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Effect of amino acids on sperm motility, velocity parameters, plasma membrane integrity and lipid peroxidation levels at cooled and post–thawed ram epididymal semen

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ABSTRACT

This study is focused to look into the possibility of including amino acids during cryopreservation to improve the quality of frozen semen in sheep. Cauda epididymal semen was collected from 2-3 year-old Bannur crossbred rams slaughtered at the local abattoir. The semen samples were diluted in tris egg yolk glycerol diluent along with different additives and made into 7 aliquots. Aliquot 1 served as control, L-alanine was added @ 100 and 135mM in the aliquot 2 and 3, L-glutamine was added @ 20 and 25mM in the aliquot 4 and 5 and L-proline was added @ 25 and 50mM in the aliquot 6 and 7, respectively. Diluted semen was filled in 0.25 ml French straws, sealed manually and equilibrated at 4ºC for 2 h, cooled in LN_2 vapour before being plunged into LN_2 till further evaluation. Inclusion of L-proline and L-glutamine in the diluent significantly increased the percent live sperm, total motility, lipid peroxidation and maintained higher functional membrane and acrosomal integrity than the control group. In contrast, L-alanine decreased the percentage of total motility, fast progressive spermatozoa and increased the percentage of immotile spermatozoa. It can be concluded that 20mM L-glutamine and 25mM L-proline can be used as semen additive to freeze ram epididymal semen as they prevented cryo injuries to sperm and improved the pre-freeze and post-thaw semen characteristics.

Key words: Amino acids, CASA, Freezing, HOST, Lipid peroxidation, Ram epididyaml semen

Artificial insemination (AI) with frozen thawed semen results in a low fertility rate, which limits the practical application of this technique in sheep. The potential use and wider application of frozen ram semen can result in production of superior germplasm. It is essential to minimize the losses that occur during cryopreservation. There is a need to improve the quality of frozen semen to achieve better fertility rate through AI in sheep. The toxicity and /or injury caused by cryoprotectants are poorly understood phenomenon which is the major obstacle in achieving fullest cryopreservation of semen (Fahy *et al*. 1990). Mammalian spermatozoa are highly sensitive to lipid peroxidation, which occurs as a result of the oxidation of the membrane lipids by the partially reduced oxygen molecules such as superoxide, hydrogen peroxide and hydroxyl radicals (Aitken 1984, Alvarez and Storey 1989). Ram sperm are vulnerable to oxidative damage caused by ROS as they have a higher PUFA to saturated fatty acids ratio and lower cholesterol to phospholipid molar ratio (Kenan Coyan *et al*. 2012). The testis of the ram secretes considerable amounts of amino acids (200, µmoles / day) and the principal amino acid is glutamate (Setchell *et al*. 1967). Epididymis and testis are the most likely sources of amino acids in bovine seminal plasma and that the accessory glands produce only small quantities (Hopwood and Gassner 1962). Studies have shown that amino acids have cryoprotective effect in freezing of sperm in rams (Sanchez-partida *et al.* 1998), stallions (Koskinen *et al.* 1989) and humans (Renard *et al*. 1996). Amino acids may act electrostatically with phosphate group of the sperm plasma membrane phospholipids, thereby forming a layer on the sperm surface as amino acids are charged molecules (Anchordoguy *et al.* 1988). However, the exact mechanism of cryoprotection provided by amino acids is not fully understood and still unclear. Alanine is a non-essential amino acid and at higher concentrations affects the motility and viability of sperm (Koskinen *et al*. 1989). Proline has been reported to improve the motility and velocity, preserves the structural and functional integrity of biological membranes during freezing and dehydration (Heber *et al*.

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1971, Crowe and Crowe 1986, Scanchez-partidata *et al.* 1998) and probably by protecting free-radical-induced damage (Smirnoff and Cumbes 1989). Proline may act as an antioxidant, reduce the lipid peroxidation and able to penetrate the spermatozoa and inhibit intracellular ice formation. Glutamine has been shown to improve motility of the human and stallion sperm (Renard *et al.* 1996, Khilfaoui *et al.* 2005). Epididymal sperm, never been exposed to seminal plasma, may potentially respond differently than ejaculated sperm with respect to capacitation, their ability to fertilize oocytes *in vitro* and cryopreservation (Stout 2012). The antioxidant properties of the amino acids may help to protect the sperm cell against cold shock at equilibration time as well as during freezing. The aim of this study was to evaluate the effect of amino acids *i.e.* L-alanine, L-Glutamine and L-proline on epididymal sperm characters at pre-freeze and post-thaw stage in ram.

MATERIALS AND METHODS

Epididymal semen collection from slaughter house samples: The present work was carried out at the Reproductive Physiology Laboratory of ICAR- National Institute of Animal Nutrition and Physiology, Bangalore. Intact testes of 2-3 year old Bannur crossbred rams (n=6) were obtained from the abattoir (Bengaluru, Karnataka, India) transported in normal saline at 37ºC within 1-2 h of slaughter. The testes samples were washed with 70% alcohol, followed by tap water and finally with distilled water. Excess tissues were trimmed and cauda epididymides were isolated from testes, several incisions were made on the cauda epididymis. The sperm rich fluid gushing out was collected in petridish containing 1 ml of PBS solution (1 ml). The concentration of spermatozoa (million/ml) in the epididymal semen was determined by haemocytometer method (Salisbury *et al.* 1978).

Composition of dilutor: The semen samples were diluted in tris egg yolk glycerol diluent along with different additives incorporated in this study. The dilutor was checked for its pH before the dilution of semen. Tris egg yolk glycerol diluent composed of Tris 3.630 g, citric acid 1.99 g, fructose 0.5 g, egg yolk 20 ml, glucerol 5 ml, penicillin 100,000 units, streptomycin 100 mg, was used for the study (Evans and Maxwell 1987). The dilutor with this composition for 10 ml was made freshly for each sample. A total of seven equal aliquots were made of 1 ml each after diluting the semen. Aliquot 1 served as control, L-alanine was added @ 100 and 135 mM in the aliquot 2 and 3, L-glutamine was added @ 20 and 25 mM in the aliquot 4 and 5, L-proline was added @ 25 and 50mM in the aliquot 6 and 7, respectively. One concentration of each amino acid was selected as per the earlier works (Ali Al Ahmad *et al*. 2008, Scanchez-partidata *et al*. 1998, Koskinen *et al*. 1989) and another concentration was used arbitrarily. After dilution the samples were filled in 0.25 ml straws and sealed, and placed in the cold handling unit for the equilibration at 4ºC for 2 h.

Pre-freeze evaluation: computer assisted semen analysis (CASA): The semen straws were selected from each aliquot and thawed for 30 sec in 37ºC water for both pre-freeze and post-thaw evaluation. The semen sample (10 µl) was diluted in 0.5 ml of PBS solution. A drop of diluted semen was taken on to a pre-warmed slide, covered with cover slip and observed under 10X in phase contrast microscope connected to the CASA software computer. Five field images were randomly taken which were analysed later and report was saved for each aliquot as described earlier (Selvaraju *et al.* 2012). The parameters analysed using CASA were individual progressive motility, curvilinear velocity (VCL), average path velocity (VAP), straight-line velocity (VSL), linearity (LIN), beat-cross frequency (BCF), amplitude of lateral head displacement, straightness, wobbling and mean angular displacement.

Per cent live spermatozoa: By adopting the procedure described by Tomar (1997), the percentage of live and dead spermatozoa was determined by using Eosin-Nigrosin stain. The stained smear was observed under a phase contrast microscope (40×) for unstained and stained spermatozoa. A total of 100 spermatozoa were counted to determine the percentage of live and dead spermatozoa.

Hypoosmotic swelling and giemsa test (HOST-G): Combined test of hypoosmotic swelling (HOS) test and acrosome integrity was carried out as per the method adopted by Selvaraju *et al.* (2008). The stained slides were thoroughly washed under tap water and examined under oil immersion object of phase contrast microscope (100×). Two hundred cells were counted, the spermatozoa were classified into four subpopulations based on the reaction to HOST-G. The various pattern of sperm swelling to HOS solution were calculated.

H^+A^+ Functional membrane integrity positive and acrosome integrity positive (Both membrane and acrosome intact).

H^+A^- Functional membrane integrity positive and acrosome integrity negative (Only membrane intact).

H^-A^+ Functional membrane integrity negative and acrosome integrity positive (Only acrosome intact).

H^-A^- Functional membrane integrity negative and acrosome integrity negative (Both membrane and acrosome integrity lost).

Lipid peroxidation test (LPO): Lipid peroxidation test was performed as per Selvaraju *et al*. (2008) with slight modifications in sperm concentration and amount of reagents used. The lipid peroxidation level of spermatozoa was determined by measuring the MDA production. The MDA concentration was determined by the specific absorbance coefficient (1.56×10^5 mol^{-1} cm^{-3}).

$$\text{MDA produced } (\mu\text{mol}/30 \text{ million cells}) = \frac{OD \times 10^6 \times \text{total volume}}{OD \times 10^5 \times \text{test volume}} = \frac{OD \times 10 \times 1.5}{1.56 \times 0.5}$$

The TBA-TCA reagent consists of tricarboxylic acid (TCA) 15 % W/V, thiobarbituric acid (TBA), 0.375%, HCL 0.375% and the final volume made 100ml.

Freezing of semen: Freezing of semen was performed as per the method adopted by Ari *et al.* (2012). After 2 h of equilibration, the straws were frozen horizontally on a rack about 4 cm above the liquid nitrogen (LN_2) held in an insulated container for 15 min. Then the straws were plunged into LN_2 for 2 min and transferred to LN_2 containers at -196°C. The straws were stored in LN_2 until evaluation 24 h post-freezing.

Post-thaw evaluation: After the freezing in LN_2, straws were selected from each aliquot and thawed for 30s in 37°C water for the post-thaw semen evaluation. Post-thaw evaluation was done for various sperm motility and velocity parameters by CASA, per cent live sperm, combined test of hypoosmotic sperm swelling test and acrosomal integrity (HOST-G) and lipid peroxidation test. The methods described for the prefreeze evaluation was used for the post-thaw evaluation of the said parameters.

Statistical analysis: The results were expressed as mean (±SE). The percentage data were arcsin transformed before analysis. The data for different spermatozoa parameters was analysed using two way analysis of variance (ANOVA), followed by Duncan's post hoc test to determine the significant difference among the semen additives and within different concentration of semen additives using SPSS/PC computer programme (Version 16.0). Statistical significance were considered with values of ($P<0.05$).

RESULTS AND DISCUSSION

The average volume of epididymal semen was 0.77±0.08 ml (0.6 to 1.0 ml) and average sperm concentration was 3504.10±67.40 × 10^6/ml (3216 to 3659.2 × 10^6/ml).

Total motility, fast progressive spermatozoa and sperm velocity: The influence of L-alanine, L-glutamine and L-proline on standard semen parameters at pre-freeze and following the freeze–thawing process was evaluated in 6 independent experiments on cauda epididymal spermatozoa. As shown in Tables 1 and 2, the freezing extender supplemented with L-glutamine and L-proline led to higher percentages ($P<0.05$) of total motility and fast progressive motilities, when compared to L- alanine supplemented and control group during pre-freeze and post thawing process. 20 mM L-glutamine and 25 mM L-proline significantly ($P<0.05$) increased total and fast progressive motility compared to 25 mM L-glutamine and 50 mM L-proline. On the other hand alanine significantly decreased total motility. Supplementation of L-glutamine and L-proline in the semen extender significantly ($P<0.01$) increased the amplitude of lateral head (ALH) displacement and beat cross frequency (BCF) in the post-thaw semen. L-proline also significantly ($P<0.05$) decreased the wobbling motility of spermatozoa in the post-thaw semen, while at higher concentration of L-alanine (135 mM) significantly ($P<0.05$) decreased BCF in post-thaw semen.

Percent live spermatozoa: Both L- glutamine and L-proline added to semen extender significantly ($P<0.001$) increased the per cent of live spermatozoa during the pre-freeze stage and post-thaw semen. However, 20 mM of L-glutamine did not significantly alter the per cent of live spermatozoa in post-thaw semen compared to that of control. On the other hand, L- alanine, significantly decreased per cent of live spermatozoa at both stages (Fig. 1a).

Combined functional membrane and acrosome integrity of spermatozoa: Both L- glutamine and L-alanine significantly ($P<0.001$) increased the functional membrane and acrosome integrity of the spermatozoa during the pre-freeze and post-thaw stages. On the other hand, L –alanine significantly ($P<0.001$) decreased the integrity of functional membrane and acrosome (Fig. 1b).

Lipid peroxidation: Supplementation of L-glutamine and L-proline significantly ($P<0.001$) decreased the formation of malondialdehyde indicator of lipid peroxidation due to

Table 1. Effect of different concentrations of amino acids as semen additives on CASA parameters of epididymal ram semen during prefreeze stage

Parameter	Control	L-Alanine		L-Glutamine		L-Proline	
		100mM	135mM	20mM	25mM	25mM	50mM
Sperm motility							
Total motile (%)	69.8±5.2^c	70.73±5.5^c	69.13±6.2^c	80.22±6.7^b	87.43±6.5^a	90.5±6.3^a	83.85±6.2^b
Fast progressive motile (%)	32.18±3.9	21.95±2.7	20.61±3.8	32.15±5.3	38.16±5.4	32.98±2.8	28.79±5.8
Immotile (%)	30.19±5.2^a	29.25±6.9^a	30.86±6.7^a	19.77±19.7^c	12.57±3.0^b	9.48±2.3^b	16.14±3.1^c
Sperm Velocity							
VCL (µm/s)	117.67±9.7	105.04±13.6	119.1±16.1	131.02±9.7	123.18±8.3	130.8±10.1	133.21±5.5
VSL (µm/s)	74.2±6.4	52.73±8.0	50.91±11.0	74.56±14.0	65.96±13.7	62.97±7.0	60.35±10.0
VAP (µm/s)	96.73±8.7	78.31±12.6	82.71±9.0	102.7±12.9	94.52±13.4	95.55±10.9	97.94±7.7
LIN (%)	64.58±6.7	50.92±4.8	43.68±7.2	55.93±8.1	52.24±8.0	47.75±2.9	45.27±6.9
STR (%)	77.87±5.2	70.16±5.7	58.99±5.4	71.63±6.9	67.84±5.1	66.48±2.5	60.81±7.0
WOB (%)	82.27±4.0	72.98±4.7	72.50±6.7	76.90±4.9	75.76±7.0	71.97±3.9	73.15±3.6
ALH (µm)	3.15±0.3	2.72±0.3	2.68±0.3	3.07±0.5	3.35±0.6	3.00±0.4	3.02±0.2
BCF (Hz)	8.87±1.3	8.05±1.6	6.08±1.0	7.66±1.3	8.85±0.5	7.64±0.6	7.39±1.1

Values bearing different superscripts in a same row are significantly different at P value Immotile ($P<0.01$), TM ($P<0.001$).

oxidative stress during pre-freeze and post-thaw stages. However, L alanine did not have any significant effect (Fig. 1c).

Cryopreservation of semen leads to an overall reduction in morphological and physical properties of the spermatozoa, viz. viability, motility, velocity and integrity of plasma membrane and acrosome. This affects the probability of conception rate following AI. The major factors/attributes that reduced motility and viability of cryopreserved sperm are the membrane changes due to the toxicity effect of the cryoprotective agents present in the freezing diluent (Slavik 1987, Watson *et al*. 1992), variation in the freeze–thaw process (Maxwell and Watson 1996) and induced lipid peroxidation by the reactive oxygen free radicals (Bell *et al*. 1993, B¨uy¨ukleblebici *et al*. 2014). The present study showed the effect of the selected amino acids to overcome these deleterious effects in the epididymal semen characters during the pre-freeze and post-thaw stages. Studies demonstrated about the cryoprotective effect of amino acids during the freezing of sperm from rams (Scanchez-partidata *et al*. 1998), stallions (Koskinen *et al*. 1989) and men (Renard *et al*. 1996). In combination with glycerol, amino acid protected the sperm cells by calcium ATPase during the changes of state of freezing medium (Lalonde *et al.* 1991). These amino acids form a layer over the sperm surface due to electrostatic interaction with phosphate group of the sperm plasma membrane phospholipids (Anchordoguy *et al*. 1988), and as a cushion for damage against ice crystals formation and prevents thermal shock (Kundu *et al*. 2001). Supplementation of L- glutamine and L-proline in the extender improved total and fast progressive motilities, in compared to the controls and L-alanine during pre-freeze and post-thawing process. Among the additives studied, 20 mM L-glutamine and 25 mM L-proline showed significantly ($P<0.05$) higher total motility and fast progressive spermatozoa per cent than the

Table 2. Effect of different concentrations of amino acids as semen additives on CASA parameters of epididymal ram semen at post-thaw stage

Parameter	Control	L-Alanine		L-Glutamine		L-Proline	
		100mM	135mM	20mM	25mM	25mM	50mM
Sperm motility							
Total motile (%)	42.49±2.8^{c}	50.63±3.1^{b}	35.51±3.0^{c}	61.04±6.2^{a}	57.28±3.5^{a}	64.29±9.6^{a}	48.6±4.2^{b}
Fast progressive motile (%)	13.70±1.7^{b}	14.09±2.7^{b}	7.96±1.2^{c}	21.83±3.8^{a}	19.50±1.5^{ab}	20.38±2.4^{ab}	16.07±1.8^{ab}
Immotile (%)	57.49±4.6^{ab}	49.37±5.7^{bc}	64.54±6.0^{a}	38.95±7.7^{bc}	42.71±4.3^{c}	35.72±5.6^{c}	51.38±6.2^{ab}
Sperm velocity							
VCL (µm/s)	93.23±14.2	73.98±6.3	71.96±9.8	84.25±7.05	80.73±8.7	85.14±6.6	92.41±3.5
VSL (µm/s)	61.54±7.2	49.06±3.4	46.20±3.7	58.47±4.0	59.95±5.6	48.63±3.6	60.10±4.6
VAP (µm/s)	82.80±12.9	63.34±6.0	60.20±8.9	71.18±6.6	69.86±7.1	64.09±6.4	76.78±3.9
LIN (%)	68.40±4.8	67.39±4.6	66.54±4.3	70.10±3.0	75.72±3.8	57.69±3.9	65.02±4.5
STR (%)	77.20±5.1	78.75±4.2	79.69±4.1	83.59±4.1	86.77±3.2	76.96±3.4	78.39±5.2
WOB (%)	88.60±1.4^{a}	85.60±3.2^{a}	83.53±2.9^{a}	84.17±2.5^{a}	87.11±1.6^{a}	74.87±3.1^{b}	82.91±1.4^{a}
ALH (µm)	2.18±0.2^{b}	2.56±0.1^{ab}	2.59±0.1^{ab}	2.92±0.2^{a}	2.65±0.2^{a}	3.26±0.3^{a}	2.87±0.1^{a}
BCF (Hz)	6.98±0.6^{c}	9.00±0.3^{ab}	8.45±0.3^{bc}	10.43±1.0^{a}	8.80±0.5^{b}	8.99±0.2^{b}	10.11±0.6^{ab}

Values bearing different superscripts in a same row are significantly different at P value TM, FP($P<0.001$), WOB, ALH (P 0.01), Immotile, BCF ($P<0.05$).

Table 3. Effect of different concentrations of semen additives on functional membrane and acrosmomal integrities of epididymal ram semen during prefreeze and post-thaw stages

Parameters	Control	L-Alanine		L-Glutamine		L-Proline	
		100mM	135mM	20mM	25mM	25mM	50mM
Prefreeze stage							
Functional membrane integrity (%)	64.17±1.0^{b}	53.50±0.6^{c}	51.50±0.4^{c}	85.17±0.6^{a}	84.67±0.6^{a}	86.67±0.3^{a}	92.00±0.8^{a}
Acrosmomal integrity (%)	64.83±0.6^{c}	51.66±0.6^{d}	55.00±0.3^{d}	75.50±0.4^{b}	75.00±0.3^{b}	84.83±0.3^{a}	94.33±0.5^{a}
Post -thaw stage							
Functional membrane integrity (%)	44.83±1.1^{c}	33.50±0.6^{d}	37.83±0.8^{d}	64.50±0.9^{b}	65.16±0.9^{b}	73.00±0.9^{a}	75.83±0.7^{a}
Acrosmomal integrity (%)	46.16±0.8^{c}	32.00±0.9^{d}	35.00±1.0^{d}	64.66±0.8^{b}	65.66±1.3^{b}	73.83±1.4^{a}	75.33±0.8^{a}

Values bearing different superscripts in a same row for H^{+}, A^{+}, differ significantly ($P<0.001$) .

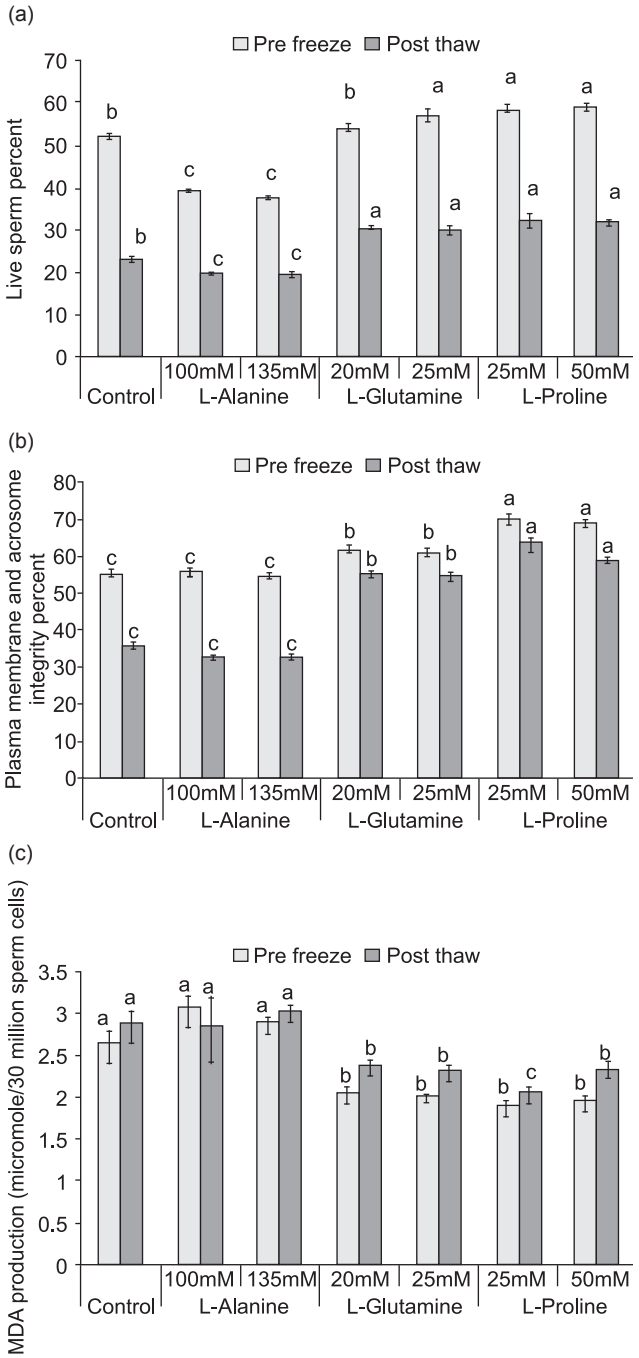

Fig. 1a–c: Effect of different concentrations of amino acids as semen additives on per cent live sperm of epididymal ram semen during pre-freeze and post-thaw stages. The data are expressed as per cent (n = 6). Different letters above the bars indicate significant differences ($P < 0.001$) between different and control groups. **1b**. Effect of different concentrations of amino acids as semen additives on hypoosmotic swelling and giemsa combined test of epididymal ram semen during pre-freeze and post-thaw stages. The data are expressed as per cent (n = 6). Different letters above the bars indicate significant differences ($P < 0.001$) between different and control groups. **1c.** Effect of different concentration of amino acids as semen additives on Lipid Peroxidation of epididymal ram semen during prefreeze and post-thaw stages. The data are expressed as the mean ± standard error of the mean (n = 6). Different letters above the bars indicate significant differences ($P < 0.001$) between different and control groups.

25mM L-glutamine and 50mM L-proline, respectively. This observation is similar with the earlier works of Kundu *et al*. (2001 and 2002), whose results confirmed the improvement of buck cauda epididymal spermatozoa exposure to amino acids prior to freezing. In the present study amino acid proline improved sperm motility and velocity parameter, and also preserved the structural and functional integrity of biological membranes during freezing and dehydration which is in agreement with earlier confirmation (Heber *et al*. 1971, Crowe and Crowe 1986, Scanchez-partidata *et al*. 1998). Proline also protected sperm cells against free-radical-induced damage, which is in agreement with Smirnoff and Cumbes (1989), indicating an overall protective effect on the spermatozoa during cryopreservation. Proline interacts with phospholipid bilayers and stabilizes membrane structure and function during freezing (Rudolph and Crowe 1986). Thus, proline might have interacted with the sperm membranes that protect the sperm cells from the stresses caused by the osmotic changes occurring during freezing. Proline also limits membrane lesions and plasmolysis through its action in the mechanism of membrane stabilization.Thus, it is possible that the proline is able to penetrate the spermatozoa and inhibit intracellular formation of ice crystal. In this study, inclusion of proline @ 25 and 50mM to the freezing extender reduced the lipid peroxidation which might have correspondingly increased the percent live spermatozoa during pre-freeze and post-thaw stages. These finding suggests that proline could have a probable antioxidant activity during sperm cryopreservation (Table 3).

Glutamine is known to play a regulatory role in several cell specific processes, including the metabolism, cell integrity, protein synthesis, and degradation, redox potential, gene expression (Curi *et al*. 2005). Glutamine acts at the extracellular level, improving the motility of the human, stallion sperm (Renard *et al*. 1996, Khilfaoui *et al*. 2005), ram (Bucak *et al*. 2009) and fertilizing ability of human (Renard *et al*. 1996) and stallion sperm (Kenan *et al*. 2003). The hypothesis from this present study is, glutamine provides cryoprotection to ram sperm as it prevented lipid peroxidation both at pre-freeze as well as post-thaw semen, which is in agreement with earlier report of Kenan Coyan *et al.* (2012). Ram sperm is more vulnerable to oxidative damage caused by ROS as it has a higher polyunsaturated fatty acid (PUFA) to saturated fatty acids ratio and lower cholesterol to phospholipid molar ratio.The observed overall improvement in membrane stabilization as well as more acrosome intact sperm cells during pre-freeze and post-thaw stages, has shown glutamine to be a potential additive in semen extender. Minimization of lipid peroxidation level along with increased plasma membrane and acrosome integrity observed in this study is in agreement with earlier reports in human (Renard *et al*. 1996) and stallion (Trimeche *et al*. 1995, 1999). Supplementation of alanine in the freezing extender resulted in lower cryoprotection. 135mM alanine showed maximum cryoprotection potential and motility recoveries were relatively higher in presence of

alanine than glutamine or glycine Kundu *et al*. (2001). In the present study, it was found that inclusion of 100 and 135 mM alanine resulted minimization of sperm motility and velocity and increased the per cent of immotile and non-progressive spermatozoa in ram. The exact mechanism of alanine action in sperm cells is poorly understood. However, we suggest that, it might be the higher concentration of alanine resulted in the deleterious effects as higher concentration of amino acids in the diluents result in lower post-thaw motility and viability which is in agreement with earlier report (Koskinen *et al.*1989). Though the epididymal sperm cells are having the fertilizing potential, it has not exposed to accessory gland secretion, hence some of components present in that group of fluid mixture is thought to be important and involved in sperm membrane stabilization and acrosome reaction. It concluded that L- proline or L-glutamine can be added in the semen extender to improve the freezability of ram semen.

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