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Effect of reduced glutathione supplementation in semen extender on tyrosine phosphorylation and apoptosis like changes in frozen thawed Hariana bull spermatozoa

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ABSTRACT

To provide new insights into the mechanisms through which reduced glutathione (GSH) is able to protect spermatozoa, we tested the hypothesis that cryocapacitation and apoptosis like changes can contribute to the negative effect of freezing and thawing on bull spermatozoa, and that GSH prevent this damage. Having known protective effects of GSH in terms of a potent antioxidant, we evaluated capacitation, tyrosine phosphorylation and apoptosis like changes in bull spermatozoa after freezing and thawing in egg yolk tris glycerol extender containing (0.5 m M-GSH-T1 & 1 mM GSH-T2) and without GSH serving as the control (C). Forty ejaculates were collected from four Hariana bulls and were pooled due to non significant variations among the bull ejaculates for the evaluation of sperm attributes. Capacitation like changes, tyrosine phosphorylation, localization of tyrosine phosphorylated proteins, apoptosis like changes in terms of mitochondrial transmembrane potential and DNA fragmentation after final dilution, 4 h of equilibration at 4 °C and 24 h after freezing and thawing were evaluated. GSH supplementation at 0.5 mM showed significant reduction in B- and AR- pattern spermatozoa during all stages of semen freezing and thawing. Immunoblot revealed six proteins which were tyrosine phosphorylated and protein of 30 and 75 kDa (p30, p75) were the major tyrosine phosphorylted proteins. On further analysis, the p30 showed differential variation in intensity in all the three groups after freezing and thawing. Positive immune reactivity for tyrosine phosphorylated proteins was found in neck, middle piece and post-acrosomal regions of spermatozoa. Addition of 0.5 mM GSH decreased percentage of spermatozoa showing fragmented DNA and increased the percentage of spermatozoa having high transmembrane mitochondrial potential ($P < 0.05$). This study demonstrates that GSH favours survival of bull spermatozoa by interfering with apoptotic and cryocapacitation pathways, and thereby protects the spermatozoa from deleterious effects of cryopreservation. The findings of the study indicated that GSH at 0.5 mM can be effectively used as an additive in bull semen extender for freezing and thawing.

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1. Introduction

Cryopreserved mammalian semen is generally acknowledged due to lower post-thaw viability and sub-lethal dysfunction in a proportion of the surviving subpopulation (Watson, 2000). This leads to decreased sperm quality, altered sperm function and reduced fertilizing competence of spermatozoa (Aziz et al., 2004). It is now well accepted that oxidative stress is an inevitable phenomenon occurs during cryopreservation of almost all of the mammalian spermatozoa (Tatone et al., 2010; Bansal and Bilaspuri, 2011). Growing evidence also suggests that oxidative stress as a result of excessive production of free radicals is one of the major contributing factor (Aitken et al., 2016).

Spermatozoa are highly vulnerable to oxidative stress because of limited intrinsic antioxidant protection system and high content of polyunsaturated fatty acids in the plasma membrane (Aitken et al., 2016). The susceptibility to oxidative attack is further exacerbated due to dilution of seminal antioxidant enzymes during extension of semen and an excessive generation of free radicals at freezing and thawing (Chatterjee et al., 2001). Oxidative stress causes defective sperm function by disrupting the integrity of sperm DNA and damage to proteins and lipids in the sperm plasma membrane (Aitken et al., 2014). Further, under stressful conditions they default to an intrinsic apoptotic pathway characterized by mitochondrial ROS generation, loss of mitochondrial membrane potential, caspase activation, phosphatidylserine exposure and oxidative DNA damage (Aitken et al., 2016). Free radical also induce capacitation, acrosome reaction and apoptosis like changes in spermatozoa during freezing and thawing and thus limits the fertilizing potential of spermatozoa (Agarwal et al., 2003; Aitken et al., 2016). Considering the above, development of antioxidant formulations is becoming increasingly urgent to ameliorate the oxidative damage of spermatozoa (Aitken et al., 2016).

To date, a number of strategies have been developed to reduce the free radical mediated damage to spermatozoa (Bucak et al., 2007; Bucak et al., 2009). Among those, the exogenous addition of antioxidant in the form of enzymes, vitamins, and amino acids to extenders has been tried in several studies to enhance the post thaw quality of spermatozoa. Further, the beneficial effects of antioxidants against the free radical mediated damage to the sperm cells of are also reported in bull (Bucak et al., 2007; Gadea et al., 2008), buck (Bucak et al., 2009; Atessahin et al., 2008), ram (Uysal et al., 2007) and swine (Gadea et al., 2005; Satorre et al., 2007; Funahashi and Sano, 2005).

Reduced glutathione (GSH) is synthesized in a controlled manner intra-cellularly in all mammalian cells and is involved in detoxification, antioxidant defense, and modulation of cell proliferation (Lu, 2009). It directly reacts with free radicals and catalyses the reduction of toxic H_2O_2 in to water and hydro peroxides (Bilodeau et al., 2001). Both removal as well as dilution of seminal plasma during semen freezing is reported to cause reduction in GSH content. This results in to reduced GSH availability to spermatozoa as they fails to synthesize GSH by own (Gadea et al., 2011). Further, in a study it is also shown that supplementation of GSH in the extenders prior to freezing minimized the oxidative damage caused due to free radical generation (Gadea et al., 2005). In the present study, we attempted to determine the effect of exogenous addition of GSH in the extender prior to freezing on capacitation, acrosome reaction, and apoptosis like changes in spermatozoa of Hariana bull.

2. Materials and methods

2.1. Ethical permission

Ethical permission was taken from Institutional Animal Ethics Committee for collection of semen from bulls using artificial vagina (AV). Due care was taken not to provide any stress to the bulls during the period of semen collection by AV.

2.2. Chemicals and reagents

FITC- PSA, Chlortetracycline hydrochloride, DAB system (Sigmafast Tablets), goat anti mouse IgG-HRP, Anti mouse IgG- FITC conjugate, DABCO, HEPES were purchased from (Sigma, St Louis, USA). PBS, total cell lysis protein isolation buffer (Amresco, USA), DMSO (Merck, Germany, USA), protein quantification kit (Gennei, Merck, India), Mito Capture Apoptosis detection kit (Merck-Millipore, Germany). All other chemicals for electrophoresis and western blotting were procured from Amresco, USA unless and until stated.

2.3. Collection of semen

Semen samples were collected during morning hours between 8–9 a.m. using AV as per the standard procedure from four apparently healthy Hariana bulls, maintained under semi intensive system of rearing. Total forty ejaculates were collected (ten ejaculate from each bull). Immediately after collection, tubes containing semen were placed in water bath at 32–34 °C for further processing in the laboratory.

2.4. Processing of semen for initial evaluation

Screening of 8 ejaculates from each bull revealed that the bulls were not showing significant variation among the ejaculates. Further, in order to eliminate individual differences in bulls, the ejaculates were mixed in a pool for balancing the sperm contribution of each bull (Chauhan et al., 2017; Yadav et al., 2017). During the study, the ejaculates having mass motility more than 3.5, progressive motility more than 80%, and live per cent more than 90 were selected. Sperm concentration of pooled bull ejaculates were determined using automatic sperm counter and dilution was carried out with glycerolated egg yolk tris extender having 20%