RESEARCH ARTICLE

Correlations Amongst Functional and Morphological Attributes and Oxidative Markers of Fresh and Cryopreserved Semen of Gir and Murrah Bulls

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Abstract

This study was undertaken during the winter season on healthy mature Gir cattle and Murrah buffalo bulls (n=3 each). The semen samples (6 ejaculates/bull, total 36 ejaculates) collected in the morning using artificial vagina were evaluated for routine seminal attributes, including acrosomal and plasma membrane integrity. The samples were then diluted @ 100 million sperm/ml with tris fructose yolk glycerol extender without and with sericin @ 0.1, 0.25, 0.5 and 1.0% (w/v), filled in French mini-straws, and frozen in LN2 using biofreezer as per standard freezing protocol. Straws were thawed in water bath at 37°C for 30 sec and evaluated for post-thaw quality, *viz.,* motility, viability, morphology, acrosome integrity and plasma membrane integrity (HOST). Lipid peroxidation (malondialdehyde - MDA production) and activities of enzymes superoxide dismutase (SOD) and glutathione peroxidase (GPx) were assessed as oxidative markers in seminal plasma of freshly diluted and frozen-thawed semen samples. Sericn at 0.5% level significantly (p<0.01) improved the post-thaw sperm quality with reduced oxidative stress in both the species. The breed-wise correlation coefficients (r) among sperm quality attributes and oxidative markers were studied in fresh and frozen-thawed semen of each species, and also for fresh with frozen-thawed semen. The findings revealed significant interrelationships amongst most of the attributes of fresh as well as post-thawed semen and also of fresh semen attributes with those of cryopreserved semen including oxidative markers in both the species. Sperm motility estimation in fresh, pre-freeze and post-thawed semen was a legitimately good indicator of quality of spermatozoa at various steps of semen processing/freezing, and its fertilizing potential. Thus, the sperm motility, HOS test and either MDA or SOD/GPx activity alone may be used as valuable and practical tools for routine assessment of bovine semen quality considering significant correlations found between them.

Key Words: Bovine semen, Correlations, Fresh semen, Frozen-thawed semen, Oxidative markers, Spermatozoal attributes.

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Introduction

Semen analysis is done as a routine to evaluate male fertility potential (Rana and Dhami, 2003; Patel and Dhami, 2016). Fertility of male is an important factor in bovine reproduction since a single bull is generally bred to thousands of cows through AI. However, no single test or combinations of tests have been proved to be totally reliable for accurate prediction of semen quality in relation to fertility. The correlations of the physical characters of bovine semen reported with its fertility are highly variable and poor (Shelke and Dhami, 2001; Tiwari *et al*., 2009; Chaudhari *et al.,* 2014; Chaudhary *et al.,* 2017). There are numerous factors including species, breed, dilutor, season and freezing-thawing protocol that may affect the motility, plasma membrane integrity, morphology and viability of fresh and frozen-thawed semen. Recently more attention is being focused to evaluate the functional sperm plasma membrane integrity through HOS test rather than simply evaluating the structural integrity of acrosome by Giemsa or triple stain (Lodhi *et al.,* 2008; Pathak *et al.,* 2018). Significant correlations if established between fresh and cryopreserved semen would help to select a few most valid simple traits of fresh semen to predict freezability/post-thaw quality and thereby fertility of such ejaculates. Hence, the interrelationships of spermatozoal attributes of fresh as well as frozen-thawed semen including oxidative markers, and also of fresh semen to those of frozen-thawed semen of cattle and buffalo bulls were evaluated while evaluating antioxidant-antifreeze properties of sericin (silkworm protein) at different concentration in the standard Tris extender.

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Materials and Methods

The study was conducted during winter season on semen of three mature Gir and three Murrah bulls, aged 5-7 years, maintained at Sperm Station of the College. All the bulls were healthy and were maintained in nearly identical nutritional and managerial conditions throughout the period of study. They were under regular weekly twice semen collection schedule using AV. For the present study, the ejaculates (6/bull, 18 per breed) were evaluated once in a week for routine physico-morphological attributes. The ejaculates with >75% initial motility were divided into five equal aliquots, and were extended with standard tris-citrate-fructose-yolk-glycerol (TFYG) extender without and with antioxidant Sericin - a silk worm protein (Sigma-Aldrich, USA) @ 0.10, 0.25, 0.50 and 1.00 % W/V.

The French mini straws were filled and sealed from each aliquot using automatic filling and sealing machine (IS4 System, IMV Technologies, France) and were cooled to 4-5°C within 60-90 minutes and further equilibrated at the same temperature for 4 hrs in cold handling cabinet (IMV, France). Just before freezing, the samples were evaluated for pre-freeze sperm motility. Freezing of the straws was carried out using a programmable bio-freezer (Digitcool 5300 CE ZH 350, IMV, France) using a previously tested freezing curve for bovine semen. After 18 hrs of frozen storage, the straws were thawed in a water bath at 37°C for 30 seconds. Post-thaw motility was assessed under phase contrast microscope (40x) fitted with a biotherm.

The freshly diluted and frozen-thawed semen samples (2.0 ml each) of each aliquot were centrifuged at 1000 g for 10 minutes. The supernatant (plasma) separated was stored at -20° C. Lipid peroxidation in terms of malondialdehide (MDA) production and the activities of antioxidant enzymes, *viz.,* glutathione peroxidase (GPx) and superoxide dismutase (SOD) in pre- and post-thaw seminal plasma were determined using commercial kits (Cayman Assay Kits, USA, Cat No. 706002, 705003 & 703102) according to the instructions of manufacturer. The analyses for correlation matrix within freshly diluted and cryopreserved semen including oxidative markers, and between fresh and frozen-thawed sperm attributes of each breed/species, irrespective of levels of additive sericin, were done according to Snedecor and Cochran (1994).

Results and Discussion

The correlation matrix analysis (r) done amongst various sperm attributes and oxidative markers in freshly diluted and post-thawed samples of Gir cattle and Murrah buffalo semen (extended and frozen in TFYG extender without and with Sericin, a silkworm protein from Sigma, @ 0.1, 0.25, 0.5 and 1.0% w/v) revealed significant interrelationships amongst most of the traits of fresh as well as post-thawed semen and also of fresh semen attributes with those of cryopreserved semen in both the species. For both the species, sericin @ 0.5% was found to improve significantly (p<0.01) the sperm quality attributes with reduced oxidative stress in cryopreserved semen. The details of these findings have already been reported earlier (Patel *et al.,* 2019).

Correlations Amongst Fresh Sperm Quality Attributes

In freshly diluted Gir bulls semen (Table 1), the initial motility had highly significant (p<0.01) positive correlations with HOS reactive sperm, live sperm, pre-freeze motility and GPx activity (r= 0.847, 0.880, 0.582 and 0.348, respectively) and significant (p<0.05) negative correlations with sperm abnormalities (-0.287) and MDA activity (-0.270). Similarly, the HOS reactive sperm showed significant positive correlations with live sperm, intact acrosome, pre-freeze motility and GPx activity (0.725, 0.208, 0.623, 0.255) and negative correlations with sperm abnormalities (-0.284). The live sperm per cent in fresh semen was positively correlated with pre-freeze motility and GPx (0.551, 0.358) and negatively with MDA concentration (-0.281). Total sperm abnormalities had significant negative and positive correlations with intact acrosome (-0.318) and MDA (0.263), respectively. Sperm with intact acrosome in fresh semen showed significant positive correlations with

Table 1: Correlations among spermatozoal attributes and oxidative markers of freshly diluted semen of Gir and Murrah bulls in TFYG

	Initial Motility	*Initial HOST*	*Live Sperm*	*Tail Ab.sp.*	*Total Ab.sp.*	*Intact Acrosome*	*Prefreeze Motility*	*Initial MDA*	*Initial SOD*	*Initial GPX*
Correlations: Gir bull semen										
Initial Sperm Motility	1	0.847**	0.880**	-0.232*	-0.287**	0.186	0.582**	-0.270*	0.081	0.348**
HOS Reactive Sperm (%)	0.865**	1	0.725**	-0.215*	-0.284**	0.208*	0.623**	-0.121	0.057	0.255*
Live Sperm (%)	0.958**	0.840**	1	-0.083	-0.160	0.182	0.551**	-0.281**	0.059	0.358**
Tail Abnormalities (%)	-0.175	-0.001	-0.136	1	0.800**	-0.227*	-0.146	0.116	0.101	0.035
Total Sp Abnormalities	-0.334**	-0.212*	-0.331**	0.793**	1	-0.318**	-0.165	0.263*	-0.165	-0.104
Intact Acrosome (%)	0.301**	0.048	0.289**	-0.509**	-0.437**	1	0.462**	-0.161	0.011	0.245*
Pre-freeze Motility (%)	0.745**	0.605**	0.679**	-0.073	-0.202	0.311**	1	-0.102	-0.081	0.312**
Malondaldehide (MDA)	-0.228*	-0.169	-0.223*	0.306**	0.423**	-0.076	-0.207	1	-0.242*	-0.325**
Superoxide Dismutase	0.281**	0.314**	0.281**	-0.065	-0.357**	0.019	0.246*	-0.497**	1	0.192
Glutathione Peroxidase	0.337**	0.187	0.324**	-0.179	-0.084	0.246*	0.271**	-0.230*	0.003	1
Correlations: Murrah buffalo semen										

*Significant at the 0.05 level, ** Significant at the 0.01 level (2-tailed), Number of paired observations = 90.

pre-freeze motility and GPx (0.462, 0.245); pre-freeze motility with GPx (0.312,) and MDA levels had negative correlations with SOD and GPx (-0.242, -0.325). SOD activity in freshly diluted seminal plasma had significant correlations only with MDA production (-0.242) in Gir bulls.

In freshly diluted Murrah bulls semen (Table 1), most of the above correlations were highly significant, and all three oxidative markers also showed significant correlations among themselves and with all the functional and morphological attributes of sperm. The SOD activity revealed highly significant positive correlations with initial motility, HOS reactive sperm, live sperm and pre-freeze motility (0.281, 0.314, 0.281, 0.246), while it had negative correlations with total sperm abnormalities and MDA activity (-0.357, -0.497) in Murrah bulls.

Correlations Amongst Post-thaw Sperm Quality Attributes

In post-thawed semen of Gir bulls (Table 2), sperm motility was highly significantly (p<0.01) and positively correlated with post-thaw HOS reactive sperm, live sperm and intact acrosome (0.847, 0.923, 0.247) and negatively correlated with total sperm abnormalities (-0.302). The post-thaw HOS reactive sperm had significant positive and negative correlations only with post-thaw live sperm (0.782) and total sperm abnormalities (-0.209), while post-thaw live sperm showed negative correlations with post-thaw sperm abnormalities (-0.318) and positive correlation with intact acrosome (0.285). Post-thaw total sperm abnormalities showed negative correlations with intact acrosome and SOD (-0.234, -0.421). The post-thaw seminal plasma MDA and GPx activity did not show significant correlation with any of the post-thaw sperm functional and morphological traits, but GPx had significant positive correlation with SOD (0.288) and negative correlation with MDA (-0.237). As compared to Gir bulls, *in Murrah bulls* most of the post-thaw traits were highly significantly (p<0.01) interrelated, and the activity of MDA had inverse correlations to those of SOD and GPx activity with all the sperm functional and morphological attributes (0.225 to 0.926) (Table 2).

Correlations of Fresh Semen with Post-thaw Quality Attributes

The associations of sperm quality attributes and oxidative markers of freshly diluted semen to those of post-thawed semen of *Gir bulls* evaluated (Table 3) revealed that initial motility had highly significant (p<0.01) positive correlations with post-thaw motility, live sperm and intact acrosome (0.301, 0.329, 0.317), post-thaw SOD & GPX activity (0.214, 0.292), and negative correlations with post-thaw sperm

Table 2: Correlations among spermatozoal attributes and oxidative markers of post-thawed semen of Gir and Murrah bulls in TFYG

Post-thaw Semen Traits	*PT Motility*	*PT HOST*	*PT Live Sp*	*Tail Ab*	*Total Abn*	*Intact Acrosome*	*PT MDA*	*PT SOD*	*PT GPX*
Correlations: Gir bull semen									
Post-thaw (PT) Motility	1	0.847**	0.923**	-0.269*	-0.302**	0.247*	0.010	0.077	0.063
Post-thaw HOS Test	0.926**	1	0.782**	-0.152	-0.209*	0.021	0.010	0.048	0.091
Post-thaw Live Sperm	0.895**	0.771**	1	-0.337**	-0.318**	0.284**	-0.046	0.098	0.171
PT Tail Abnormality	-0.324**	-0.266*	-0.314**	1	0.779**	-0.264*	0.081	-0.291**	-0.111
PT Total Abnormality	-0.447**	-0.379**	-0.450**	0.857**	1	-0.234*	0.085	-0.421**	-0.071
PT Intact Acrosome	0.286**	0.280**	0.378**	-0.334**	-0.418**	1	-0.147	0.169	0.189
PT LPO/MDA	-0.235*	-0.153	-0.232*	0.441**	0.419**	-0.102	1	-0.007	-0.237*
PT SOD activity	0.316**	0.306**	0.273**	-0.086	-0.229*	0.244*	-0.371**	1	0.288**
PT GPX activity	0.498**	0.374**	0.514**	-0.373**	-0.362**	0.111	-0.277**	0.037	1
Correlations: Murrah buffalo semen									

*Significant at the 0.05 level, **Significant at the 0.01 level (2-tailed), Number of paired observations = 90.

Table 3: Interrelationships of spermatozoa traits and oxidative markers between freshly diluted and frozen-thawed semen of Gir bulls

	Post-thaw (PT) quality attributes								
Freshly diluted semen	*PT Motility*	*PT HOST*	*PT Live Sperm*	*PT Tail Abnormality*	*PT Total Abnormality*	*PT Intact Acrosome*	*PT MDA*	*PT SOD*	*PT GPX*
Initial Sperm Motility	0.301**	0.148	0.329**	-0.427**	-0.441**	0.317**	-0.064	0.214*	0.292**
HOS Reactive Sperm	0.382**	0.357**	0.359**	-0.386**	-0.468**	0.198	0.015	0.174	0.208*
Live Sperm Per cent	0.321**	0.191	0.340**	-0.333**	-0.288**	0.342**	-0.106	0.185	0.298**
Tail Abnormalities	-0.254*	-0.093	-0.213*	0.462**	0.422**	-0.143	-0.132	-0.025	0.066
Total Sp Abnormalities	-0.280**	-0.140	-0.247*	0.468**	0.500**	-0.274**	0.002	-0.246*	-0.100
Intact Acrosome	0.352**	0.386**	0.535**	-0.402**	-0.269*	0.159	-0.109	0.069	0.316**
Pre-freeze Motility	0.620**	0.666**	0.662**	-0.321**	-0.284**	0.119	-0.042	0.031	0.300**
Malondialdehyde	-0.179	-0.014	-0.181	0.160	0.051	-0.468**	0.428**	-0.196	-0.337**
Superoxide Dismutase	0.010	0.025	0.013	-0.155	-0.288**	0.150	-0.061	0.902**	0.240*
Glutathione Peroxidase	0.112	0.130	0.203	-0.119	-0.084	0.155	-0.255*	0.252*	0.905**

*Significant at the 0.05 level, **Significant at the 0.01 level (2-tailed), Number of paired observations = 90.

abnormalities (-0.441). HOS reactive sperm in fresh semen was positively correlated with post-thaw motility, HOS reactive sperm, live sperm and GPx activity (0.382, 0.357, 0.359, 0.208) and negatively correlated with post-thaw sperm abnormalities (-0.468), while initial live sperm revealed significant positive correlations with post-thaw motility, live sperm, intact acrosome and GPx activity (0.321, 0.340, 0.342, 0.298) and negative correlations with post-thaw sperm abnormalities (-0.288). The total sperm abnormalities of fresh semen revealed significant negative correlations with post-thaw motility, live sperm, intact acrosome and SOD activity (-0.280, -0.247, -0.274, -0.246) and positive correlations with post-thaw sperm abnormalities (0.500). The intact acrosome in fresh semen had significant positive correlations with post-thaw motility, HOS reactive sperm, live sperm and intact acrosome (0.352, 0.386, 0.535, 0.316) and negative correlations with post-thaw sperm abnormalities (-0.269). Pre-freeze motility also showed significant positive correlations with post-thaw motility, HOS reactive sperm, live sperm and GPx activity (0.620, 0.666, 0.662, 0.300) and negative correlations with abnormalities (-0.284). The correlations of all three oxidative markers of fresh semen with post-thaw functional and morphological attributes were negligible in Gir bulls. However, the MDA concentration in fresh semen revealed significant positive correlation only with post-thaw MDA levels (0.428) and negative correlations with post-thaw intact acrosome and GPx activity (-0.468, -0.337), while SOD activity in fresh semen showed negative correlations with post-thaw sperm abnormalities (-0.288) and positive correlations with post-thaw SOD and GPX activity (0.902, 0.240). The GPx profile in fresh plasma had significant negative correlations with post-thaw MDA levels (-0.255) and positive correlations with post-thaw SOD and GPx activity (0.252, 0.905).

In *Murrah bulls,* correlations evaluated (Table 4) showed that initial motility, HOS reactive sperm and live sperm of freshly diluted semen had significant ($p<0.01$) positive correlations with post-thaw motility (r= 0.640, 0.633, 0.556 resp.), post-thaw HOS reactive sperm (0.568, 0.617, 0.470), post-thaw live sperm (0.624, 0.588, 0.555), post-thaw intact acrosome (0.267, 0.145, 0.237), post-thaw SOD (0.317, 0.390, 0.345) and post-thaw GPX activity (0.376, 0.267, 0.356 resp.), and negative correlations with post-thaw sperm abnormalities (-0.399, -0.259, -0.394). The total sperm abnormalities in fresh semen had significant negative correlations with post-thaw live sperm, intact acrosome and SOD activity (-0.263, -0.293, -0.402) and positive correlations with post-thaw MDA levels (0.384). The intact acrosome in fresh semen had significant positive correlations with post-thaw live sperm and intact acrosome (0.295, 0.390). Pre-freeze motility also showed highly significant positive correlations with post-thaw motility, HOS reactive sperm, live sperm, intact acrosome (0.757, 0.641, 0.716, 0.326), post-thaw SOD and GPx activity (0.216, 0.358). Moreover, the MDA concentration in fresh semen of buffalo bulls revealed significant negative correlation with post-thaw motility, HOS reactive sperm, live sperm (-0.306, -0.217, -0.278), post-thaw SOD and GPx activity (-0.478, -0.286) and positive correlations with post-thaw sperm abnormalities (0.464) and MDA levels (0.929), while SOD and GPx activity in freshly diluted seminal plasma showed significant positive correlations with post-thaw motility (0.314, 0.397), HOS reactive sperm (0.301, 0.283), live sperm (0.237, 0.452), intact acrosome (0.223, 0.151) and negative correlations with post-thaw sperm abnormalities (-0.232, -0.381), and post-thaw MDA levels (-0.415, -0.205). The SOD activity in fresh and post-thaw semen (0.907) and GPX activity in fresh and post-thaw semen (0.910) were significantly and positively interrelated (Table 4).

These findings on correlation coefficients observed among functional and morphological attributes of sperm in Gir and Murrah buffalo semen corroborated well with many of the previous reports, particularly of Shelke and Dhami (2001), Lodhi *et al.* (2008), Tiwari *et al.* (2009), Patel *et al.* (2012), Mahmoud *et al.* (2013), Chaudhari *et al.* (2014) and Pathak *et al.* (2018) in bovine semen. However, it was hard to find any report of direct correlation between sperm quality

Table 4: Interrelationships of spermatozoa traits and oxidative markers between freshly diluted and frozen-thawed semen of Murrah bulls

	Post-thaw (PT) quality attributes								
Freshly diluted semen	*PT Motility*	*PT HOST*	*PT Live Sperm*	*PT Tail Abnormality*	*PT Total Abnormality*	*PT Intact Acrosome*	*PT MDA*	*PT SOD*	*PT GPX*
Initial Sperm Motility	0.640**	0.568**	0.624**	-0.255*	-0.399**	0.267*	-0.150	0.317**	0.376**
HOS Reactive Sperm	0.633**	0.617**	0.588**	-0.092	-0.259*	0.145	-0.083	0.390**	0.267*
Live Sperm Per cent	0.556**	0.470**	0.555**	-0.233*	-0.394**	0.237*	-0.142	0.345**	0.356**
Tail Abnormalities	-0.074	-0.109	-0.160	0.250*	0.255*	-0.295**	0.337**	-0.089	-0.111
Total Sp Abnormalities	-0.198	-0.202	-0.263*	0.216*	0.356**	-0.293**	0.384**	-0.402**	-0.028
Intact Acrosome	0.205	0.176	0.295**	-0.241*	-0.211*	0.390**	-0.094	0.069	0.182
Pre-freeze Motility	0.757**	0.641**	0.716**	-0.402**	-0.506**	0.326**	-0.158	0.216*	0.358**
Malondialdehyde	-0.306**	-0.217*	-0.278**	0.429**	0.464**	-0.174	0.929**	-0.478**	-0.286**
Superoxide Dismutase	0.314**	0.301**	0.237*	-0.115	-0.232*	0.223*	-0.415**	0.907**	0.036
Glutathione Peroxidase	0.397**	0.283**	0.452**	-0.389**	-0.381**	0.151	-0.205	0.015	0.910**

*Significant at the p<0.05 level, **Significant at the p<0.01 level (2-tailed), Number of paired observations = 90.

attributes and oxidative markers in fresh or frozen-thawed bovine semen supplemented with Sericin.

Raval and Dhami (2010) recorded highly significant (p<0.01) positive correlation for initial motility with mass activity and negative correlations for total abnormal sperm with initial motility and live sperm in crossbred bulls. Patel *et al.* (2012) found significant (p<0.01) positive interrelationships between individual sperm motility, viability and plasma membrane integrity both in fresh and post-thawed semen. Rao *et al.* (2012) found fertility rate to first insemination to be positively correlated with mass activity, initial motility and sperm concentration in semen of Ongole bulls. A significant positive correlation existed between intact acrosome, motility and live sperm (Agrawal, 1997). Likewise, a high correlation of percent intact acrosome with fertility has also been indicated earlier (Saacke and White 1972). Acrosomal integrity was found positively correlated with post-thaw livability and motility in crossbred bull semen (Prasad *et al.,* 1999). A highly significant positive correlation (0.43) between HOS reacted sperms after dilution and after freezing of bull semen was found by Shukla *et al.* (2011).

Present findings also corroborated with those of Rana and Dhami (2003), Chaudhari *et al.* (2017) and Pathak *et al.* (2018), who found significant (p<0.01) interrelationships for the percentages of motile, live, abnormal sperms, intact acrosome and HOS reactive spermatozoa of fresh semen with post-thawed semen of bovine and bubaline species (0.17 to 0.90). Perumal *et al.* (2011) recorded significant (p<0.05) correlations for post-thaw sperm progressive motility (r=0.4) and acrosomal integrity (r=0.8) of bull sperm with field fertility results. Further, the results of present study showed that sperm motility, viability, HOS reactive sperm and MDA production may be good markers for semen quality considering significant correlations found between them. These observations are also in agreement with the opinion of Mahmoud *et al.* (2013). Dhami and Sahni (1994) recorded highly significant positive correlations (0.68 to 0.98) for the sperm motility traits of liquid and frozen-thawed semen of HF bulls at various processing steps, and concluded that freezability of semen could be predicted based on its initial quality and keeping quality at 5°C.

Conclusions

In the present study significant correlations of progressive sperm motility, viability sperm abnormalities, as well as acrosome and plasma membrane integrity with oxidative markers were observed in both fresh, post-thawed and fresh with post-thawed semen of Gir cattle and Murrah buffalo semen together with HOST. These findings in general suggest that motility estimation in fresh, pre-freeze and post-thawed semen is a legitimately good indicator of quality of spermatozoa at various steps of semen processing/ freezing, and its fertilizing potential. Hence, sperm motility and HOS reactivity with either MDA or SOD/GPx activity alone can be adopted in routine assessment of semen quality, rather than going into the time consuming clumsy staining procedures for evaluation of viability, morphology and acrosomal integrity, or for ELISA based oxidative multi-marker assays, which in fact are not always correlated with *in vivo* fertility. The seminal plasma MDA and SOD/GPx revealed inverse correlations themselves and MDA with most of the spermatozoa functional and morphological attributes of fresh, frozen and fresh with frozen-thawed semen, hence it was concluded that any one of these markers in addition to motility and HOST could be a valuable and practical tool to know the functional capacity of fresh and cryopreserved cattle and buffalo spermatozoa.

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