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COMPARISON OF FRESH SEMEN PARAMETERS WITH FROZEN THAWED SEMEN FOLLOWING INCORPORATION OF TREHALOSE

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ABSTRACT:

The research work was conducted to study comparison of fresh Kankrej bull's semen parameters with frozen thawed semen following incorporation of different Trehalose concentrations, on the semen samples collected from three Kankrej bulls through a period of 12 weeks. The collected semen samples were pooled and examined for fresh semen and oxidative stress parameters and then diluted with Tris-based extender containing different Trehalose concentrations viz. 50mM, 100mM, 150mM and control, and evaluated for semen and oxidative stress parameters at frozen thawed stage of cryopreservation. Results clearly indicated that, cryopreservation led to a significant ($P<0.05$) decrease in frozen semen parameters in comparison with fresh semen. Therefore, 100mM Trehalose incorporated group showed significantly ($P<0.05$) better values in comparison to that of fresh semen, 50mM Trehalose, 150mM Trehalose and control groups, respectively. Howover, the sperm abnormality was significantly ($P<0.05$) lower in fresh semen and frozen thawed 100mM Trehalose semen, when compared to

other and the control groups, respectively. Biochemical assays showed that, Malondialdehyde (MDA) level was significantly (P<0.05) higher in fresh semen in comparison to that of frozen thawed semen and Glutathione reductase (GSH) level was significantly (P<0.05) lower in fresh semen in comparison to that of frozen thawed semen. Conclusively, supplementation of 100mM Trehalose in the Tris-based extender is best concentration for cryopreservation of Kankrej bull's semen.

KEY WORD: *Trehalose, Kankrej bull, Oxidative stress, Malondialdehyde, Glutathione reductase*.

INTRODUCTION:

Gujarat state is well known for different breeds of cattle viz. Dangi, Gir, Kankrej and crossbred, but Kankrej is powerful draft cattle with fair milk production. Kankrej cattle at Livestock Research Station, Sardarkrushinagar has shown a noticeable potential with respect to milk production and disease resistance (Annual Progress Report, 2009). Many farmers in the state maintain 2-3 female cattle, but are unable to avail the sires with superior germplasm for the breeding. Artificial insemination helps in disseminating the frozen semen to rural areas for improvement of native cattle. Some of the semen characteristics such as sperm motility, viability, concentration, etc. are found to have significant correlation with freezability and/or fertility of bovine semen and hence are currently being used as routine tests for the assessment of semen quality (Bhoite *et al.*, 2005).

Various effects were observed when different sugars that are not capable of diffusing across a plasma membrane, such as lactose, sucrose, raffinose, trehalose, or dextrans are added to the diluent. The sugars create an osmotic pressure, inducing cell dehydration and results in lower incidence of intracellular ice formation. These sugars also interact with the phospholipids in the plasma membrane, reorganizing the membrane which results in sperm that is better suited to surviving the cryopreservation process (Uysal *et al.*, 2007). Addition of trehalose to bull semen extenders is known to provide a modest improvement in fertility when used in combination with glycerol (Badr *et al.*, 2010).

Cryopreservation of spermatozoa is associated with an oxidative stress induced by free radicals. In recent years, antioxidants have been used to protect spermatozoa from the deleterious effects of cryopreservation and free radicals are eliminated by antioxidants (Umut *et al.*, 2013). Sperm cells have a high content of unsaturated fatty acids in their membranes and they lack a significant cytoplasmic component containing antioxidants. Therefore, sperm cells are highly susceptible to lipid peroxidation (LPO) by O_2 and H_2O_2 When trehalose was added in hypertonic conditions, it showed a synergic effect with glycerol used as a cryoprotectant in order to avoid intracellular ice crystal

formation (Badr *et al.*, 2010). Semen contains appreciable amounts of antioxidants that balance lipid peroxidation and prevent excessive peroxide formation (Muzafer *et al.*, 2012). Glutathione, a naturally occurring tri-peptide in semen plays an important role in scavenging reactive oxygen intermediates and other radicals with the help of the glutathione reductase/peroxidase cycle. Glutathione can influence cell metabolism through detoxication and by preventing the formation of free radicals in spermatozoa (Serpil *et al.*, 2009). So, cryoprotectants are incorporated in tris-based extender to reduce the damaging effects during the process of freezing (Badr *et al.*, 2010; Purdy, 2006).

In view to the facts above, the present investigation was carried out to know frozen thawed characteristics of Kankrej bull semen and to determine suitable concentration of trehalose, which might help in improving preservability of Kankrej bull semen.

MATERIALS AND METHODS:

Total three Kankrej bulls aged between 4 to 5 years, maintained in good health under uniform veterinary care and in identical sanitary conditions of Dama semen production Unit, Banas dairy, Palanpur were used for research study. Total number of 36 ejaculates, 12 ejaculates from each bull was obtained once in a week using Artificial Vagina (Danish Model) maintained at 41^{o}C. Immediately after collection, semen collection tubes were placed in water bath at 37^{o}C until their fresh assessment in the laboratory. Tris-Fructose Egg Yolk Citrate Glycerol (TFYG) buffer was prepared for dilution of semen as described by Foote (1980) during the study. Ejaculates of semen with > 70 per cent initial motility were used for the study. The collected semen samples were pooled to split further into 4 equal aliquots and each one was diluted with Tris-Fructose Egg Yolk Citrate Glycerol (TFYG) freezing extender containing different Trehalose concentrations viz. 50mM, 100mM, 150mM and no additive (control) so as to have a final sperm concentration of 80 million sperms per ml. Extended semen aliquots were filled, sealed and printed in French Mini Straw of 0.25 ml capacity using automatic machine (IS-4, IMV-France) and were stored in Liquid Nitrogen at -196^{0}C. After cryopreservation period of 24 hrs, straws were thawed at 37^{0}C for 30 seconds in a water bath to evaluate frozen thawed individual motility, sperm viability, sperm abnormality, osmotic resistance test (HOST) and acrosomal integrity (MSP, GOI).

Individual motility was subjectively evaluated using the standard method (Bearden and Fuquay, 2000). The sperm viability and sperm abnormality were calculated using eosin-nigrosin stain as per the standard method (Evans and Maxwell, 1987). The acrosomal integrity of spermatozoa was

evaluated by means of modified giemsa staining technique (Manokaran *et al.*, 2010) with minor modification and was assessed by counting a total of 200 spermatozoa under phase contrast microscope. The per cent intact or damaged acrosomes were counted in different fields. The osmotic resistance test (HOST) was done to evaluate the functional integrity of the sperm membrane, based on curled and swollen tails as per the standard method (Revell and Mrode, 1994).

The seminal plasma was separated from fresh as well as frozen thawed semen straws by centrifugation at 5000 rpm for 10 min. and stored at -20° C before being assayed. The seminal plasma samples were thawed before analyzing the lipid peroxidation and glutathione reductase levels. Membrane peroxidative damage in seminal plasma was determined in terms of malondialdehyde (MDA) by using the standard method (Placer *et al.*, 1966). The values of MDA were expressed as µmol/ml. The GSH content of sperm was measured using the standard method (Sedlak and Lindsay, 1968). The values of GSH were expressed as U/L. The data were statistically analyzed using Completely Randomized Design (CRD) and Duncan New Multiple Range Test to determine levels of significance. The interrelationship was worked out as per the standard procedure (Snedecor and Cochran, 1994).

RESULTS AND DISCUSSION:

The semen physical parameters like individual motility, sperm viability, osmotic resistance test (HOST), acrosomal integrity in fresh and frozen thawed semen were assessed. The overall mean fresh semen per cent individual motility, sperm viability, sperm abnormality, osmotic resistance test (HOST) and acrosomal integrity of fresh semen were 88.75 ± 0.25, 89.69 ± 0.32, 3.08 ± 0.15, 86.80 ± 0.24 and 90.72 ± 0.25, respectively. The overall mean per cent individual motility, using different concentrations of Trehalose in frozen thawed semen were 58.00 ± 0.42 in 50mM; 64.16 ± 0.52 in 100mM, 57.58 ± 0.35 in 150mM Trehalose and 53.91 ± 0.57 in control group. The overall mean per cent sperm viability, using different concentrations of Trehalose in frozen thawed semen were 65.58 ± 0.35 in 50mM; 71.41 ± 0.31 in 100mM, 65.83 ± 0.40 in 150mM Trehalose and 60.33 ± 0.45 in control group. The overall mean per cent sperm abnormality, using different concentrations of Trehalose in frozen thawed semen were 8.58 ± 0.31 in 50mM; 5.75 ± 0.35 in 100mM, 9.58 ± 0.31 in 150mM Trehalose and 9.67 ± 0.35 in control group. The overall mean per cent HOST reactive sperm, using different concentrations of Trehalose in frozen thawed semen were 67.25 ± 0.32 in 50mM; 73.91 ± 0.35 in 100mM, 69.91 ± 0.22 in 150mM Trehalose and 66.50 ± 0.55 in control group. The overall mean per cent acrosomal integrity, using different concentrations of Trehalose in frozen

thawed semen were 72.41 ± 0.37 in 50mM; 80.91 ± 0.43 in 100mM, 72.91 ± 0.39 in 150mM Trehalose and 68.83 ± 0.38 in control group.

Cryopreservation led to a significant ($P<0.05$) decrease in semen physical parameters. These aforementioned values for 100mM Trehalose group were significantly ($P<0.05$) better as compared to that of fresh semen, 50mM Trehalose, 150mM Trehalose and control groups, respectively.

These findings are in accordance with Chhillar *et al.* (2012) in Karan-Fries bulls who have reported a significant ($P<0.05$) decrease in post-thaw motility, sperm viability and membrane integrity in frozen thawed semen as compared to fresh semen. Similarly, Badr *et al.* (2010) in buffalo bulls, Hu *et al.* (2010) in bovine bulls and Kumar *et al.* (2012) in buffalo (Murrah) and cattle (Karan Fries) bulls have also reported that the post thaw individual motility and sperm viability were significantly ($P<0.05$) decreased, whereas per cent sperm abnormality significantly ($P<0.05$) increased in frozen thawed semen as compared to fresh semen.

The semen oxidative stress parameters like lipid peroxidation, glutathione reductase in fresh and frozen thawed semen were assessed. The overall mean Malondialdehyde (MDA) and glutathione reductase (GSH) of fresh semen were 49.96 ± 0.06 µmol/ml and 30.14 ± 0.06 U/L respectively. The overall mean Malondialdehyde (MDA) values, using different concentrations of Trehalose in frozen thawed semen were 32.15 ± 0.09 µmol/ml in 50mM; 20.06 ± 0.13 µmol/ml in 100mM, 28.04 ± 0.08 µmol/ml in 150mM Trehalose and 31.98 ± 0.07 µmol/ml in control group. The overall mean glutathione reductase (GSH) values, using different concentrations of Trehalose in frozen thawed semen were 62.15 ± 0.08 U/L in 50mM; 84.00 ± 0.16 U/L in 100mM, 57.07 ± 0.06 U/L in 150mM Trehalose and 62.04 ± 0.07 U/L in control group.

Cryopreservation led to a significant ($P<0.05$) increase in Malondialdehyde (MDA) values and significantly ($P<0.05$) decrease in glutathione reductase (GSH) levels in comparison to that of fresh semen. These aforementioned values for 100mM Trehalose group were significantly ($P<0.05$) better as compared to that of fresh semen, 50mM Trehalose and 150mM Trehalose groups and control group, respectively.

The present investigation was in accordance with Chhillar *et al.* (2012) in Karan-Fries bulls who have reported that the post-thaw lipid peroxidation was significantly ($P < 0.05$) higher as compared to fresh but upon supplementation of 100mM Trehalose to freezing extender lipid peroxidation was significantly ($P < 0.05$) decreased. Similarly, Badr *et al.* (2010) in buffalo bulls and Hu *et al.* (2010) in bovine bulls have shown that supplementation of 50mM and 100mM Trehalose in egg yolk based extender improves sperm quality and oxidative stress parameters in frozen thawed bovine semen.

Trehalose has a protective action related to the osmotic effect and to specific interactions with membrane phospholipids, which renders the media hypertonic, thereby minimizing the degree of sperm cell injury during the freeze-thaw process (Kumar *et al.* 2012). The functional integrity of sperm acrosomal membrane and plasma membrane associated with sperm motility can be expected to have been destroyed by high doses of Trehalose.

In the present study, the highest protective effects of Trehalose were at the concentration of 100mM, and a much reduced extent at 150mM. The latter concentration resulted in a high osmolarity of the extender was in itself deleterious to the sperm cells. When Trehalose concentration was 150mM, the percent sperm motility, intact-acrosomal membrane, and intact-plasma membrane sperm of frozen-thawed bovine semen were decreased. Hu *et al.* (2010) shown in his research that antioxidant additives exhibited cryoprotective activity on certain sperm parameters in moderate doses, but increasing doses of antioxidant additives would result in a hypertonic property of extender and impair sperm function viz. sperm motility, membrane integrity and fertility.

The extender supplemented with 100mM trehalose resulted in the highest sperm motility, acrosomal membrane integrity, and plasma membrane integrity in this study. There was an increase in the levels of GSH and decrease in LPO values in Trehalose supplemented groups during the cryopreservation of semen in the present study.

CONCLUSIONS:

Fresh semen parameters viz., individual motility, sperm viability, plasma membrane integrity and acrosomal integrity were better in fresh Kankrej bull's semen than all other cattle breeds in India. The frozen thawed semen supplemented with 100mM Trehalose reduced the oxidative stress provoked by cryopreservation and improves individual motility, acrosomal membrane integrity and plasma membrane integrity, except abnormal sperm count which was lower than other additive groups. Hence, the optimum concentration of 100mM Trehalose was determined to be effective for Kankrej bull's semen.

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Table 1. COMPARISION OF FRESH SEMEN PARAMETERS WITH FROZEN THAWED SEMEN (MEAN ± SE) (n=36)

Semen Additive Concentration	Individual Motility	Sperm Viability	Sperm Abnormality	Osmotic Resistance test	Acrosomal Integrity
Overall (Fresh)	89.02±0.19	89.97±0.21	3.08±0.15	86.80±0.24	90.72±0.25
Trehalose 50mM	58.00±0.42^{b}	65.58±0.35^{b}	4.75±0.18^{a}	67.25±0.32^{a}	72.41±0.37^{b}
Trehalose 100mM	64.16±0.52^{c}	71.41±0.31^{c}	5.00±0.21^{a}	73.91±0.35^{c}	80.91±0.43^{c}
Trehalose 150mM	57.58±0.35^{b}	65.83±0.40^{b}	4.50±0.15^{a}	69.91±0.22^{b}	72.91±0.39^{b}
Control	53.91±0.57^{a}	60.33±0.45^{a}	5.08±0.22^{a}	66.50±0.55^{a}	68.83±0.38^{a}

- **Means with different superscripts within column differ significantly at (P<0.05) level.**

Table 2. COMPARISON OF FRESH SEMEN BIOCHEMICAL PARAMETERS WITH FROZEN THAWED SEMEN (MEAN ± SE) (n=36)

Semen Additive Concentration	Lipid Peroxidation	Glutathione Reductase
Overall (Fresh)	**49.96 ± 0.06**	**30.14±0.06**
Trehalose 50mM (n=36)	**32.15 ± 0.09^{c}**	**62.15 ± 0.08^{b}**
Trehalose 100mM (n=36)	**20.06 ± 0.13^{a}**	**84.00 ± 0.16^{c}**
Trehalose 150mM (n=36)	**28.04 ± 0.08^{b}**	**57.07 ± 0.06^{a}**
Control (n=36)	**31.98 ± 0.07^{c}**	**62.04 ± 0.07^{b}**

Means with different superscripts within column differ significantly at (P<0.05) level

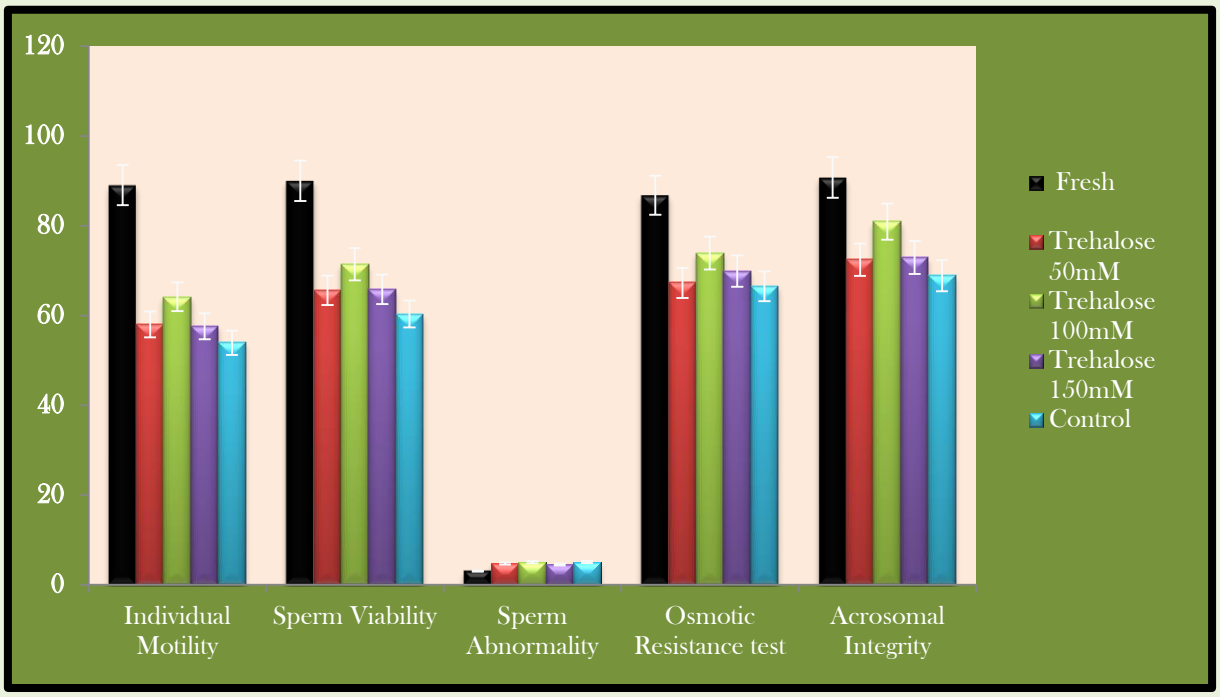

Fig.1. GRAPHICAL REPRESENTATION OF COMPARISION OF FRESH SEMEN PARAMETERS WITH FROZEN THAWED SEMEN

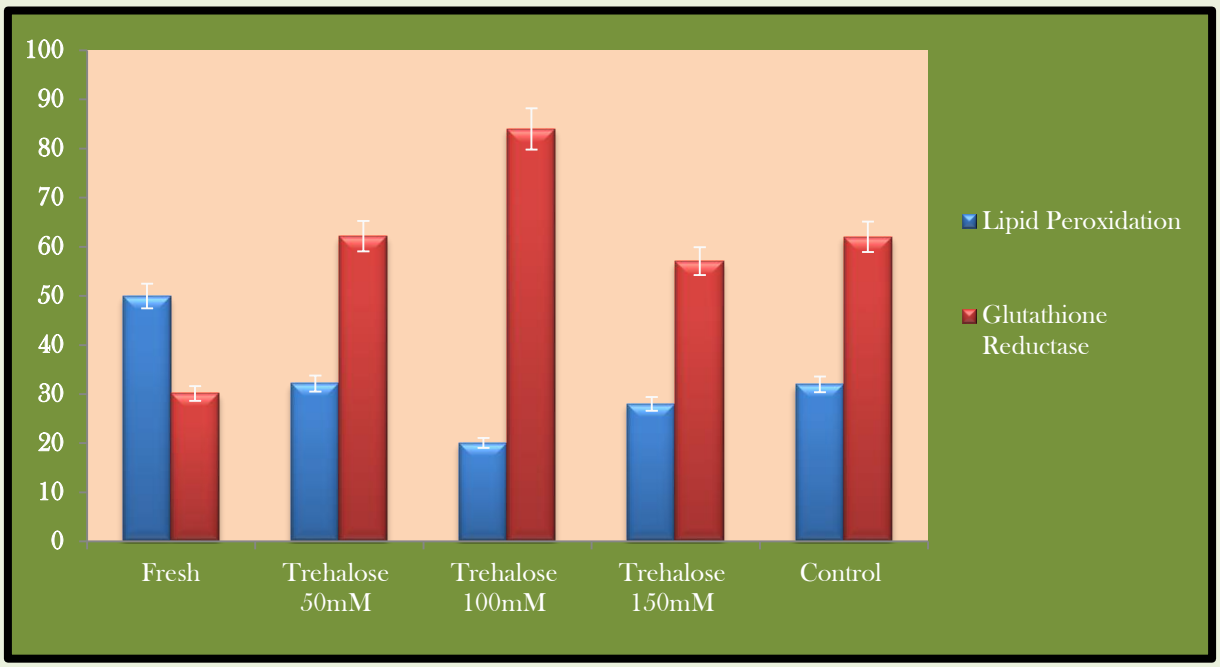

Fig. 2. GRAPHICAL REPRESENTATION OF COMPARISON OF FRESH SEMEN BIOCHEMICAL PARAMETERS WITH FROZEN THAWED SEMEN