Fret-TALP medium (control), or Fret-TALP supplemented with 25, 50 and 100 µM ascorbic acid; 2, 3- or 5-mM glutathione; or 10^{-4}, 10^{-5} and 10^{-6}M melatonin and sperm motility and viability were determined every hour. Results showed that addition of 10^{-5} M melatonin to Fret-TALP medium can support sperm motility and viability up to 3 h of incubation. In conclusion, addition of melatonin to IVF medium of buffalo spermatozoa improves sperm motility and viability.

Keywords:

Buffalo spermatozoa, In vitro fertilization medium, Antioxidants, Motility and viability.

INTRODUCTION

Reproductive performance in buffaloes is dramatically affected by the adverse environmental conditions. In addition, the application of assisted reproductive technologies such as in vitro embryo production (IVEP) and embryo transfer (ET) are slower in buffaloes than in cattle. During IVEP, the culture conditions showed higher oxygen concentrations than in vivo environment, leading to an increased level of reactive oxygen species (ROS), which in turn cause lipid peroxidation and cellular damage.

Furthermore, oxidative stress (OS) has been reported to be one of the most important factors that are incriminated for poor quality semen **(Bucak *et al.,* 2010).** ROS due to OS might lead to a rapid loss of intracellular ATP contents and axonemal damage, that was associated with decreased sperm motility, viability and increased mid-piece sperm morphological changes and decreased sperm capacitation and acrosome reaction viability **(Bansal and Bilaspuri 2011)**. In vitro fertilization rate was negatively correlated to ROS in human IVF trials **(Agarwal *et al.,* 2005)**. Therefore, controlling of ROS could be essential for the development of capacitation and hyper-activation, which is necessary to ensure fertilization(**De Lamirande and Gagnon 1993).** Several antioxidants have been used to decrease the impact of OS, thereby improving sperm quality and consequently its ability to fertilize an egg **(Jang *et al.,* 2010**). However, during in vitro fertilization procedures, supplementation of antioxidants improved sperm quality ensured normal pronuclear formation and embryo development to the blastocysts stage in human being **(Goncalves *et al.,* 2010)**. There was a lack of literatures regarding the effect of addition of antioxidants to a fertilization medium on sperm motility and viability in animals. Therefore, the present work was conducted to evaluate the direct effect of addition of different concentrations of antioxidants (Ascorbic acid, glutathione and melatonin) to in vitro fertilization medium on sperm motility and viability in buffaloes.

MATERIAL AND METHODS

All chemicals used in the present study were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise mentioned**.**

Semen collection: Frozen buffalo bull semen of proven fertility was purchased from Animal Reproduction Research Institute, Giza, Egypt.

Statement of Animal Rights:

All animal studies in the present work were conducted in accordance with the requirements of the Institutional Animal Care Committee and were reviewed and approved by the Animal Ethics Committee of the National Research Centre of Egypt (NRC, ID: 12/1/7).

Experimental Design:

The effect of addition of different concentrations of antioxidants (vitamin C, glutathione and melatonin) to fertilization medium on motility and viability of frozen thawed buffalo semen.

In three replicates, buffalo frozen semen straws that were thawed at 37ºC for 40 seconds, washed two times using Spa-TALP medium then the number 0f sperms was counted using

hemocytometer. The final sperm concentration was adjusted to 2 x 10^6 sperm / ml using Fert-TALP medium supplemented with 5 mg/ml BSA (Fatty acid free) and 50 µg/ml gentamycin. Sperm suspension was divided into ten parts, each contains five straws: The first part 1, was without antioxidant and served as control. Groups 2, 3, 4 were ascorbic acid groups. Sperm suspension was supplemented with 25, 50 and 100 µM ascorbic acid, respectively. In groups 5, 6 and 7 (Glutathione groups) sperm suspension was supplemented with 2, 3 and 5 mM glutathione, respectively. Moreover, in groups 8, 9, 10 (Melatonin group), sperm suspension was supplemented with 10^{-4}M, 10^{-5}m and 10^{-6}M melatonin, respectively. In all groups, sperm suspension was kept in CO_2 incubator at 38°C for three hours. Sperm motility and viability were checked every one hour.

Evaluation of sperm motility:

Sperm motility was assessed using a phase contrast microscope (100 x), with a warm stage maintained at 37 °C. A wet mount was made using a 5-µL drop of semen placed directly on a microscope slide and covered by a coverslip. Sperm motility estimations were carried out in three different microscopic fields for each semen sample. The mean of the three successive estimations were recorded as the final

Assessment of sperm viability:

One drop of the sperm suspension was placed on a tempered glass slide and mixed with one drop of eosin nigrosine solution then left to dry. The samples were examined using light microscope. Eosin penetrates non-viable cells which appeared red in color. Nigrosine offers a dark background facilitating the detection of viable-non-stained cells.

Statistical analysis:

Data were expressed as mean ± SEM. Statistical analyses was performed using unilabiate analysis of variance (ANOVA) with the aid of SPSS 20.0 statistical software. Duncan's multiple range tests were used to differentiate between significant and nonsignificant means at $P< 0.05$.

RESULTS

The direct effect of addition of different concentration of ascorbic acid, glutathione and melatonin to in vitro fertilization medium on motility of frozen-thawed buffalo spermatozoa is illustrated in Table 1. After the first hour of buffalo sperm incubation, sperm motility was significantly ($P<0.05$) higher for buffalo spermatozoa incubated in the presence of 25 µM/ml

ascorbic acid and all concentrations of melatonin than in control or the other concentrations of ascorbic acid or glutathione. Furthermore, after the second hour of incubation, sperm motility was significantly higher ($P<0.05$) in 10^{-4}M and 10^{-5}M melatonin groups than in control or glutathione and ascorbic acid groups. After the third hour, buffalo spermatozoa that were incubated in the presence of 10^{-5}M melatonin showed the highest ($P<0.05$) sperm motility than the control or the other examined groups.

Table (1): The effect of addition of antioxidant into vitro fertilization medium on sperm motility of buffalo spermatozoa (Mean ±SEM).

Incubation time	Control	Ascorbic acid			Glutathione			Melatonin		
		25µM	50µM	100µM	2mM	3mM	5mM	10^{-4}M	10^{-5}M	10^{-6}M
0.0 time	71.0± 1.2	71.0± 1.2	71.0± 1.2	71.0± 1.2	71.0± 1.2	71.0± 1.2	71.0± 1.2	71.0± 1.2	71.0± 1.2	71.0± 1.2
1st h	51.9± 0.9^{b}	68.1± 2.1^{a}	50.6± 1.1^{b}	46.3± 1.6^{b}	43.6± 1.3^{c}	56.3± 1.6^{b}	41.3± 0.8^{c}	68.4± 1.6^{a}	73.8± 2.3^{a}	70.6± 1.8^{a}
2nd h	35.0± 1.6^{c}	48.1± 2.1^{b}	49.4± 1.1^{b}	26.9± 2.0^{c}	22.1± 0.9^{c}	51.3± 1.3^{b}	35.6± 2.0^{c}	65.6± 4.2^{a}	66.3± 2.3^{a}	56.3± 0.9^{b}
3rd h	21.3± 0.8^{c}	43.1± 2.5^{b}	40.6± 2.0^{b}	18.8± 1.8^{c}	20.7± 0.7^{c}	21.9± 0.9^{c}	30.0± 1.8^{c}	49.4± 3.2^{b}	58.1± 2.6^{a}	48.8± 3.5^{b}

a, b, c superscripts within the same row differ significantly at P< 0.05.

Sample no. in each group= 5 straws.

The effect of addition of different concentrations of ascorbic acid, glutathione and melatonin to fertilization medium on viability of frozen thawed buffalo sperm was demonstrated in (Table 2). The results showed that after the first hour of sperm incubation, the percentages of viable sperm were significantly ($P< 0.05$) higher in groups incubated with 10^{-5}M melatonin, 10^{-4}M melatonin and 50 µM ascorbic acid than the other concentrations of ascorbic acid or melatonin, control and glutathione groups. During the second and third hours of sperm incubation, the percentage of sperm viability was significantly ($P<0.05$) higher for sperm incubated in Fert-TALP medium in the presence of 10^{-5}M or 10^{-4}M melatonin compared with the other groups.

Table (2): Effect of addition of different doses of antioxidants in vitro fertilization medium on viability of frozen thawed- buffalo semen (Mean ± S.E).

Treatments	Incubation time/hour			
	0 h	1st h	2nd h	3rd h
Control	70.7±2.3	68.3±1.5^{c}	55.7±1.5^{c}	28.3±2.3^{d}
25 µM Ascorbic acid	70.7±2.3	73.0±1.5^{b}	60.7±2.4^{b}	36.3±3.8^{c}
50 µM Ascorbic acid	70.7±2.3	84.0±3.2^{a}	54.7±4.4^{c}	41.3±1.8^{c}
100 µM Ascorbic acid	70.7±2.3	73.7±2.4^{b}	64.7±2.9^{b}	30.0±3.1^{d}
2mM Glutathione	70.7±2.3	61.0±2.1^{c}	38.0±2.3^{d}	39.3±1.8^{c}
3mM glutathione	70.7±2.3	64.3±3.0^{c}	50.0±2.0^{c}	27.3±2.9^{d}
5mM Glutathione	70.7±2.3	73.3±2.9^{b}	36.0±4.2^{d}	25.3±2.0^{d}
10^{-4}M Melatonin	70.7±2.3	86.0±1.5^{a}	71.0±2.0^{a}	60.7±1.8^{a}
10^{-5}M Melatonin	70.7±2.3	89.0±2.1^{a}	76.7±2.7^{a}	67.0±4.2^{a}
10^{-6}M Melatonin	70.7±2.3	79.0 ± 3.8^{b}	60.7±2.9^{b}	51.3±1.8^{b}

a, b, c, d Superscripts within the same column differ significantly at P< 0.05.

Sample no. in each group= 5 straws.

DISCUSSION

Mammalian spermatozoa are vulnerable to OS due to the high concentration of polyunsaturated fatty acids (PUFAs) in sperm membranes, that induced damage mediated by lipid peroxidation **(Sariozkan *et al.,* 2010)**. In the present work, supplementation of in vitro fertilization medium with 10^{-5}M or 10^{-4}M melatonin (P<0.05) increased significantly buffalo sperm motility and viability up to 3 hours of incubation when compared with control on and ascorbic acid or glutathione groups. Melatonin prevented free radical mediated damage **(Reiter *et al.,* 2014)**. Previous studies showed that melatonin modulate the activity of many pro- and anti-oxidant enzymes, leading to reduction of oxidative damage to lipids, proteins, and DNA **(Manchester *et al.,* 2015; Pang *et al.,* 2016)**. More specifically, melatonin could directly improve sperm characteristics, through enhanced total motility, progressive motility, viability rates **(Ortiz *et al.,* 2011)**; increased sperm membrane and DNA integrity **(Succu *et al.,* 2011; Ashrafi *et al.,* 2013)** and reduced membrane lipid peroxidation **(Jang *et al.,* 2010).** On the contrary, addition of 10^{-6}M melatonin to bovine semen samples reduced the

percentage of viable sperms with intact acrosomes, and increased the incidence of DNA fragmentation and DNA oxidation. The finding that agrees with those of **(Cheuqueman *et al.,* 2015)**. Such discrepancy could be attributed to the effect of dose, batch or the species difference in response to melatonin supplementation. The present investigation showed that the presence of 50 µM ascorbic acid increases sperm motility significantly ($P<0.05$) during the first hour of incubation, however it did not support sperm motility and viability during the rest of incubation time. Moreover addition of glutathione to IVF medium did not achieve any improvement in buffalo sperm motility and viability compared with control group.

The present findings may indicate that to carry on buffalo spermatozoa estimation of the proper concentration of glutathione should be known.

In conclusion, the addition of 10^{-5}M melatonin to IVF medium improved buffalo sperm motility and viability up to three hours of incubation in vitro.

Acknowledgements:

The authors would like acknowledge to gratefully the financial support offered by the National Research Centre (Pro. 12/1/7).

We gratefully also acknowledge **Prof. Dr. Omaima M. Kandil** director to Embryo and Genetic Resource Conservation Bank in National Research Centre, financially supported by STDF (CB grant ID: 2339) for support us with melatonin used in this work financially supported from academy of scientific research and technology through the project in tittle "Assessment the viability of cryopreserved buffalo embryo and semen"**.**

REFERENCES

AgarwalA,GuptaS,SharmaRK.(2013): Roleofoxidative stress in female reproduction. *Reproductive Biology and Endocrinology*. 2005;3,article 28-Ashrafi I, Kohram H, Ardabili FF. Antioxidative effects of melatonin on kinetics,microscopic and oxidative parameters of cryopreserved bull spermatozoa. Animal Reprod. Sci 139:25-30.

Bansal A.K. and Bilaspuri G.S.(2011): Impacts of oxidative stress and antioxidants on semen functions. Veterinary Medicine International Volume 2011, Article ID 686137, 7 pages doi:10.4061/2011/686137.

Bucak MN, Sariözkan S, Tuncer PB, *et al.* (2010): The effect of antioxidants on post-thawed Angora goat (Capra hircus ancryrensis) sperm parameters, lipid peroxidation and antioxidant activities. *Small Ruminant Research*. ; 89 (1):24-30.

Cheuqueman C, Arias ME, Risopatron J, Felmer R, Alvarez J, and Mogas T, Sanchez R. (2015): Supplementation of IVF medium with melatonin: Effect on sperm functionality and in vitro produced bovine embryos. Andrologia 47:604-615.

De Lamirande E, Gagnon C. (1993): A positive role for the superoxide anion in triggering hyper activation and capacitation of human spermatozoa. *International Journal of Andrologia*. ; 16 (1):21–25.

Goncalves F, Barrette LSS, Aruba RP, Perry SHV, Minot GZ. (2010): Effect of antioxidants during bovine *in vitro* fertilization procedures on spermatozoa and embryo development *Reproduction in Domestic Animals*. ; 45(1):129-135.

Jang HY, Kim YH, Kim BW, Park IC, Cheong HT, Kim JT, Park CK, Kong HS, Lee HK, Yang BK. (2010): Ameliorative effects of melatonin against hydrogen peroxide-induced oxidative stress on boar sperm characteristics and subsequent in vitro embryo development. Reprod Domestic Animals 45:943-950

Manchester LC, Coto-Montes A, Boga JA, Andersen LP, Zhou Z, Galano A, Vriend J, Tan DX, Reiter RJ. (2015): Melatonin: An ancient molecule that makes oxygen metabolically tolerable. J Pineal Res 59:403-419.

Ortiz A, Espino J, Bejarano I, Lozano GM, Monllor F, Garcia JF, and Pariente JA, Rodriguez AB. (2011): High endogenous melatonin concentrations enhance sperm quality and short-term in vitro exposure to melatonin improves aspects of sperm motility. J Pineal Res 50:132-139.

Pang YW, Sun YQ, Jiang XL, Huang ZQ, Zhao SJ, Du WH, Hao HS, Zhao XM, Zhu HB. (2016): Protective effects of melatonin on bovine sperm characteristics and subsequent in vitro embryo development. Moll Reprod Dev. 2016 Nov; 83 (11):993-1002. Doi: 10. 1002 /mrd. 22742. Epub Oct 10.

Melatonin and stable circadian rhythms optimize Reiter RJ, Tan DX, Korkmaz A, Rosales-Corral SA. (2014): maternal, placental and fetal physiology. Hum Reprod Update 20:293-307.

Sariözkan S, Bucak MN, Tuncer PB, Taşdemir U, Kinet H, Ulutaş PA. (2010): Effects of different extenders and centrifugation/washing on post thaw microscopic-oxidative stress parameters and fertilizing ability of Angora buck sperm. Theriogenology. Feb; 73 (3):316-23.doi: .1016/ j. theriogenology.2009.09.015. Epub 2009 Oct 31.

Succu S, Berlinguer F, Pasciu V, Satta V, and Leoni GG, Naitana S. (2011): Melatonin protects ram spermatozoa from cryopreservation injuries in a dose-dependent manner. J Pineal Res 50:310-318.