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Influence of Adding Manganese to Tris Extender on some Post-Cryopreservation Semen Attributes of Holstein Bulls

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Abstract: This study was conducted to explore the adding effect of manganese (Mn^{+2}) to Tris extender on post cooling and cryopreservation semen attributes of Holstein bulls for different periods. Seven Holstein bulls of 3.5-4.5 years old were used in this study. Pooled semen was divided into three groups. The first group was diluted with Tris only (control group, G1). While the 2nd (G2) and 3rd (G3) groups were added 0.7 and 0.9 mM Mn^{+2}respectively. The effect of these additions on Holstein bulls semen quality was studied during different periods (cooling at 5 Celsius, 72 hrs.,1st , 2nd and 3rd post cryopreservation, PC). Addition of Mn^{+2} led to significant increases of total ($\times 10^6$) plasma membrane integrity of sperms and total function sperm fraction as compared with control groups during cooling and PC periods. It can be concluded that the addition two levels of acid Mn^{+2} led to enhanced PC semen quality of bulls. This will in turn improved fertility rate of cows and owner's economic income.

Keywords: Mn^{+2}, Cryopreservation, Holstein Bulls Semen

1. Introduction

Plasma membrane and acrosome are important sites for the changes caused by cryopreservation(associated with oxidative stress) process. Cryopreservation affects the lipid architecture of plasma membrane [1 and] and metabolism due to the high amount of polyunsaturated fatty acids (PUFAs) with significantly less cytoplasmic components containing antioxidants [2]. The PUFAs plays an important role in regulating sperm membrane fluidity and spermatogenesis [3]. Alteration of the membrane fluidity during freezing-thawing processes will cause accumulation of calcium ion which consequently impaired sperm motility and eventually endanger the sperm survival [4]. Bovine sperm themselves have only few amounts of endogenous antioxidants for the protection against reactive oxygen species (ROS) and the main antioxidant source is the seminal plasma [5 and 6]. Several ROS produced by both sperm and leukocytes contaminating the seminal fluid, adversely affect sperm motility and impair fertilizing ability [7]. Antioxidative mechanisms protect the sperm from the damage caused by free radicals [8].

Manganese is an essential ultra-trace element, needed for a variety of physiological processes ranging from the regulation of reproduction to normal brain functions [9]. The M^{+2} is an important cofactor of mitochondrial superoxide dismutase, an antioxidant enzyme which scavenges oxygen free radicals [10]. It inhibits LPO produced by a free radical producing system, as potent antioxidant against oxidative stress at low doses [11]. Mn^{+2} supplementation reduced the leakage of lipids and phospholipid sperm contents under normal and induced oxidative stress conditions [12]. Mn^{+2} is able to penetrate the cell easily and help sperm to maintain or recover appropriate ion balance. Thus, it suffer less from the freezing and thawing procedures [13]. Bansal et al [14] suggest the role of Mn^{+2} supplementation in improving the quality of bull semen by its scavenging property, through reduction of ROS production during its incubation at 37°C for capacitation or storage at 4°C . Moreover, Eidan et al [15] recorded that addition of Mn^{+2} to Tris extender led to significant increases of total ($\times 10^6$) motile spermatozoa, acrosomal integrity of sperms and normal morphology of sperms as compared with control groups at different PC periods

The previous studies did not identify the accurate concentrations of these relevant antioxidants, and contradictory results have been reported. Higher concentrations of Mn^{+2} may give better PC semen

characteristics of Holstein bulls. Therefore, the objective of this study was to investigate effect of adding Mn^{+2} to Tris extender on some PC semen characteristics of Holstein bulls.

2. Materials and Methods

Seven Holstein bulls, 3.5- 4.5 years old and 550-770 kg live body weight, were trained for semen collection using artificial vagina. The experimental bulls have good health and free of disease, being under the veterinarian supervision permanently. Animals fed 4 kg concentrate ration/ bull /day composed of 33% wheat bran, 20% soybean, 35% barley, , 10% yellow corn, 0.5% salt 0.5% limestone, and 1% vitamins and minerals. Total crude protein and energy contents were 18% and 3323 kcal / kg respectively. Green fodder (51-61 kg / bull / day) alfalfa hay (8-9 kg/bull /day) were also introduced. Pooled semen was taken from each bull and combining together to remove the individual variations among bulls. The extender was prepared according to the method of Salamon and Maxwell [16]. Semen was divided equally to three groups. First group was considered as a control group (G1) diluted with Tris only. Manganese (Gailad Chemical Company\UK) was added to G2 (0.5 mM) and G3 (1.0 mM) treatments respectively. Semen evaluation was done on each treatment following cooling (5˚ C) and 72 hours, 1st , 2nd and 3rd PC periods. Semen characteristics involve total plasma membrane integrity of sperms(TPMIS), total osmotic shock of sperms(TOSS) and total function sperm fraction (TFSF) in each straw (20×10^6 sperm) were determined according to Jeyendran et al [17], Zavos [18] and Johnston et al [19]. The statistical computations were performed using SAS program [20] based on completely randomized design (CRD) to study the effect of different factors on the studied characteristics. Means with significant differences were compared using Duncan multiple range test [21].

3. Results and Discussion

Highly significant ($p\leq0.01$) effect of two Mn^{+2} levels was noticed on TPMIS among different preservation periods (cooling,72 hrs.,1st, 2nd and 3rd PC) as compared with control group (Table 1). Furthermore, TPMIS was significantly greater ($p\leq0.01$) in G3 treatment ($14.61\pm0.25\times10^6$) in comparison with G2 ($13.22\pm0.46\times10^6$) treatment at 72 hrs. PC period. No significant differences were observed in TPMIS at different preservation periods within G1 and G2 treatments. On the other hand, G3 treatment exhibited significant ($p\leq0.05$) differences in TPMIS ($14.6\pm0.2\times10^6$) during 72 hrs. as compared with both 2nd ($13.6\pm0.2\times10^6$) and 3rd ($13.6\pm0.2\times10^6$) month PC (Table 1).

The TOSS was decreased ($p\leq0.01$) in G3 ($1.20 \pm 0.06\times10^6$) and G2 ($1.50 \pm 0.07\times10^6$) groups as compared with G1 group ($1.74 \pm0.06\times10^6$) at cooling. The G3 treatment exhibited lower ($p\leq0.01$) TOSS value ($1.23\pm 0.06\times10^6$) in comparison with G1($1.59 \pm 0.09\times10^6$) at 72 hrs. PC period (Table 2). On the other hand, no significant differences were recorded between G3($1.23\pm 0.06\times10^6$) and G2 ($1.44\pm 0.07\times10^6$) at 72 hrs. PC period. G3 treatment exhibited lower TOSS value in comparison with both G1 and G2 at 1st ,2nd and 3rd PC periods(Table 2). No significant differences were noticed among the overall experimental periods (5˚ C,72 hrs.,1st 2nd and 3rd PC period) within each treatment (Table 2).

The G2 ($6.54\pm0.44\times10^6$) and G3 ($7.27\pm0.41\times10^6$) groups exhibited higher ($p\leq0.01$) TFSF values as compared with G1 group ($3.79\pm0.27\times10^6$) at cooling period (Table 3). The G3($6.37 \pm 0.33\times10^6$) and G2 ($5.65 \pm 0.38\times10^6$) groups recorded highly significant differences ($p\leq0.01$) in TFSF values as compared with G1 group ($2.88 \pm 0.18\times10^6$) at 72 hrs. PC period (Table 3). The G2 (4.20 ± 0.51- 4.93 ± 0.37×10^6) and G3 (5.09 ± 0.32- 5.56 ± 0.29×10^6) groups exhibited higher ($p\leq0.01$) TFSF values as compared with G1group (2.07 ± 0.25 - 2.25 ± 0.18×10^6) during 1st , 2nd and 3rd PC periods (Table 3).

Highly significant differences ($p\leq0.01$) were noticed in TFSF ($\times10^6$) values between cooling and the overall PC periods within group G1 (Table 3). Within the relevant characteristic and group, no significant differences were noticed among 1st , 2nd and 3rd month PC (Table 3). On the other hand, TFSF ($\times10^6$) was greater ($p\leq0.01$) at cooling (6.54 ± 0.44) in comparison with 1st (4.93±0.37), 2nd (4.48 ± 0.37) and 3rd (4.20 ± 0.51) month PC within G2 group (Table 3). Meanwhile, no significant differences were noticed among 1st , 2nd and 3rd month PC within G2 group (Table 3). Cooling (7.27 ± 0.41), 72 hrs. (6.37±0.33) and 1st (5.56±0.29) month PC were superior ($p\leq0.01$) in TFSF ($\times10^6$) values as compared with those at 2nd (5.12 ± 0.37) and 3rd (5.09 ± 0.32) month PC within G3 group (Table3).

Table 1. *The Effect of adding Mn+2 to Tris extender on the total plasma membrane integrity of sperms (TPMIS $\times10^6$) at cooling and different post-cryopreservation (PC) periods in Holstein bulls.*

Period Treat.	5˚ C	72 hrs. PC	1st mo. PC	2nd mo. PC	3rd mo. PC	Sig.
G1	10.7 ± 0.5 B a	10.8 ± 0.5 C a	10.2 ± 0.6 B a	9.9 ± 0.5 B a	9.8 ± 0.5 B a	ns
G2	13.2 ± 0.6 A a	13.2 ± 0.4 B a	12.8 ± 0.5 A a	12.8 ± 0.5 A a	12.6 ± 0.6 A a	ns
G3	14.3 ± 0.3 A ab	14.6 ± 0.2 A a	14.0 ± 0.2 A ab	13.6 ± 0.2 A b	13.6 ± 0.2A b	*
Sig.	**	**	**	**	**	

Means with capital superscripts within each column indicate comparison among treatments and small superscripts within each row indicate comparison among periods within each treatment. mo.: month. Sig.: significance. ns: no significant. **($p\leq0.01$). G1: Tris extender (Control group). G2: Tris extender + 0.7mM Mn^{+2}. G3: Tris extender + 0.9 mM Mn^{+2}.

The Addition of two Mn^{+2} levels had an obvious effect ($p\leq0.01$) in improving TPMIS and TFSF as compared with control groups during cooling and PC periods. Some of these results were in line with Lapointe et al [13] who found that

the addition of 0.1 mM Mn^{+2} to semen extender enhanced PC individual motility (53%) as compared with control group (28%). The current results also agreed with Cheema et al [22] who found that the addition of Mn^{+2} (150 µM) to egg-yolk-citrate extender increased PC individual motility (58.3%) in comparison with those of control group (45%). Similar authors found that the addition of Mn^{+2} to

Table 2. *The Effect of adding Mn+2 to Tris extender on the total osmotic shock (TOSS×10⁶) at cooling and different post-cryopreservation (PC) periods in Holstein bulls.*

Period Treat.	5° C	72 hrs. PC	1st mo.PC	2nd mo. PC	3rd mo.PC	Sig.
G1	1.74 ± 0.06 A a	1.59 ± 0.09 A a	1.61 ± 0.08 A a	1.60 ± 0.08 A a	1.69 ± 0.08 A a	ns
G2	1.50± 0.07 B a	1.44± 0.07 AB a	1.53 ± 0.07 A a	1.72 ± 0.15 A a	1.54 ± 0.06 A a	ns
G3	1.20± 0.06 C a	1.23± 0.06 B a	1.23± 0.09 B a	1.20± 0.07 B a	1.29± 0.09 B a	ns
Sig.	**	**	**	**	**	

Means with capital superscripts within each column indicate comparison among treatments and small superscripts within each row indicate comparison among periods within each treatment. mo.: month. Sig.: significance. ns: No significant. **(p≤0.01). G1: Tris extender (Control group). G2: Tris extender with 0.7mM Mn^{+2}. G3: Tris extender with 0.9 mM $Mn^{+2.}$

Table 3*. Effect of adding Mn^{+2} to Tris extender on the Total function sperm fraction (TFSF) (×10⁶) at cooling and different post-cryopreservation (PC) periods in Holstein bulls.*

PeriodTreat.	5° C	72 hrs. PC	1st mo. PC	2nd mo. PC	3rd mo. PC	Sig.
G1	3.79± 0.27 B a	2.88± 0.18 B b	2.25± 0.18 Bcb	2.15± 0.23 B c	2.07± 0.25 B c	**
G2	6.54 ± 0.44 A a	5.65 ± 0.38 A ab	4.93 ± 0.37 Abc	4.48 ± 0.37 A bc	4.20 ± 0.51 A c	**
G3	7.27 ± 0.41 A a	6.37±0.33 A ab	5.56 ± 0.29 Aab	5.12 ± 0.37 A c	5.09 ± 0.32 A c	**
Sig.	**	**	**	**	**	

Means with capital superscripts within each column indicate comparison among treatments and small superscripts within each row indicate comparison among periods within each treatment. mo.: month. Sig.: significance. ns: No significant. **(p≤0.01). G1: Tris extender (Control group). G2: Tris extender with 0.7mM Mn+2. G3: Tris extender with 0.9 mM Mn+2.

Tris extender improved PC plasma membrane integrity (30.1%) in comparison with the control group (19.7%). Moreover, these data were agreed with those of Eidan et al "in press" [23] and Eidan and Sultan "unpublished" [24] who observed that addition of 0.7 and 0.9 mM of Mn^{+2} to Tris extender have improved individual motility, live sperm, sperm plasma membrane integrity, freezing ability percentages and decreased Malondialdehyde (MDA) concentrations at cooling and post-cryopreservation of semen.

However, excluding data of Eidan and Sultan "unpublished" [24], the current Mn^{+2} concentrations are higher than those pointed out in the previous studies. Many preliminary trials were conducted to select these concentrations based on their significant effects. It is worthy to mention that the concentrations used by the previous studies had negative effects on PC semen characteristics when used in the preliminary trials. Therefore, it is the first study that describes the effect of high Mn^{+2} concentrations (0.7 and 0.9 mM) as added to Tris extender on post-cryopreservation semen quality in Holstein bulls. Manganese is an important cofactor of mitochondrial superoxide dismutase antioxidant enzyme which scavenges oxygen free radicals [25 and 26]. Manganese may stimulate the enzymes of glutathione cycle and affect the total thiols, glutathione reduced and glutathione oxidized contents in bull and human spermatozoa [12 and 27]. It acts as a potent antioxidant in protection against OS [24 and 28]. Currently, Mn^{+2} enhanced TFSF may be return to stimulates the activity of sperm adenylate cyclase enzyme to maximum extent [29]. Manganese is a potent stimulator of adenylate cyclase activity in the sperm cells, and cyclic adenosine monophosphate (cAMP) concentrations are correlated with the cell motility [13]. Mann and Mann [30] and Eddy and O'Brien [31] have described that energy transduction within sperm tail, made possible by the molecular diffusion of mitochondrial adenosine triphosphate (ATP) along the flagellum for rhythmic flagellar movements. This energy, according to these workers is generated along the flagellum by a mechanic-chemical process coupled to enzymatic dephosphorylation of ATP. However, this ATP dephosphorylation is not entirely irreversible. Some of the dephosphorylated ATP can be resynthesized as a result of axonemal adenylate kinase activity. Addition of Mn^{+2} to Tris extender enhanced total plasma membrane integrity and total function of sperm fraction through chain-breaking antioxidant as it is able to quench peroxyl radicals and decreasing the OS [24, 25 and 27]. It decreases the production of thiobarbituric acid reactive substances [24 and 28]. High intracellular manganese provides protection against oxidative damage through unknown pathways [32]. The efficacy of manganese as an antioxidant has been drastically reduced in cells with hyper accumulates phosphate [32]. It is well known, that Mn^{+2} is a potent inhibitor of LPO in a variety of systems, while it also exerts a superoxide radical

scavenging action, which may account for the inhibition of LPO [24]. Some studies proposed that Mn^{+2} might forming a complex with unsaturated lipids making them more resistant to attack by peroxides[33]. Mn^{+2} inhibits the free radical chain which follows the formation of hydro peroxides and formation of malondialdehyde consequently[24]. Mn^{+2} is able to form complexes with $O2^{\bullet}$ and OH^{-} giving rise to complexes like MnO_2^{+2} and $Mn(OH)^{+2}$. It has been reported that Mn^{+2} is able to scavenge the superoxide anion. The interaction of Mn^{+2} with the reported free radical species supports the hypothesis of general antioxidant action that might occur through the reduction of lipid free radicals making them unable to carry on the process of LPO [24 and 33]. Mn^{+2} has also proved to be the best antioxidant in reducing the ferrous ascorbate-induced LPO in bull spermatozoa [34]. Manganese has beneficial influences on sperm survival during capacitation and acrosome reaction [12 and 15]. The manganese stabilizes the plasma membrane, thereby maintaining the membrane integrity and viability [15, 24 and 35].

In conclusion, addition of Mn^{+2} to Tris extender inhibits lipid peroxidation, and thus improving the total sperm membrane integrity, total function sperm fraction and the remaining semen characteristic at cooling and PC periods. This will certainly lead to enhance fertility of artificially-inseminated cows. The minimum sperm number necessary to obtain acceptable fertility rates is still a great challenge for the bovine artificial insemination industry. One of the alternatives to increase the efficiency of this technology for single and multiple ovulations is the increase total sperm membrane integrity, total function sperm fraction per insemination dose post-cryopreservation. This strategy could be especially interesting when associated with protocols for estrus synchronization or fixed-time artificial insemination, resulting in higher pregnancy per insemination and, consequently, higher economic return.

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