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HOLESTEROL CONTENT OF BULL SPERMATOZOA ALTERS SURVIVAL AT ULTRA-LOW TEMPERATURES

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ABSTRACT

Though cryopreservation bestows many advantages, it decreases tolerance to stress of temperature variations, reducing survival of spermatozoa to a great extent. A gradual reduction in cholesterol content of spermatozoa during cryopreservation was reported. Thus the objective of this investigation was to evaluate relationship of cholesterol content of spermatozoa with surviving ability, biochemical integrity of membrane and in vitro fertility (IVF) of spermatozoa following cryopreservation. From each ejaculate (n=12) aliquot was taken for evaluation of semen quality parameters and thereafter it was processed for cryopreservation. Cholesterol content, viability, motility and biochemical integrity of membrane (Hypo osmotic swelling, HOS response) of spermatozoa at fresh, pre-freeze and frozen-thaw stages and IVF parameters at frozen-thaw stage were evaluated. Values were fitted in prediction equation to predict cryosurvivability and biochemical integrity of spermatozoa membrane. Study indicated that cholesterol content of fresh spermatozoa can be used to predict viability at pre-freeze and frozen-thaw stages of cryopreservation protocol with medium level of accuracy ($p<0.05$). Cholesterol content of fresh, pre-freeze and frozen-thaw spermatozoa can be used to predict HOS response with medium level of accuracy ($p<0.05$) at respective and forthcoming stages. However cholesterol content of spermatozoa was not found to be significant predictor of individual motility and in vitro fertility (penetration percent and penetration index). This study revealed evidence that cholesterol content of fresh, pre-freeze as well as frozen-thaw bull spermatozoa can be a good predictor of viability and biochemical integrity of spermatozoa membrane following preservation at ultra-low temperature.

Keywords: Cryo-survival, Membrane integrity, Hypoosmotic swelling, Viability, In vitro fertility.

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Contribution/ Originality

This study contributes by way of showing importance of sperm cholesterol in cryopreservation. The assays used were currently in vogue. The equation is new one and not many studies have been done on this aspect. The paper contributes in emphasizing that sperm cholesterol content has an important bearing on cryosurvival.

1. INTRODUCTION

Cryopreservation of semen renders spermatozoa less tolerant to temperature fluctuations resulting in low survival post-thaw. Volumetric changes [1], oxidation of the sperm membrane lipids [2] and damages to the selective permeability mechanism of the membrane are the most important stress factors in freezing. Phospholipid fatty acids are major structural component of biological membranes and affect both the biophysical properties and functioning of the cell [3]. Holt [4] reported sperm sensitivity to cold shock is determined by membrane phospholipid (P) composition and membrane cholesterol (C) to phospholipid ratio. In another study involving bull semen efflux of sperm cholesterol following cooling and after freezing-thawing and strong evidence linking cholesterol content of fresh as well as frozen-thaw bull spermatozoa with level of cryoinjury was reported [5]. Cholesterol has stabilizing effect on spermatozoa membrane; hence any change in its content is expected to induce reorganization or destabilization of membrane [6]. Thus present study was designed to evaluate relationship of cholesterol content of spermatozoa with cryo-survival and biochemical integrity of sperm membrane at different stages of cryopreservation, and *in vitro* fertility (IVF) of post-thaw spermatozoa.

2. MATERIALS AND METHODS

2.1. Experimental Animals and Collection of Semen

In this investigation relationship of cholesterol content of spermatozoa with semen quality parameters was evaluated. Thus of the two aliquot, cholesterol content of fresh spermatozoa was estimated from first whereas second was utilized for evaluating semen quality parameter. There after values were fitted in regression equation for final conclusion.

A total of 12 ejaculates were collected randomly from six cross bred (Friesian x Jersey x Brown Swiss x Haryana) bulls, aged between 4 to 6 years maintained at Germ Plasm Centre, Indian Veterinary Research institute, Izatnagar. Semen was collected during morning hours, between 8 AM to 9 AM, using artificial vagina as per the standard procedure. For uniformity, only those ejaculates that had a mass activity of 3 and above (on a scale of 0 to 5) and individual motility of 70 per cent or above were selected for further processing. From each ejaculate, 1.0 mL of semen was used for estimation of cholesterol content of spermatozoa whereas remaining was processed for cryopreservation. Cryopreserved semen was used later for evaluation of semen quality parameters.

2.2. Estimation of Cholesterol Content of Spermatozoa

Washing of spermatozoa was necessitated for estimation of cholesterol content at fresh, pre-freeze and post-thaw stages. Immediately after initial evaluation, fresh spermatozoa were washed using Percoll density gradient [7] to remove egg yolk particles, dead cells and debris. This procedure was repeated for pre-freeze and post-thaw spermatozoa by thawing of semen straws at 37° C for 30 seconds for evaluation of cholesterol content. Thereafter an aliquot of 1.0 mL (in duplicate) of all the ejaculates in cryovials was made and stored at -20°C till used for estimation of cholesterol. Method of Bligh and Dyer [8] for cholesterol extraction followed by cholesterol assay kit (Span Diagonstics Ltd. India) was employed to estimate cholesterol content of spermatozoa.

2.3. Evaluation of Semen Quality Parameters

In fresh semen samples, the concentration of spermatozoa (millions/mL) was determined by using haemocytometer. Other parameters viz. individual motility, viability and sperm plasma membrane integrity (Hypo Osmotic Swelling, HOS, response) of spermatozoa in fresh, pre-freeze and at post-thaw stage were assessed. Semen straws were thawed at 37° C for 30 sec before post-thaw evaluation. Individual motility was assessed by placing a drop of the diluted semen on a clean, grease free glass slide mounted on a stage maintained at 37°C under high power magnification (400X). The semen sample was extended so that approximately 15 to 20 spermatozoa were visible under the visual field of microscope the per cent live spermatozoa was determined by Eosin-Nigrosin stain whereas the HOS response was assessed as described by Jeyendran, et al. [9].

2.4. *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. [10] 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 and penetration index, PI). At least 60 oocytes (with four repetitions) were used to estimate the zona-binding of frozen-thawed spermatozoa.

2.5. Statistical Analysis

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 using PROC REG procedure of SAS 9.2. The following prediction equation was fitted to predict sperm quality parameters of fresh or post the stage of cryopreservation using cholesterol content of fresh spermatozoa.

$Y_i = ß_0 + ß_1 + X_i + e_i$

Where Y_i = Dependent variable viz. sperm quality parameters of fresh or post thaw stage semen

$ß_0$= Intercept of the prediction equation.

$ß_1$=Intercept of fresh pre-freeze or post-thaw cholesterol content of spermatozoa

X_i=Observation of cholesterol content of spermatozoa at fresh or post thaw semen samples, and

e_i= Error(normally and independently distributed with mean 0 and variance σe^2

To minimize variation in subjective scoring system followed in present investigation, each sample was evaluated by two co-authors and their average was mentioned in the manuscript.

3. RESULTS AND DISCUSSION

In the present investigation, cholesterol content of fresh spermatozoa was 21.95±0.97 µg/100 million cells. As the processing of semen progressed, a reduction in cholesterol content of spermatozoa from fresh to pre-freeze and from pre-freeze to post-thaw was observed. Our results are in agreement with Srivastava, et al. [5] who reported that sperm cholesterol level decreased after cooling and subsequently after cryopreservation. Though the mechanism of loss of cholesterol is not completely understood, it is supposed most of the cholesterol loss is due to slow diffusion from cell and a net transfer of cholesterol from bovine spermatozoa to the surrounding medium [11].

Table-1. Prediction equations of semen quality parameters using cholesterol content of spermatozoa (n=12)

Y_i	Mean±SE	$ß_0$	$ß_1$	R^2 (%)	Significance
Chol. Content of fresh spermatozoa vs.					
Pre-freeze					
Viability	75.25±1.84	76.83	-0.07	0.0	0.91
HOS Res	67.83±1.15	52.65	0.69	0.34	0.04*
Frozen-thaw					
Viability	60.92±1.76	51.70	0.42	0.51	0.02*
HOS Res	59.50±0.79	58.50	4.82	0.44	0.02*
Chol. Content of pre-freeze spermatozoa vs.					
Pre-freeze					
Viability	75.25±1.84	84.05	-0.92	0.35	0.05*
HOS Res	67.83±1.15	58.89	0.93	0.30	0.05*
Frozen-thaw					
Viability	60.92±1.76	59.50	0.15	0.45	0.02*
HOS Res	59.50±0.79	56.70	0.30	0.32	0.04*
Chol. Content of frozen-thaw spermatozoa vs.					
Frozen-thaw					
Viability	60.92±1.76	54.78	0.82	0.44	0.02*
HOS Res	59.50±0.79	60.16	-0.07	0.35	0.04*
IVF assay					
PP	57.83±2.05	60.26	-0.32	0.00	0.83
PI	45.61±2.96	29.93	2.11	0.01	0.32

Cholesterol content = 21.95±0.97, 9.58±0.45 and 7.44±0.44 µg/100 million fresh, pre-freeze and post-thaw spermatozoa, respectively. PP = Penetration percent, PI = Penetration Index, *-p<0.05.

Though evaluation of quality parameters of fresh semen is routinely practised in semen laboratories world over, cryo-survival and fertility of spermatozoa remains unpredictable following freezing-thawing. No single sperm attribute exists that is highly and significantly correlated to cryosurvival of spermatozoa. Protocols with most refined freezing procedures controlled freezing conditions and diluents modifications do not achieve much more than 50% motility after freezing whereas the fertilizing ability of the spermatozoa is reduced about seven fold [12]. This study revealed that cholesterol content of fresh, pre-freeze or post-thaw spermatozoa can be used to predict viability and HOS response of spermatozoa of subsequent stages of cryopreservation protocol with medium ($p<0.05$) level of accuracy (Table). However post-thaw cholesterol content was not found to be a significant predictor of *in vitro* fertility of frozen-thaw bull spermatozoa.

Amorim, et al. [13] reported higher cholesterol content of a bull spermatozoa prior to cryopreservation resulted in increased percentage of viable and motile cells after thawing. Higher level of cholesterol content of fresh spermatozoa was also related to increased acrosome integrity of post-thaw spermatozoa [14]. Spermatozoa sensitivity to cryodamage is determined by cholesterol and other lipid composition of cell membrane [4]. Our study also support above line of observation revealing that fresh, pre-freeze as well as frozen-thaw cholesterol content of spermatozoa are related to higher number of viable cells with intact membrane. This finding is further supported by the observation [15] that cholesterol has a profound effect on the thermodynamic and mechanical properties of lipid bilayers, and influences stability and fluidity of sperm. In agreement, several studies reported sequestration of detrimental protein causing efflux of membrane cholesterol improves freezing [16] and minimizes cryoinjury [5]. Thus it is evident that sperm cells with high cholesterol content prior to cryopreservation are less prone to die and show higher sperm membrane integrity.

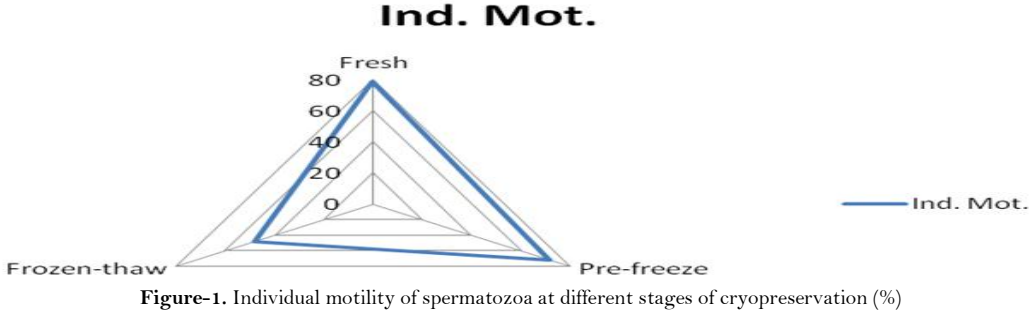

Figure-1. Individual motility of spermatozoa at different stages of cryopreservation (%)

However, in this study, cholesterol content of fresh, pre-freeze and frozen-thaw spermatozoa was found to be a low predictor of individual motility (Figure). In order to reduce spermatozoa damage incurred during cryopreservation osmotic tolerance of the cells may be widened. Bull spermatozoa has narrow osmotic tolerance limits [17] and rapidly become immotile when exposed to anisosmotic conditions less than 270 mOsm or greater than 370

mOsm. Having higher cholesterol content of spermatozoa before cryopreservation widens the osmotic tolerance limits of the cell after freezing-thawing. Though cholesterol content of post-thaw spermatozoa was not found to be a significant predictor of *in vitro* fertility, it could be argued that once viability as well as biochemicl membrane integrity of spermatozoa are predicted using cholesterol content, it can be taken as an indicator of improved parameters of IVF assay. Other workers [18] had reported higher choleterol content of spermatozoa results in reduction in spermatzoa membrane injury following processing and storage at ultra-low temperatures.

4. CONCLUSION

In conclusion, higher cholesterol content in spermatozoa increases tolerance to osmotic changes which subsequently improves cryo-survival and biochemical integrity of spermatozoa membrane with possible beneficial effect on *in vitro* fertility parameters.

4.1. Conflict of Interest Statement

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

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