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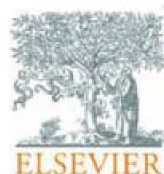

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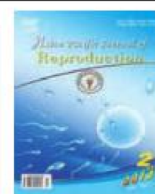

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Acrosome membrane integrity and cryocapacitation are related to cholesterol content of bull spermatozoa

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ABSTRACT

Objective: To evaluate the cryoinjury prediction of spermatozoa in relation to its cholesterol content at fresh and frozen–thaw stages. **Methods:** Ejaculates (n=12) were processed for cryopreservation, acrosome integrity (fluorescent and giemsa stains), cryoinjury (distribution of non capacitated, capacitated and acrosome reacted, pattern F, B and AR, respectively of Chlortetracycline, CTC assay), *in vitro* fertiltiy (IVF) and cholesterol content of spermatozoa at fresh, pre–freeze and frozen–thaw stages were evaluated. Values were fitted in prediction equation to predict acrosome integrity (AI) and cryoinjury. **Results:** Study indicated that cholesterol content of fresh spermatozoa can be used to predict cholesterol content of spermatozoa at pre–freeze and frozen–thaw stages of cryopreservation protocol with medium to high level of accuracy ($P<0.05$ and $P<0.01$, respectively). Cholesterol content of fresh spermatozoa can be used to predict AI, pattern B and AR and Penetration Index (PI) of IVF with medium level of accuracy ($P<0.05$) at frozen–thaw but not at pre–freeze stage. Similarly cholesterol content of frozen–thaw spermatozoa can be used to predict AI and pattern AR of frozen–thaw spermatozoa with medium level of accuracy ($P<0.05$). **Conclusion:** This study revealed strong evidence that cholesterol content of fresh as well as frozen–thaw bull spermatozoa can be a good predictor of level of cryoinjury following preservation at ultra low temperature.

1. Introduction

The cryopreservation of bull semen is one of the most widely used technologies for rapid dissemination of superior germplasm. However cryopreservation drastically reduces frozen–thaw semen quality by destabilization of spermatozoa plasma membrane [1] and loss of sperm motility and function [2]. Many study show that spermatozoa patho–morphology is highly correlated with fertility in man [3, 4], stallion [5] and Bull [6]. Such detrimental effects arise due to destabilization of the lipid architecture of the plasma membrane. The affected spermatozoa have reduced ability to bind and penetrate the zona pellucida [7] and are predisposed for premature capacitation and spontaneous acrosome reaction [8]. Maintenance of optimum fertility depends on the acrosome being structurally and biochemically intact, which contains the enzymes necessary to penetrate through the outer layers of the ovum, and achieve fertilization. Hence assessing the sperm subpopulation positive for functional membrane integrity and acrosome integrity may be important indicators of spermatozoa fertility.

Membrane cholesterol has stabilizing effect on spermatozoa membrane; hence any change in its content is expected to induce reorganization or destabilization of membrane [9, 10]. Membrane destabilization occurs when the membrane undergoes thermotropic lipid phase transition,

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from the fluid phase to the gel phase, as temperature is decreased. At least some of this membrane damage can be diminished by adding lipids, in the form of egg yolk, to the sperm prior to cooling and freezing [11]. Alternately, when cholesterol was added to liposomes, the lipid phase transition temperature of the liposomal membranes was reduced, and if sufficient cholesterol was added, the phase transition was eliminated [12].

Post-thaw semen quality parameters following cryopreservation vary greatly among bulls, and also between different ejaculates of same bull. This may be attributed to varying concentration of membrane cholesterol, thus fresh spermatozoa with higher cholesterol content is expected to resist destabilization of membrane following cryopreservation [12]. In agreement, [13] demonstrated sperm subpopulation is capacitated as a result of cryopreservation (i.e. cryocapacitation). As cryopreservation alters sperm membrane integrity, sperm with more cholesterol content prior to cryopreservation will resist membrane damage and hence effect of membrane damage and cryocapacitation would be less evident in these spermatozoa. With this hypothesis, the objective of present study was to evaluate acrosome integrity and cryocapacitation in relation to cholesterol content of fresh and frozen-thaw spermatozoa of bulls.

2. Materials and methods

2.1. Animals and experimentation

Study was carried out at Germ Plasm Centre, Indian Veterinary Research Institute, Izatnagar. A total of 12 ejaculates were collected randomly from six crossbred (Vrindavani, Friesian x Jersey x Brown Swiss x Haryana) bulls aged between 4 to 6 years with proven fertility. Semen was collected during morning hours, between 8-9 AM, using artificial vagina as per the standard procedure. For uniformity, only those ejaculate that had a mass activity of 3+ and above (on a scale of 0 to 5) and individual motility of 70 percent or above were selected for further processing. From each ejaculate, 0.5 mL of semen was used for estimation of cholesterol content of fresh spermatozoa whereas remaining was processed for cryopreservation. For cryopreservation of ejaculates protocol as described by Srivastava *et al.* [14] was followed. Cryopreserved semen was used later for evaluation of cholesterol content, acrosome integrity and cryocapacitation status of frozen-thaw spermatozoa.

2.2. Cholesterol content of spermatozoa

Washing of spermatozoa was necessitated for estimation of cholesterol content at fresh, pre-freeze and frozen-thaw stages. Immediately after initial evaluation, fresh spermatozoa were washed using Percoll density gradient [15] to remove egg yolk particles, dead cells and debris. This procedure was repeated for pre-freeze and frozen-thaw spermatozoa by thawing of semen straws at 37 ℃ for 30 seconds for evaluation of cholesterol content.

For washing of spermatozoa, 1.0 mL layer of 40 per cent Percoll (V/V, Sigma Aldrich, St. Louis, USA) in non-capacitating medium (NCM, pH 7.4 containing KCl, 0.201 g; H_2PO_4, 0.04 g; Na_2HPO_4, 1.150 g; NaCl, 8.00 g; Glucose, 0.999 g and Pyruvate, 0.11 g in 1 000 mL of double distilled water) was pipetted over a 1.0 mL layer of 80 percent Percoll (V/V in NCM) in a disposable 15 mL centrifuge tube. One mL fresh or thawed semen was gently layered on top of the two steps Percoll column. This content was centrifuged at 400 rpm for 30 min. After centrifugation, the pellets were washed once again with NCM and re-suspended in NCM to make 100 million spermatozoa per mL. This procedure was followed to make an aliquot of 1.0 mL (in duplicate) of all the ejaculates in cryovials and stored at -20 ℃ till used for estimation of cholesterol.

Further, pellet of approximately 100 million washed spermatozoa were taken in a 10 mL vial. The sperm pellet was extracted with 20 volume of chloroform : methanol (1:1, V/V) solution and vortexed for 20 seconds. Subsequently, it was centrifuged at 800 rpm for 5 min followed by evaporation to dryness under liquid nitrogen. At the time of estimation, 0.5 mL of chloroform was added to each vial and cholesterol was estimated by cholesterol assay kit (Span Diagnostics Ltd, Surat, Gujarat, India).

2.3. Evaluation of initial semen quality

The concentration of spermatozoa (millions/mL) in fresh semen samples was determined by using haemocytometer [16]. At the end of equilibration period (pre-freeze) and after cryopreservation for a minimum of 24 hour (frozen-thaw), semen straws from each group were thawed at 37 ℃ for 30 s in water bath and were evaluated for individual motility of spermatozoa. To minimize variation in subjective scoring system followed in present investigation, each sample was evaluated by two co-authors and their average was only mentioned in the manuscript.

2.4. Acrosome integrity

Acrosome integrity of spermatozoa at each stage was assessed by giemsa stain [17] as well as fluorescein labeled lectin from the peanut plant, Arachis hypogaea (FITC-PSA) using a slightly modified version of the procedure [18].

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Modification included use of PBS instead of HEPES buffer and removal of excess Propidium Iodide (PI) by diluting the contents several fold and centrifugation instead of filtration of the solution. Slides were examined within two hours under the fluorescence (Nikon Microphot FXA EPI–FL3; Nikon, New York, USA) with filter set at 40 X magnification. A total of 200 spermatozoa were counted per slide. Cells which retained staining of the equatorial segment were considered fully acrosome reacted as these cells were considered totally devoid of PSA staining. The PI–positive cells were excluded from the estimate of acrosome intact and AR live spermatozoa. Only PSA positive and PI negative spermatozoa were considered as acrosome intact live.

2.5. Chlortetracycline (CTC) fluorescence assay

Chlortetracycline fluorescence as an assay of sperm cryocapacitation [19] was used. The CTC solution (pH 7.8) was used for determination of capacitation and acrosome reaction. 10 μL of spermatozoa suspension was mixed with equal volume of CTC solution on a previously cleaned grease free slide at room temperature. After five seconds, 1.5 mL of gluteraldehyde (12.5 per cent v/v in 20 mM Tris–HCl, pH 7.4) was added to the samples. Finally a drop of 0.22 M, 1,4– diaza–bicyclo (2,2,2) octane dissolved in glycerol: Phosphate–buffered saline (9:1) was added to retard the fading of CTC fluorescence. The slides were covered with cover slips and examined on the same day. The CTC fluorescence was observed with a microscope equipped with phase contrast and epifluorescent optics. At least 200 spermatozoa per slide were examined and classified as Pattern F: Uniform bright fluorescence over the whole head, characteristic of non–capacitated cells, Pattern B: Fluorescence–free band in post–acrosomal region (capacitated cells) and Pattern AR: Dull fluorescence over the whole head except for a thin punctate band of fluorescence along the equatorial segment (acrosome reacted cells). No fluorescence was observed when CTC was omitted from the preparation.

2.6. *In vitro* fertility of spermatozoa

Spermatozoa of each ejaculate were subjected to heterologous (buffalo oocyte) zona–binding assay after freezing–thawing. The method of Fazeli *et al.*[20] was followed. After 18–20 h of co–incubation of oocytes with spermatozoa, the penetrated oocytes were washed and processed further for counting number of oocyte bound with spermatozoa (penetration per cent, PP). At least 60 oocytes (with four repetitions) were used to estimate the zona–binding of frozen–thawed spermatozoa.

2.7. Statistical analysis of data

For statistical analysis of data, ANOVA was carried with PROC GLM of SAS 9.2 for estimation of mean and standard error of parameters under investigation. The predictions of equations were developed for predicting sperm quality parameters. The following prediction equation was fitted to predict sperm quality parameters of fresh, pre–freeze or frozen–thaw stage of cryopreservation using cholesterol content of fresh spermatozoa.

$$Y_i = \beta_0 + \beta_1 + X_i + e_i$$

Where Y_i = Dependent variable viz. sperm quality parameters of fresh, pre–freeze or pos–thaw stage semen

β_0 = Intercept of the prediction equation

β_1 = Intercept of fresh, pre–freeze or frozen–thaw cholesterol content of spermatozoa

X_i = Observation of cholesterol content of spermatozoa at fresh, pre–freeze or frozen–thaw semen samples, and

e_i = Error (normally and independently distributed with mean 0 and variance $\sigma^2 e$

3. Results

Evaluation of cholesterol content of spermatozoa (μg/100 million spermatozoa) at three stages revealed significant ($P<0.05$) reduction in cholesterol content as cryopreservation progresses (Figure 1). Results show that cholesterol content of fresh spermatozoa can be used to predict cholesterol content of pre–freeze spermatozoa with high accuracy of 67 percent ($P<0.01$) whereas for post thaw spermatozoa, accuracy of prediction was low at 29 percent. Acrosome membrane integrity and pattern F of fresh sperm cells approximates viability value whereas per cent distribution of capacitated and acrosome reacted sperm cells are minimal as revealed by evaluation of spermatozoa in fresh ejaculates (Figure 2). Though cholesterol content of fresh spermatozoa was not found to be a reliable value to predict spermatozoa characteristics at pre–freeze stage, it can be used to predict acrosome integrity, as revealed by fluoroscent and giemsa stain, and pattern B and AR, and PI of *in vitro* fertilization assay with medium level of accuracy ($P<0.05$) in frozen–thaw spermatozoa (Table 1). Further evaluation revealed that cholesterol content of frozen–thaw spermatozoa can be used to predict acrosome membrane integrity and pattern AR with medium level of accuracy ($P<0.05$, Table 2).

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Table 1

Prediction equations of acrosome integrity and cryocapacitation status using cholesterol content of fresh spermatozoa (n=12).

Yi	Mean±SE	β0	β1	R2 (%)	P Value
Pre-freeze Acrosome integrity					
Giemsa	72.25±1.49	66.41	0.27	0.03	0.59
FITC PSA	71.00±1.48	67.17	0.17	0.01	0.72
CTC pattern F	29.67±1.14	35.27	0.26	0.05	0.50
B	48.83±2.06	41.45	0.34	0.03	0.62
AR	21.50±1.84	23.28	−0.08	0.00	0.89
Frozen-thaw Acrosome integrity					
Giemsa	61.25±2.00	46.96	0.65	0.48	0.02*
FITC PSA	61.58±1.70	58.47	0.14	0.39	0.03*
CTC pattern F	15.83±1.02	25.45	−0.44	0.17	0.18
B	53.58±1.27	71.21	−0.80	0.38	0.03*
AR	30.58±1.85	3.34	1.24	0.43	0.02*
IVF assay PP	57.83±2.05	66.57	−0.39	0.03	0.56
PI	45.61±2.96	−2.12	2.18	0.51	0.01*

Cholesterol content=(21.95±0.97) μg/100 million fresh spermatozoa, *-P<0.05, CTC pattern F: non-capacitated, B: capacitated, AR: acrosome reacted sperm cells.

Table 2

Prediction equations of acrosome integrity and cryocapacitation status using cholesterol content of frozen-thaw spermatozoa (n=12).

Yi	Mean±SE	β0	β1	R2 (%)	P Value
Acrosome integrity Giemsa	61.25±2.00	52.38	1.19	0.44	0.02*
FITC PSA	61.58±1.70	58.81	0.37	0.45	0.02*
CTC pattern F	15.83±1.02	25.68	−1.33	0.32	0.55
B	53.58±1.27	−1.24	62.84	0.18	0.16
AR	30.58±1.85	11.48	2.57	0.37	0.04*

Cholesterol content=(7.44±0.44) μg/100 million Frozen-thaw spermatozoa,*-P<0.05, CTC pattern F: non-capacitated, B: capacitated, AR: acrosome reacted sperm cells.

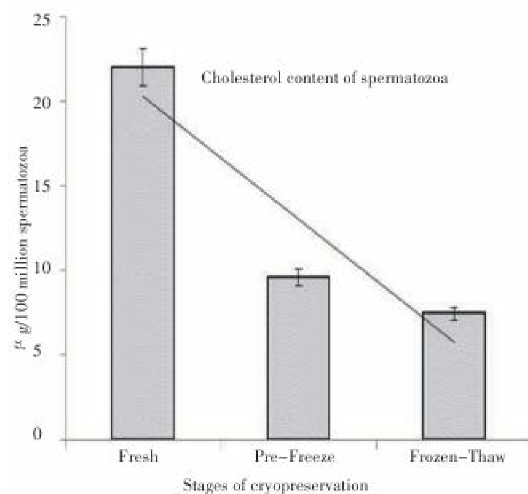

Figure 1. Cholesterol content of spermatozoa (μg/100 million spermatozoa) at different stages of cryopreservation.

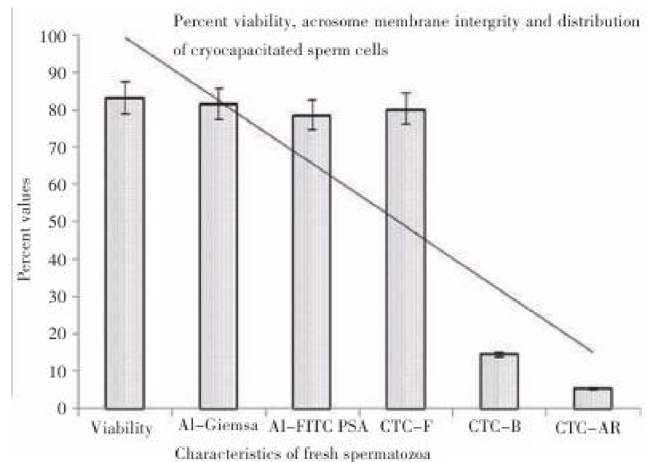

Figure 2. Characteristics of spermatozoa in fresh ejaculates of a bull. Acrosome membrane integrity and pattern F (non capacitated) of fresh sperm cells approximates viability value whereas percent distribution of capacitated and acrosome reacted sperm cells are minimal.

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4. Discussion

The problem with frozen-thaw sperm evaluation is that semen samples with very high or very low quality may be relatively predictable, but for medium quality, no single sperm attribute exists that is highly and accurately correlated either with *in vivo* fertility or with other vital sperm quality characteristics. Protocols with most refined freezing procedures, controlled freezing conditions and diluents modifications do not achieve much more than 50% motility after freezing [21] whereas the fertilizing ability of the spermatozoa is reduced about seven fold [22]. Besides, the fertilizing potential is strongly affected by the decrease in lifespan, the sub lethal dysfunction in the proportion of the surviving subpopulation and the decreased ability to interact with female tract [23]. One of the reasons for loss of fertility is oxidation of the spermatozoa membrane lipids [24] and loss of sperm cholesterol level after cooling and again after cryopreservation [25, 26]. In agreement this study report significant decrease in cholesterol content of spermatozoa at pre-freeze and frozen-thaw stages following cryopreservation. Spermatozoa sensitivity to cryodamage is determined by cholesterol and other lipid composition of cell membrane [27]. Drobnis *et al.* [28] suggested that sperm membranes are the primary site for structural and functional cold shock injury in sperm. More recently, Amorim and others [29] observed higher cholesterol content of post-thaw spermatozoa results from higher cholesterol content of spermatozoa prior to cryopreservation. Our study also support above line of observation revealing that fresh as well as frozen-thaw cholesterol content of spermatozoa are related to acrosome membrane integrity and these values can be used to predict acrosome membrane integrity with medium level of accuracy (P<0.05). This finding is supported by the observation of Sparr and co workers [30] that cholesterol has a profound effect on the thermodynamic and mechanical properties of lipid bilayers, and influences stability and fluidity of sperm. Thus it is evident that sperm cells with high cholesterol content prior to cryopreservation are less prone to sperm membrane damages.

Spermatozoa with intact membrane could undergo capacitation and acrosome reaction [31]. Capacitation is a collective term for changes a sperm undergoes when it comes into contact with the female reproductive tract [32]. Capacitation and subsequent acrosome reaction (AR) are important events in the process of fertilization. However, induction of premature capacitation or capacitation-like processes (spontaneous AR) following cryopreservation, termed as cryoinjury has detrimental effect on sperm longevity [33]. Cholesterol plays a key role in this mechanism [34] and its loss from the acrosome membrane is an obligatory step in the sperm capacitation process. The direct relationship between proportion of non capacitated, capacitated and AR spermatozoa in pre-freeze as well as frozen-thaw spermatozoa with cholesterol content of fresh and frozen-thaw spermatozoa is evident in present study. This is further supported by observations of Gadella and co-workers [35], suggesting that rate of capacitation depends on the rate of cholesterol depletion from the sperm plasma membrane. Thus, spermatozoa with high cholesterol are slow to undergo spontaneous capacitation and AR whereas sperm with lower cholesterol concentration seem to capacitate faster. This could be the reason for fresh spermatozoa with high cholesterol content showing greater PI values in IVF assay in our study.

Although our study has not shown association of cholesterol content of fresh spermatozoa with individual motility after freezing-thawing, Purdy and Graham [36] had observed association between higher cholesterol content of fresh spermatozoa with improved recovery of viable and motile spermatozoa after freezing-thawing.

This study revealed strong evidence that cholesterol content of fresh spermatozoa can be a good predictor of acrosome membrane integrity and cryoinjury to frozen-thaw spermatozoa. A relationship between proportion of non-capacitated and acrosome reacted spermatozoa at frozen-thaw stages of cryopreservation with cholesterol content of fresh and frozen-thaw spermatozoa was established. Though a much wider study is needed for a final say, this study points to cholesterol content of fresh spermatozoa as a reliable laboratory protocol to predict frozen-thaw spermatozoa quality characteristics and thus can be used to predict level of cryoinjury following preservation at ultra low temperature.

Conflict of interest statement

The authors do not have any conflict of interests in this experiment.

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