



## Prognostic value of post thaw semen quality parameters, mitochondrial integrity and cholesterol content of sperm membrane vis-à-vis conception rate in Frieswal bulls

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### ABSTRACT

The present study aimed to analyze the quantitative relationship of membrane cholesterol content (Chol content), mitochondrial integrity ( $\Delta\psi_m$ ), and quality parameters of spermatozoa (SQP) at post-thaw stage with conception rate (CR) and estimated relative conception rate (ERCR) in Frieswal bulls. For the experiment, frozen semen straws (32) were collected from the SF laboratory and CR (Total insemination 3482) was obtained from Field Progeny Testing units. Based on the CR, bulls were grouped into low, medium or high groups (G I, G II and G III, respectively). SQP, viz. viability (Eosin Nigrosin), post thaw motility, biochemical integrity of the membrane (HOS res), acrosome integrity (Giemsa, and fluorochromes fluorescein isothiocyanate *Pisum sativum* agglutinin and propidium iodide, respectively), chol-content, and  $\Delta\psi_m$  using fluorescent probe JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide) were determined. The values thus obtained were subjected to the stepwise regression analysis using the least squares principles for each group, and the CR and ERCR were regressed on the various SQP. Pearson's correlation coefficients were estimated between the CR and ERCR values and the various SQPs. The coefficient of determination ( $R^2$ ) moderately higher for all the models and ranged from 63.70–93.40% (high and medium group, respectively). High  $R^2$  value of the prediction equation for the herd and bulls with medium CR (75.9 and 93.4%, respectively) reveal their suitability to predict the CR and ERCR potential of the cryopreserved semen. Study results point to inclusion of cholesterol content and  $\Delta\psi_m$  estimation in routine semen analysis before long-term storage or usage for insemination purposes.

**Key words:** Cholesterol content, Conception rate, Frieswal bulls, Mitochondrial membrane potential, Quality parameters

Knowledge on the crucial relation between conception rate (CR) and semen quality parameters (SQP) would be of great practical and economic value for efficient transfer of genetic merit and consequent productivity of future animals, as well as for success of artificial insemination (AI) programme. Evaluation of seminal quality parameters readily identify low fertile animals, but are lacking in accuracy to discriminate among moderate to high fertility bulls. Seminal parameters examined routinely, viz. concentration, post thaw motility (PTM), hypo-osmotic swelling (HOS) response and sperm abnormality show

varied associations with bull fertility (Dogan *et al.* 2013). On the other hand, commonly used PTM showed poor or strong correlation with fertility (Rodriguez-Martinez 2000), sperm concentration, motility and zona binding assay were associated significantly with each other (Zhang *et al.* 1999). For the reasons of association of sperm motility with viability and indirect indicator of sperm metabolism (Vyt *et al.* 2004), assessment of mitochondrial membrane potential (MMP,  $\Delta m$ ) for motility estimates might show strong association with CR than other parameters. Since higher cholesterol content in sperm membrane enables cells to withstand osmotic changes, thereby improving cryo-survival with possible beneficial effect on *in vitro* fertility parameters (Srivastava *et al.* 2013), evaluation of membrane cholesterol content along with other SQP at post thaw stage shall provide for more accuracy of discriminating bulls with high- from that of low-CR.

The prediction of expected conception rate of young bulls prior to their induction for breeding will be helpful to reduce the cost of bull rearing through early culling of the inferior bulls. The sire conception rate (SCR) or the fertilizing ability

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of the bulls is importantly indicated by the SQPs which always increase the probability of conception. Even though, the factors altering the actual conception rate in cattle are numerous, viz. the climate, management conditions, reproductive health status of females etc., the SQP of bulls are considered as the major determining factor and hence exploiting the SQP for predicting the CR will be helpful in gaining the prior knowledge on the breeding soundness of the bulls.

With above observations in sight, the objective of present study was to investigate relationship of SQP including mitochondrial functional status, and cholesterol content of sperm membrane at post thaw stage with conception rate; and to construct a prognostic equation to predict CR and estimated relative conception rate (ERCR) based on the above qualitative parameters in Frieswal bulls.

#### MATERIALS AND METHODS

*Sample collection:* Cryopreserved semen samples of Frieswal bulls packed in 0.25 ml French Mini straws (containing approximately 20 million cells/straw) were collected from the Semen Freezing Laboratory, ICAR-Central Institute for Research on Cattle, Meerut. At the time of evaluation, two straws were thawed in a water bath at 37°C for 60 sec, content evacuated in a 1.5 ml Eppendorf tube. Aliquots were taken from thawed samples maintained in water bath at 37°C for evaluation of different parameters namely PTM, HOS, acrosome integrity (Giemsa stain), viability (using eosin-nigrosin, and fluorescent probes propidium iodide and lectin bound with fluorescein isothiocyanate staining), mitochondrial functional status, and sperm membrane cholesterol content. The entire chemical for the experiment were procured from Sigma Aldrich (New Delhi, India) unless otherwise mentioned.

*Evaluation of semen quality parameters:* For examination of post thaw motility, a small drop of semen sample was placed on a clean glass slide, over which a cover slip was placed gently in a slanting manner. The cover slip was pressed lightly to extrude air bubbles and smear was examined under 20× phase contrast microscope. Viability was assessed by adding aliquot (20 µl) of thawed semen sample with the same volume of eosin-nigrosin staining solution, a smear was prepared on slides and 200 sperm were examined under 400×. Eosin penetrated dead, and damaged spermatozoa with disrupted membrane, imparting red colour, whereas nigrosin provided a dark background for easy differentiation. The HOS response was performed as described by Jeyendran *et al.* (1984). The hypo osmotic solution consisted of 7.35 and 13.51 g/l sodium citrate and fructose respectively. The final osmolarity was adjusted to 150 mOsm/l with pH 7.2. Thawed semen samples (10 µl) were mixed with 500 µl of the prewarmed (37°C) hypo-osmotic solution in 1.5 ml tubes and incubated at 37°C for 30 min. After incubation, a 10 µl aliquot was pipetted on to a slide, a cover slip was placed on top of the droplet, and the preparation was observed by phase-contrast microscopy at 400×. At least 200 spermatozoa in five different fields

were examined in each preparation. The acrosome integrity (Giemsa stain) assay was performed as described by Watson (1975).

*Estimation of viability and acrosome integrity using fluorescent probes (FITC+PI):* The spermatozoa viability and acrosome integrity in semen samples was determined using fluorescein labelled lectin from the peanut plant, *Pisum sativum* agglutinin (FITC) in combination of propidium iodide (PI) using a slightly modified version (Sukardi *et al.* 1997). In the modified procedure, PBS was used instead of HEPES buffer, and excess PI was removed by diluting the contents 7–8 times followed by centrifugation instead of filtration. Slides were examined within two hours under the fluorescence microscope (Nikon Microphot FXA EPI-FL3, Japan) with FITC filter set at 40× magnification. Positive spermatozoa showing green to yellowish green fluorescence were considered live, whereas red nucleus (PI positive) was indicative of dead/damaged spermatozoa. Spermatozoa that retained staining of the equatorial segment were considered fully acrosome reacted because such cells were totally devoid of staining. While counting, the PI-positive cells were excluded from the estimate of acrosome intact and acrosome reacted live spermatozoa. Spermatozoa showing positive and PI negative fluorescence were considered as acrosome intact live. For the purpose of this experiment, a total of 200 spermatozoa were counted per slide and categorized as positive and PI negative, acrosome intact live (AIL); positive and PI positive, acrosome intact dead (AID); negative and PI negative, acrosome reacted live (ARL) and negative and PI positive, acrosome reacted dead (ARD).

*Determination of mitochondrial functionality of spermatozoa:* Functional status of thawed sperm mitochondria (membrane potential) was evaluated employing the procedure described by Kasai *et al.* (2002). An aliquot of 10 million thawed sperm was washed twice with warm (37°C) HTF-HEPES (IMV, France) followed by dilution with 200 µl of same buffer in an amber colour centrifuge tube. To this, 20 µl of JC 1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide) working solution was added and incubated at 37°C for 15 min. After incubation, a small drop of stained sample was placed on the glass slide, covered with a coverslip and observed under fluorescent microscope (Eclipse, Nikon). Spermatozoa with high membrane potential ( $\Delta\psi_m$ ) showed red to orange fluorescence (Green filter) whereas spermatozoa with low mitochondrial potential appeared green (Blue filter). Identical images on both the filters were merged to get a single image containing spermatozoa with either high or low Dm. At least 200 cells were counted to determine percent of cells with high or low Dm.

*Estimation of cholesterol content of spermatozoa:* For estimation of cholesterol content of spermatozoa, washing of post thaw spermatozoa was carried out (Strom *et al.* 2000) using Percoll density gradient to remove dead cells, debris and egg yolk particles. For this, a 1ml layer of 40% Percoll (v/v in non-capacitating medium (NCM) containing 8.1 mM

Na<sub>2</sub>HPO<sub>4</sub>, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, 2.7 mM KCl, 137 mM NaCl, 1 mM Pyruvate, 5.55 mM Glucose, pH adjusted to 7.0, osmotic pressure 190 mosmol/kg) was pipetted carefully over a 1 ml layer of 80% Percoll (v/v in NCM) in a disposable 15 ml centrifuge tube. Over top of the two steps Percoll column, 1 ml thawed semen was gently layered. This was followed by centrifugation at 400 g 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 ml (in duplicate) in cryovials and stored at -20°C until used for cholesterol estimation. Cholesterol content of spermatozoa was estimated using a Cholesterol Quantitation Kit (Sigma-Aldrich, St Louis, USA) and expressed as (µg/100 million spermatozoa).

**Grouping of bulls according to CR:** The conception rate (%) of all the bulls included in the study were obtained from the data available at the institute. According to CR (%), the bulls were classified into three groups as low (Gr I), medium (Gr II) and high (Gr III). The mean and standard deviation estimates of CR (%) were used as the criteria to group the bulls. The bulls having CR (%) within the range of Mean±1SD were grouped as medium while the bulls with the CR (%) values lesser and higher than this range were grouped as low and higher, respectively. The prediction equations were developed independently for different groups. The semen quality parameters including MMP and cholesterol content of the spermatozoa in relation to available CR of Frieswal bulls were used to construct the prediction equation for CR. In addition to the CR, another measure called estimated relative conception rate (ERCR) was also predicted using the SQP models. The estimated relative conception rate or ERCR, was measured as the conception rate of an individual service sire relative to the average CR (%) of the service sires of herd and ERCRs are expressed as a percent difference of conception rate from the average AI service sire of herd mates (Glozzi *et al.* 2017).

**Statistical analysis:** The stepwise regression analysis was carried out using the least squares principles (Harvey 1990) for each group, and the CR and ERCR were regressed on the various SQP. The best fit regression equation was determined by using the R<sup>2</sup> (%) value or coefficient of variation as calculated below:

$$R^2 \text{ value} = \frac{\text{Total sum of squares} - \text{Error sum of squares}}{\text{Total sum of squares}} \times 100$$

The regression model which gave higher R<sup>2</sup> (%) value with minimum variables was considered as the best. The prediction accuracy was assessed as the difference between the actual and predicted CR and ERCR values. Pearson's correlation coefficients were estimated between the CR and ERCR values and the various SQPs.

## RESULTS AND DISCUSSION

The semen quality parameters including Δψm and cholesterol content of spermatozoa (µg/100 million) were

analysed after their categorization into three groups (low, medium and high) based on the conception rate. The results (Mean±SEM) are presented of experiment in which the frozen-thawed semen quality attributes of samples of Frieswal bulls were assessed by a number of laboratory assays and then correlated with the CR and ERCR, obtained by the use of cryopreserved semen samples in artificial insemination programme.

**Seminal attributes (post thaw):** Table 1 summarizes the SQP of semen samples obtained from Frieswal bulls (32). A cursory look at the Table 1 reveals that although PTM and viability (by EN stain) do not differ between bulls of different groups, other parameters showed significant differences (P<0.05) between bulls of low and high CR groups. In contrast, viability status evaluated through fluorescent probe (FITC-Live) showed significant difference (P<0.05) between Group I and III. Remarkably, a clear and significant difference (P<0.05) was observed among three groups vis-à-vis Δψm (JC 1) and cholesterol content of spermatozoa (µg/100 million).

**Effect of SQP, high Δψm, and cholesterol content of spermatozoa on CR and ERCR:** Perusal of Table 2 revealed that the conception rate is highly (P<0.01) correlated with bull spermatozoa having mitochondria with high membrane potential (r=.823), followed by cholesterol content of plasma membrane, HOS response, FITC-Live, acrosome integrity, viability, and post-thaw motility. Interestingly, and as expected, cholesterol content of spermatozoa was significantly (P<0.01) correlated with all the SQP with r value of .817 and .780 for HOS response and Δψm, respectively being highest amongst all the parameters examined. On the similar note, Δψm evaluated by fluorescent probe JC 1 showed significant (P<0.01)

Table 1. Distribution of post-thaw semen quality parameters based on conception rate of Frieswal bulls (Means±SEM)

Semen attribute	Conception rate (n=32)		
	G I (n=4, %)	G II (n=15, %)	G III (n=13, %)
CR	33.38±2.55 <sup>a</sup>	43.99±0.73 <sup>b</sup>	53.85±1.20 <sup>c</sup>
ERCR	0.72±0.05 <sup>a</sup>	0.94±0.02 <sup>b</sup>	1.15±0.03 <sup>c</sup>
PT motility	47.50±2.50	47.33±1.18	52.31±1.66
Viability	49.25±2.50	49.20±1.04	54.38±1.60
HOS res	40.25±1.31 <sup>a</sup>	43.73±1.09 <sup>a</sup>	50.08±1.03 <sup>b</sup>
Acr-Int	48.25±0.95 <sup>a</sup>	53.47±1.16 <sup>a</sup>	60.46±1.76 <sup>b</sup>
FITC PSA-Live	85.50±3.18 <sup>a</sup>	92.00±0.95 <sup>b</sup>	93.85±0.65 <sup>b</sup>
FITC PSA-Dead	14.50±3018 <sup>a</sup>	8.00±0.95 <sup>b</sup>	6.15±0.65 <sup>b</sup>
JC 1-HM	49.75±0.48 <sup>a</sup>	55.93±0.71 <sup>b</sup>	60.15±0.85 <sup>c</sup>
Chol content	17.49±0.45 <sup>a</sup>	19.42±0.42 <sup>b</sup>	23.42±0.51 <sup>c</sup>

N/n, Total number of samples; CR, Conception rate; ERCR, Estimated relative conception rate; PTM, Post thaw motility; HOS res, Hypo-osmotic swelling response; Acr-Int, Acrosome integrity; FITC-PSA Live/Dead, Fluorescein isothiocyanate-*Pisum sativum* agglutinin Live/Dead; JC 1-HM, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide -High mitochondrial membrane potential; Chol content, Cholesterol content of spermatozoa (µg/100 million). Values bearing different superscripts in a row differ significantly (P<0.05).

Table 2. Pearson's correlation coefficients between post thaw semen quality parameters and CR

Parameter	CR	ERCR	PTM	Viability	Acr-Int	HOS Res	Chol content	FITC PSA-Live	FITC PSA-Dead	JC1-HM
CR	1.00	1.000**								
ERCR	1.000**	1.00								
PTM	0.463**	0.463**	1.00							
Viability	0.480**	0.480**	0.914**	1.00						
Acr-Int	0.679**	0.679**	0.424*	0.388*	1.00					
HOS Res	0.713**	0.713**	0.517**	0.592**	0.504**	1.00				
Chol content	0.789**	0.789**	0.497**	0.519**	0.645**	0.817**	1.00			
FITC PSA-Live	0.532**	0.532**	0.30	0.476**	0.483**	0.450**	0.475**	1.00		
FITC PSA-Dead	-0.532**	-0.532**	-0.30	-0.476**	-0.483**	-0.450**	-0.475**	-1.000**	1.00	
JC1-HM	0.823**	0.823**	0.406*	0.445*	0.574**	0.780**	0.819**	0.637**	-0.637**	1.00

N/n, Total number of samples; CR, Conception rate; ERCR, Estimated relative conception rate; PTM, Post thaw motility; HOS res, Hypo-osmotic swelling response; Acr-Int, Acrosome integrity; FITC-PSA Live/Dead, Fluorescein isothiocyanate-*Pisum sativum* agglutinin Live/Dead; JC 1-HM, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide -High mitochondrial membrane potential; Chol content, Cholesterol content of spermatozoa ( $\mu\text{g}/100$  million). \*\*Correlation is significant at the 0.01 level (2-tailed). \*Correlation is significant at the 0.05 level (2-tailed).

correlation with HOS response, acrosome integrity, cholesterol content, and FITC-Live, whereas it was significantly ( $P < 0.05$ ) correlated with other two parameters namely PTM and viability evaluated by EN staining method.

*Relationship of SQP, high  $\Delta\psi_m$  and cholesterol content of spermatozoa on CR and ERCR:* The prediction of conception rate on the basis of PTM, viability, acr-Int, HOS response, cholesterol content, FITC -Live and JC 1-HM was constructed through multiple regression technique. We developed the constructed equations for prediction of CR and ERCR using a battery of laboratory tests, for whole data covering all the bulls and separately for bulls with medium and high CR following grouping of the bulls. The final equations obtained for the above data set are as follows:

The  $R^2$  values or coefficient of determination were moderately higher for all the models and ranged from 63.70 (High group) to 93.40% (Medium group). The histograms showing the frequency of standardized residual estimates of CR and ERCR are presented in Figs 1 and 2, respectively.

The SQP evaluated routinely for predicting the CR depends on number of parameters such as concentration, motility estimates, morphology and membrane integrity. However, Zavos and Centola (1990) showed futility of using these seminal attributes and have shown them as unreliable predictors of sperm fertilizing ability. Since the investigation of functional status of sperm membrane is of particular significance (Jeyendran *et al.* 1984), we included estimation of cholesterol content of spermatozoa as an indicator of membrane stability, and assessment of mitochondrial membrane potential by JC 1 for motility and metabolic indicator. Perusal of literature failed to reveal any such study in which vital sperm attributes namely cholesterol content of spermatozoa and  $\Delta\psi_m$  were combined with routine SQP to construct a prediction model for CR and ERCR for bulls. In the present investigation, we have utilized the post thaw value of the SQP to construct a prognostic equation for CR

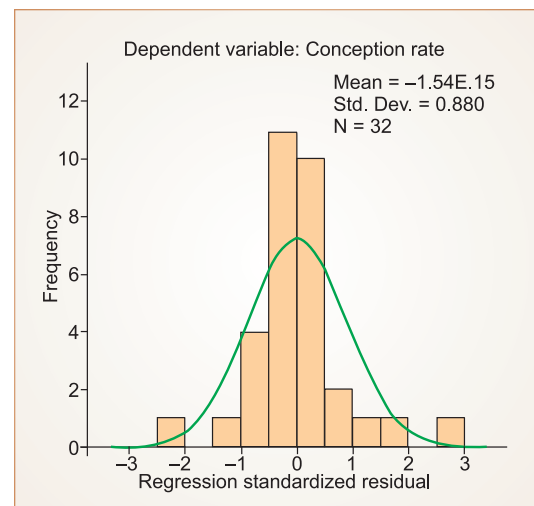


Fig. 1. Histogram showing relationship of the conception rate (CR) with semen quality parameters

and ERCR. Since process of cryopreservation is known to affect the sperm membrane by destabilizing it (Amorin *et al.* 2009) and drastically reducing the motility and vital functions (Brouwers *et al.* 2005), we opted for post thaw SQP to ensure that sperm attributes are at an end-stage of final handling before spermatozoa are used for artificial insemination.

Spermatozoa attributes such as motility, acrosome integrity (Odhiambo *et al.* 2014), sperm abnormalities (Gillian *et al.* 2008), HOS response (Tartaglione and Ritta 2004), mitochondrial activity (Thomas *et al.* 1998) have been shown to positively correlate with bull fertility. In concurrence, the results obtained show that differences in CR and ERCR level could be attributed to variations in SQP. The mean values for SQP, measured on a percentage basis, were higher for the high than for the medium or low CR group. Thus, association of SQP with CR and ERCR, and significant ( $P < 0.05$ ) positive correlation of cholesterol

Table 3. Prediction equation for conception rate (CR) and estimated relative conception rate (ERCR) for Frieswal bulls with high and medium CR and for the herd

Data set	Prediction for	Equation	Accuracy (R <sup>2</sup> value)
Overall-herd (N = 32)	CR	$-32.066 + 0.445 \times \text{Chol content} + 0.309 \times \text{Acr. Int.} - 0.100 \times \text{FITC-PSA Live} + 0.145 \times \text{Viability} - 0.037 \times \text{PTM} + 0.024 \times \text{HOS res} + 0.962 \times \text{JC 1-HM}$	0.759 or 75.90%
Overall-herd (N = 32)	ERCR	$-0.687 + 0.010 \times \text{Chol content} + 0.007 \times \text{Acr. Int.} - 0.002 \times \text{FITC-PSA Live} + 0.003 \times \text{Viability} - 0.001 \times \text{PTM} + 0.001 \times \text{HOS res} + 0.021 \times \text{JC 1-HM}$	0.759 or 75.90%
Medium group (n=15)	CR	$4.030 + 0.221 \times \text{Chol content} + 0.201 \times \text{Acr. Int.} - 0.081 \times \text{Viability} + 0.077 \times \text{PTM} + 0.261 \times \text{HOS res} + 0.384 \times \text{JC 1-HM} - 0.083 \times \text{FITC-PSA Live}$	0.934 or 93.40%
Medium group (n=15)	ERCR	$0.086 + 0.005 \times \text{Chol content} + 0.004 \times \text{Acr. Int.} - 0.002 \times \text{Viability} + 0.002 \times \text{PTM} + 0.006 \times \text{HOS res} + 0.008 \times \text{JC 1-HM} - 0.002 \times \text{FITC-PSA Live}$	0.934 or 93.40%
High group (n=13)	CR	$183.506 - 0.059 \times \text{Chol content} + 0.381 \times \text{Acr. Int.} - 0.241 \times \text{Viability} + 0.613 \times \text{PTM} - 0.623 \times \text{HOS res} + 0.977 \times \text{JC 1-HM} - 2.108 \times \text{FITC-PSA Live}$	0.637 or 63.70%
High group (n=13)	ERCR	$3.932 - 0.001 \times \text{Chol content} + 0.008 \times \text{Acr. Int.} - 0.005 \times \text{Viability} + 0.013 \times \text{PTM} - 0.013 \times \text{HOS res} + 0.021 \times \text{JC 1-LM} - 0.045 \times \text{FITC-PSA Live}$	0.637 or 63.70%

N/n, Total number of samples; CR, conception rate; ERCR, Estimated relative conception rate; PTM, Post thaw motility; HOS res, Hypo osmotic swelling response; Acr. Int, Acrosome integrity; FITC-PSA Live, Fluorescein isothiocyanate-Pisum sativum agglutinin Live; JC 1-HM, 5, 5', 6, 6'-tetrachloro-1, 1', 3, 3'-tetraethylbenzimidazolylcarbocyanine iodide –High mitochondrial membrane potential; Chol content, Cholesterol content of spermatozoa ( $\mu\text{g}/100$  million).

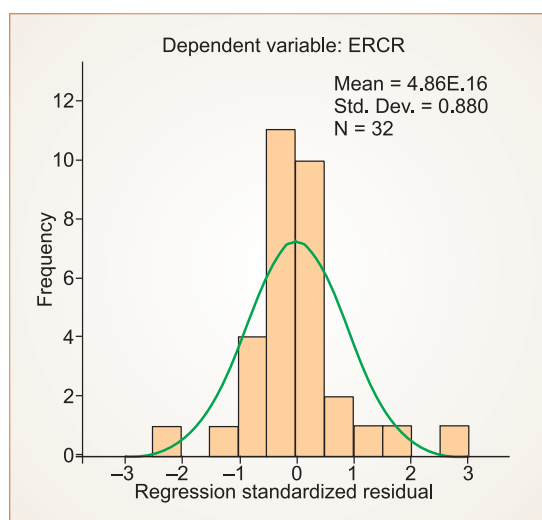


Fig. 2. Histogram showing relationship of the estimated relative conception rate (ERCR) with semen quality parameters

content of spermatozoa with conception rate, ERCR and other SQP is evident from the study. Manjunath and Therien (2002) had shown stabilizing effect of membrane cholesterol on spermatozoa, and hence it is obvious that any variation in the membrane cholesterol content would induce destabilizing or reorganizational changes affecting functioning of the spermatozoa. In support, Pesch and Bergman (2006) observed that the fertilizing potential of sperm cell with low cholesterol content is strongly affected by the sub lethal dysfunction in the proportion of the surviving subpopulation, decrease in the life span, and diminished ability to successfully interact with the oocyte.

Moreover, loss of sperm cholesterol level following cooling and subsequently again at cryopreservation was reported by Moce and Graham (2006) with diminished cholesterol content after freezing thawing. In agreement, our finding gains support from the reports of Sparr and co-workers (2002) indicating that cholesterol has a profound effect on the thermodynamic and mechanical properties of lipid bilayers, and influences stability and fluidity of sperm, thereby influencing sperm attributes. These observations support our finding that greater cholesterol content of sperm membrane at post thaw relates to possibility of improvement in SQP and consequently high CR in bulls.

For a successful pregnancy to occur, oocyte and sperm have to undergo a series of well-organized events in tandem. For spermatozoa, one of the most crucial characteristics is progressive motility, essential for transport to the waiting oocyte and penetration of zona pellucida. Mitochondria perform crucial cellular functions to maintain homeostasis within, are producers of free radicals and contribute to the process of metabolism of lipids and proteins (Cottet-Rousselle *et al.* 2011). In agreement, we report positive significant relationship of viable spermatozoa with high MMP with CR (Low, Medium and High). These findings are comparable with that of Mukhopadhyay *et al.* (2008) reporting high sperm mitochondrial activity index with high fertility cattle bulls; and Husain *et al.* (2016) showing high MMP with *in vivo* fertility in buffalo bull during low-breeding season. Since the semen samples with higher sperm motility are expected to have greater sperm mitochondrial activity, as reflected in Gr III compared to that of the low CR group values in current study, determination of  $\Delta\psi_m$  can serve dual purpose of providing

information on motility and metabolic estimates of sperm cells. In concurrence, Gopalkrishnan *et al.* (1991) suggested estimation of  $\Delta\psi_m$  as a supplementary assay to the gross SQP evaluation for accurate quality prediction.

The results of multiple regression models fitted in the study are given in Table 3 and all were statistically significant ( $P < 0.01$ ). The coefficient of determination ( $R^2$ -value), an indicative of the prediction efficiency of the model showed that the seven SQPs included in the model such as viability, PTM, HOS res, Acr-Int, FITC-Live, JC 1-HM and Chol content were reliable indicators of the conception rate in Frieswal cattle. The  $R^2$  values were 0.934, 0.637 and 0.759 for the medium SQP, high SQP and combined data of Frieswal bulls, respectively. These values indicate the CR in medium group bulls can be predicted with 93.40% accuracy while in high CR bulls, the prediction efficiency was only 63.70%. Perusal of the available literature revealed that no elaborate research has been carried out on the prediction of CR using SQPs and hence the results obtained in the study could not be ascertained with other reports. However, Mukhopadhyay *et al.* (2008) developed the regression models for predicting the CR based on *in vitro* acrosome reaction (AR) and obtained the lower prediction accuracy of 37.30% in Karan Fries crossbred cattle.

The results also revealed that the JC 1-HM, cholesterol content, acrosome integrity and viability are in general, the important SQPs in that order which alter the CR of Frieswal bulls. Thus it may be inferred that the bull superior for the above SQPs may have better chance of conception than the other bulls. The prediction models developed for ERRCR also showed the findings similar to the CR as the ERRCR was taken as the relative measure of the individual CR with the average CR of the herd. Although sparse reports are available on the predictability of cholesterol content of spermatozoa and  $\Delta\psi_m$  for CR in bovines, these assays are reliable and repeatable. Therefore, these assays can be incorporated in our routine semen analysis before using semen for long-term storage or insemination purposes. In current study, high  $R^2$  value of the prediction equation for the herd (Overall-herd) and bulls with medium CR (75.9 and 93.4%, respectively) reveal their applicability to effectively predict the CR and ERRCR potential of the cryopreserved semen.

Though a much wider study is needed for a final say, present investigation points to cholesterol content of fresh spermatozoa, and mitochondrial membrane potential assessment by JC 1 in combination with routine seminal attributes as reliable laboratory protocols to predict frozen-thaw CR and ERRCR. Though the study revealed the inadequacy of the laboratory assays employed as methods of predicting the fertility of semen samples from bulls with low CR but show that boundaries may be set outside which poor samples could be discarded. However, it is to be noted that the total number of bulls with low CR (Gr I) was less for a definite conclusion.

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