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ASSESSMENT OF MITOCHONDRIAL REDOX ACTIVITY DURING CRYOPRESERVATION PROCESS OF INDIAN RED JUNGLE FOWL (*GALLUS GALLUS MURGHI*) SPERM THROUGH MTT REDUCTION ASSAY

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ABSTRACT

The experiment was conducted to evaluate the metabolic activity through MTT (3[4,5-dimethylthiazol-2-y1]-2,5-diphenyltetrazolium bromide) reduction assay of Indian red jungle fowl sperm during cryopreservation process. In addition, MTT test was compared with sperm motility and viability data. Sperm analysis was made at pre-dilution, post-dilution, post-cooling, post-equilibration and post freeze-thawing stages. Pearson's correlation coefficient showed significant ($P<0.01$) positive association of MTT reduction rate with motility ($r=0.85$) and viability ($r=0.84$). A significant ($P<0.05$) decline was observed in motility, viability and metabolic status after each stage of cryopreservation. The highest ($P<0.05$) stage-specific percent decrease in motility and viability were observed after freeze-thawing. Nevertheless, stage-specific percent decrease in metabolic status differ ($P<0.05$) after dilution (5.93 ± 0.12), cooling (8.41 ± 0.03), equilibration (19.1 ± 0.04) and freeze-thawing (30.9 ± 0.08). The total decrease in motility, viability and metabolic activity after the complete freeze-thawing cycle were calculated as 56%, 47% and 52%, respectively. It is concluded that MTT reduction assay is simple and reliable method to evaluate mitochondrial redox activity in sperm of this threatened species.

Key words: Indian red jungle fowl, Metabolic redox activity, MTT Reduction, DNA integrity
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INTRODUCTION

The population of Indian red jungle fowl (*Gallus gallus murghi*) is decreasing in its native wild habitat and on the other hand captive populations are facing losses of genetic diversity resulting in poor survival rates (Hocking, 2009; Worley *et al*. 2010; Fernandes *et al.,* 2013). In Pakistan, it is only found in Deva Vatala National Park, Azad Jammu and Kashmir and reported to have only 109 individuals (Subhani *et al.,* 2010). Therefore, attempts are carried on establishing the semen bank for this species to have frozen semen to restore the genetic diversity in captive population through artificial insemination (Rakha *et al.,* 2016, 2017, 2018).

The successful freezing protocols that guarantee satisfactory fertility outcomes of frozen semen are fundamental in the establishment of semen banks. The fertility of the frozen semen depends upon the initial quality of fresh ejaculates. Therefore, it is necessary to screen the ejaculates using *in vitro* semen assessment techniques of high predictive value for fertility (Hazary *et al*., 2001). Commonly, bird semen is evaluated by assessing motility (visually), plasma membrane integrity (hypo-osmotic swelling test) and viability (eosin/nigrosin). These semen parameters only provide information about structural integrity and does not tells about functional status. Moreover, these techniques are less repeatable, time consuming and associated with risks of human errors and affected by experience of the examiner (McNiven *et al.,* 1992). In this regard, MTT reduction assay is simple that does not require expensive and complex instrumentation in the lab. MTT can evaluate the metabolic activity of sperm cells. This assay is based upon the ability of metabolically active cells to reduce the yellow water soluble tetrazolium salt to insoluble formazan (dark-blue) primarily by the dehydrogenase enzyme of the mitochondria (Mosmann, 1983).

The sperm MTT reduction rate of bovine sperm has shown strong positive relationship with viability, mitochondrial activity and acrosome integrity assessed through flow cytometer using SYBER-14, Rhodamine 123 and LysoTracker Green DNA-26, respectively (Aziz, 2006). The MTT reduction was positively correlated with fowl sperm ATP content, mobility, perivitelline layer interaction and fertilization potential (Hazary *et al.,*

2001). The MTT reduction rate is strongly correlated with viability assessed though eosin/nigrosin staining technique in buffalo (Iqbal *et al.,* 2010) and boar semen (Byun *et al.,* 2008). Studies have ~~been~~ reported significant damage to motility, plasma membrane integrity, viability and acrosome of Indian red jungle fowl sperm during the process of cryopreservation (Rakha *et al.,* 2016; 2017). Nonetheless, reports on metabolic status of Indian red jungle fowl semen is lacking in published literature. The present study examines the changes in metabolic activity of Indian red jungle fowl sperm, through MTT reduction assay, during different steps of freezing-thawing process.

MATERIALS AND METHODS

Study area: The nine mature cocks of Indian red jungle fowl were used in this study that were kept individually in pens of 106.68 cm x 121.92 cm and maintained photoperiod (16h L: 8hD) at Avian Research Center, Pir Mehr Ali Shah Arid Agriculture University Rawalpindi-Pakistan. The birds were offered commercially available Islamabad poultry cock breeder feed and fresh water *ad libitum*.

Experiment 1: to correlate MTT reduction assay with motility and viability of Indian red jungle fowl semen, fresh ejaculates from nine cocks were separately assessed for motility, viability and metabolic status through MTT reduction assay. Experiment 2: to compare changes in metabolic status during cryopreservation, semen collected from nine cocks was pooled, frozen in red fowl extender and metabolic status, viability and motility was assessed at pre-dilution, post-dilution, cooling, equilibration and freeze-thawing stage. The two separates experiments were repeated three times independently.

Semen was collected from cocks through abdominal massage as described by Burrows and Quinn (1935) in the graduated plastic tubes. Semen volume was measured in microlitres using micropipette. Initial sperm motility of each ejaculate was determined as described by Zemjanis (1970) by mixing 10 µl semen samples in 500 µl of phosphate buffer saline (pH 7.2, 300 mOsm/kg). The percentage of motile spermatozoa was determined by putting a drop of semen sample on a glass slide placed on the stage warmer (set at 37ºC) under the phase contrast microscope (x400, Olympus BX20, Japan). Sperm concentration was measured by taking 1µl of semen and 200 µl of formal citrate solution (prepared by adding 1mL of 37% formaldehyde in 99 mL of 2.9% (w/v) sodium citrate solution) with Neubauer haemocytometer (Marienfeld, Germany) under the phase contrast microscope (x400, Olympus BX20, Japan) (Rakha *et al.,* 2015 a;b).

Sperm viability (% live/dead sperm) was determined as described Bakst and Cecil 1997 by adding eosin-nigrosin to the Lake solution composed by sodium glutamate (0.01735g), potassium citrate (0.00128g), sodium acetate (0.0085g) and magnesium chloride (0.000676) in 100 mL distilled water. A total of 200 spermatozoa were assessed per slide under a phase-contrast microscope (1000x with oil immersion).

MTT reduction assay is a colorimetric assay for assessing cell redox activity. Under this assay, NADPH-dependent cellular oxi-reductase enzyme reflect the number of viable cells in the sample by reducing tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylytetrazolium bromide to its insoluble formazan (purple color). For this purpose, MTT stock solution was prepared by adding 5mg of MTT (Sigma-Aldrich, USA) in per ml of PBS. 10µL of stock solution were added to 100µL of semen sample (0.058 billion sperm). The absorbance was measured immediately at 550 nm with the help of spectrophotometer and then samples were incubated for 1 hour at 37ºC. After incubation absorbance was measured again. Rate of MTT reduction was calculated by following the formula given by Hazary and Wishart (2001).

MTT Reduction Rate = Initial optical density - Optical density after 1 hour of incubation at 37°C

Semen was collected from nine cocks and pooled after initial evaluation (volume, motility and concentration). The pooled semen was diluted (at 37°C) with red fowl extender (1:5) and cooled to 4 °C in two hours (0.275 min^{-1}), equilibrated for 10 min after addition of 20% glycerol at 4°C (Rakha *et al.,* 2016). After equilibration, semen was filled in 0.5 mL French straws (IMV, L'Aigle, France) and kept over liquid nitrogen vapors for 10 minutes and plunged into liquid nitrogen for storage. The straws were thawed individually at 37°C for 30 seconds in water bath for evaluation. Sperm motility, viability and metabolic status were measured before pre-dilution, after dilution, cooling, equilibration and post-thawing.

Statistical analysis: The relationship of MTT reduction rate with motility and eosin/nigrosin based viability were calculated by General Linear Bivariate Regression analysis and correlation was calculated by Person's Correlation coefficient using data on semen quality of fresh semen. The data on the effect of cryopreservation stages on motility, viability and metabolic status were analyzed using analysis of variance in completely randomized design (MegaStat Version 7.25 Mc-Graw-Hill New Media, New York). When F-ratio was found significant ($P<0.05$), post-hoc comparison between the means was done though Fisher's protected LSD test.

RESULTS

The data on the relationship of MTT reduction rates with motility and viability of Indian red jungle fowl

sperm are given in Table 1. The MTT reduction rates (nmol/min/10^9sperm) have significant (P<0.05) positive association with percentage motility (r=0.85) and viability (r=0.84) of fresh Indian red jungle fowl semen. The regression equation between MTT reduction rates and motility (dependent variable) was calculated as Y= -48 + 8281(x) with R^2=0.72. Moreover, regression equation for viability (dependent variable) was calculated Y= 7.44 + 5048(x) with R^2=0.71.

The data on the changes in motility and viability of Indian red jungle fowl semen during cryopreservation are given in Table 2. The motility and viability significantly decreased (P<0.05) after each stage of cryopreservation. The magnitude of percent loss did not differ (P>0.05) in sperm motility and viability after dilution (7.21 ± 3.6%, 12.3 ± 0.9%), cooling (11.5 ± 5.4%, 10.4 ± 1.3%) and equilibration (15.46 ± 1.7%, 12.1 ± 0.8%). Nonetheless, higher (P<0.05) percent loss in motility and viability were observed after freeze-thawing stage (34.2 ± 2.3%, 23.3 ± 1.5%) compared to percent loss after dilution, cooling and equilibration. The overall decrease in motility and viability were recorded by 56% and 47% after complete process of cryopreservation.

Table 1: Regression analysis of MTT reduction rate (nmol/min/10^9sperm) showing relationship with motility and viability of Indian red jungle semen.

Sperm quality parameters	Coefficients of regression				Correlation	t-value	R2	P-value
	Intercept	Error	Slope	Error				
Sperm motility (%)	-47.992	14.901	8281.5	1028.5	0.85	8.052	0.72	0.00
Sperm viability (%)	7.4389	9.3462	5048.2	645.09	0.84	7.83	0.71	0.00

Table 2: Sperm quality and metabolic status of Indian red jungle fowl (*Gallus gallus murghi*) semen during cryopreservation.

	Motility (%)	Percent decline	Viability (%)	Percent decline	MTT reduction rate (nmol/min/10^9sperm)	Percent decline
Pre-dilution	91.7 ± 1.7^a	--	95.0 ± 0.0^a	--	1.69 ± 0.001^a	--
Post-Dilution	85.0 ± 2.9^a	7.21 ± 3.6^b	83.3 ± 0.9^b	12.3 ± 0.9^b	1.59 ± 0.001^b	5.93 ± 0.12^d
Post-Cooling	75.0 ± 2.9^b	11.5 ± 5.4^b	74.7 ± 0.3^c	10.4 ± 1.3^b	1.46 ± 0.001^c	8.41 ± 0.03^c
Post-equilibration	63.3 ± 1.7^c	15.46 ± 1.7^b	65.7 ± 0.9^d	12.1 ± 0.8^b	1.18 ± 0.001^d	19.1 ± 0.04^b
Post-freeze thawing	41.7 ± 1.7^d	34.2 ± 2.3^a	50.3 ± 0.3^e	23.3 ± 1.5^a	0.82 ± 0.013^e	30.9 ± 0.08^a

The values (mean ± SE) with different superscript differ significantly (P<0.05) in a column.

The data on mitochondrial dehydrogenase activity of Indian red jungle fowl sperm during cryopreservation are given in Table 2. Each stage of cryopreservation significantly (P<0.05) decreased the semen metabolic status. The stage-specific magnitude of percent losses in metabolic status differs between after dilution (5.93 ± 0.12%), cooling (8.41 ± 0.03%), equilibration (19.1 ± 0.04%) and post-thawing (30.9 ± 0.08%). The overall percent decrease in metabolic activity was calculated as 52% after the cryopreservation.

DISCUSSION

MTT reduction assay allows determining the mitochondrial dehydrogenase activity in living cells (Holder *et al.,* 2012), and thus it may be a useful method to study mitochondrial function during the different stages of sperm cryopreservation process. Although spermatogonia and early spermatocytes harbor orthodox mitochondria, sperm have more condensed and metabolically more efficient forms of mitochondria (Ramalho-Santos *et al.,* 2009) helically anchored around the anterior portion of the outer dense fibres and of the axoneme, constituting the midpiece. During spermiogenesis, the loss of majority of the cytoplasm, and thus the reduction of MTT in sperm should be attributed mainly to mitochondrial enzymes, unlike somatic cells in which MTT reduction may also be catalyzed by a number of other non-mitochondrial enzymes (Bernas and Dobrucki, 2002).

The findings of the present study showed a positive association of MTT reduction rate of Indian red jungle fowl sperm with motility and viability, like a previous report in domestic chicken (Lin *et al.,* 2019). The MTT reduction has been found positively correlated with ATP content, mobility; perivitelline layer interaction and fertilization potential of the fowl spermatozoa (Hazary *et al.,* 2001). It is pertinent to mention that MTT reduction rate of bovine and equine semen has showed strong positive relationship with viability, mitochondrial activity and acrosome integrity of ~~bull~~ sperm assessed through flow cytometer using SYBER-14, Rhodamine 123 and LysoTracker Green DNA-26, respectively (Aziz *et al.,* 2005; Aziz, 2006). Several other reports demonstrated positive association between MTT

reduction rate and viability in various avian and mammalian species (Capkova *et al.,* 2000; Esfahani *et al.,* 2000; Hazary *et al.,* 2001; Park and Yi, 2002; 2004; Gaczarzewicz *et al.,* 2003; Naser- Byun *et al.,* 2008). Mitochondria are the prime organelles that provide site for electron transport chain reactions in the process of aerobic respiration (Hazary *et al.,* 2001; Aziz *et al.,* 2006). Energy required for carrying on physiological process and sperm motility is supplied by the mitochondria in the form of ATPs (Long, 2006). Active sperm mitochondria are required for fertilization, and it is believed that declining of sperm mitochondrial activity during cryopreservation is associated with the decreased levels of ATPs (Long, 2006; Blanco *et al.,* 2011). In fowl semen, MTT reduction was found positively correlated with sperm ATP content, mobility; perivitelline layer interaction and fertilization (Hazary *et al.,* 2001).

The sensitivity of the bird sperm toward freezing is species specific that depends on the physiology of the sperm plasma membrane and organelles (Blanco *et al*. 2011; 2012; 2013). In present study, motility, viability and mitochondrial dehydrogenase activity of Indian red jungle fowl semen decreased significantly after dilution, cooling, equilibration and freeze-thawing stage. The magnitude of percent loss to motility and viability did not differ after dilution, cooling and equilibration, while significant higher percent loss was observed after freeze-thawing. Nonetheless, percent losses to metabolic status differ significantly between all stages of cryopreservation, with maximum loss during freeze-thawing stage. Results showed that MTT reduction assay has more discriminating ability than eosin/nigrosin in detecting the viability of the Indian red jungle fowl. Previous reports also have revealed that maximum damage to sperm occurs during the freeze-thawing process (Rakha *et al.,* 2016; 2017). Significant decline in percentage of live fowl sperm with functional mitochondria, plasma membrane, acrosome and DNA integrity were reported during freeze-thawing process assessed through fluorescent staining with flow cytometer (Partyka *et al.,* 2010). It is suggested that motility, plasma membrane integrity and mitochondrial function are equally affected by cryopreservation. In present study, magnitude of damage to plasma membrane (viability – 47%) is very close to the loss in mitochondrial activity (52%). Therefore, it is suggested that decline in motility is associated with impairment of mitochondrial activity in sperm.

The process of cryopreservation is believed to induce alterations in lipids of the plasma and mitochondrial membrane leading to that disruption and cell death (Blesbois *et al.,* 2005; Sieme *et al.,* 2015). The ultra-structure of cell organelles, especially mitochondria was largely deteriorated in frozen-thawed fowl semen (Xia *et al.,* 1988; Partyka *et al.,* 2010). MTT reduction assay basically determine the enzymatic activity in the mitochondria that have primary role in the production of ATPs necessary for motility and physiological processes (Mahadevan *et al.,* 1997). It is known that sperm ATPs contents is reduced in the process of cryopreservation and exogenous ATPs supply improves the cryosurvival of sperm (Long, 2006; Blanco *et al.,* 2011). It is suggested that decease in MTT reduction rate in the process of cryopreservation is directly related to the decrease in mitochondrial function of the Indian red jungle fowl.

This is the first ever report that reported the dynamics in metabolic status of Indian red Jungle fowl sperm, through cryopreservation process, assessed by MTT reduction assay. The metabolic status decreased significantly after each stage of cryopreservation while the maximum percent decline was observed during freeze-thawing stage. It is concluded that maximum reduction in sperm metabolic activity occur during freeze-thawing stage. Moreover, MTT reduction assay has positive correlation with motility and viability. Although MTT reduction assay is simple, but found reliable to evaluate the semen quality of fresh and frozen-thawed Indian red jungle fowl semen.

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