



Comparative Study of Three Different Media to Improve Semen Processing and Flow Cytometric Analysis in Bovine

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ABSTRACT

The objective of this study was to compare the efficiency of three selected semen processing media: TRIS, TALP and HTF (Human Tubal Fluid) on sperm viability in post-preparation samples to improve flow cytometric analysis. Towards this aim, PI (Propidium Iodide) dye was used to evaluate sperm viability using flow cytometer. Two Cross breed (Karan Fries, KF) and Two Indian breed (Sahiwal, SW) bulls were used for different media optimization. The average sperm viability with TRIS, TALP & HTF media in cross breed (Karan fries) bulls was observed to be 92.09 ±1.93%, 82.61±2.71%, and 79.78±2.94% respectively, whereas, for Indian breeds (Sahiwal) it was 80.03±2.29%, 77.18±1.61 % and 71.32±2.51% respectively. The results revealed that viability of semen obtained from cross breed bulls (KF) is significantly higher in TRIS media (P<0.001) compared to TALP and HTF (P >0.05) whereas for Indian breed bulls (SW), viability of semen in TRIS and TALP is similar with no significant difference (P >0.05). When compared to HTF, semen of SW bull was found to have highest survivability in TRIS (80.03±2.29% vs 71.32±2.51% in TRIS and HTF respectively; P< 0.01). Hence, it can be concluded from the present study that overall TRIS medium is best suited for semen processing and flow cytometric analysis.

Keywords: Bull semen, flow cytometer, probes, fluorochromes, semen processing

One of the crucial aspects known to influence the quality of fresh bovine semen is semen preparation medium or diluter used throughout semen processing and flow cytometric assessment (Chiamchanya *et al.*, 2010). Freshly ejaculated semen from cattle bulls are frequently diluted in a diluter or extender range from a simple salt solution to a more complex buffered medium for survival of spermatozoa for extended periods. Most frequently used diluter or extender are Tris-buffered egg yolk extender (Davis *et al.*, 1963a,b; Foote, 1970), Egg yolk-phosphate (Phillips, 1939), Egg yolk-citrate (Salisbury *et al.*, 1941), homogenised whole milk (Salisbury *et al.*, 1961), fresh or reconstituted skim milk (Melrose, 1962; Foote, 1978), coconut milk (Norman, 1972). The basic constituents of these diluents used generally includes ionic or non-ionic substance that functions to provide buffering capacity along with maintaining the osmolarity of the medium, supply of lipoprotein and high molecular weight material to avoid

cold shock, glycerol or DMSO as cryoprotectant, fructose or glucose as an energy source and other preservatives such as enzymes and antibiotics (Vishwanath *et al.*, 2000). On the other hand now a day's flow cytometry is being more frequently used at semen stations and majority of specialized andrological laboratories for routine semen assessment (Martinez-Pastor *et al.*, 2010). A number of sperm attributes including integrity, viability and function can be assessed by flowcytometry using variety of fluorochromes and compounds conjugated to fluorescent probes (Gillan *et al.*, 2005). Flow cytometer rely on the ability of the equipment to detect specifically bound fluorescent molecules and resolve them from other intrinsic and non specific signals (Mosiman *et al.*, 1997). All flow cytometric analysis of semen samples requires preparation that usually includes dilution with media that ideally should not interfere with sperm bound dye fluorescence. However, all available diluters or sample

preparation medium used in most of semen stations and andrology laboratory often contains a lot of suspended particles, animal origin proteins including egg yolk granules and unwanted debris that binds to the dye or fluorophore molecules non-specifically and diminish its binding and fluorescence specificity that compromise the accuracy of flow cytometric analysis (Petrunina *et al.*, 2010). Garner and Johnson (1995) proposed that amount of debris tends to be related to the initial sperm concentration and the type of medium in which the sperm are suspended. At present scenario there is no semen preparation medium specified that meets the specific needs of flow cytometric analysis and also maintain the viability and intactness of sperm cell.

Thus, the objective of this study was proposed to compare the effects of three chemically defined transparent medium on sperm viability towards the development of optimized media that be short of animal origin products and particulate debris that not only improve flow cytometric analysis but also support the survivability of sperm cells during semen processing.

MATERIALS AND METHODS

Semen processing medium

TRIS based medium consisting of TRIS (hydroxymethyl) amino-methane pH-6.9 (Tris; 200 mM) (Cat. T6066, Sigma-Aldrich, USA) with Citric acid monohydrate (65.0 mM) and Fructose (56.0 mM), Tyrode's bicarbonate buffered medium (TALP), pH-7.4 (Cat. T2397, Sigma-Aldrich, USA) with Sodium pyruvate (1.0 mM) and Sodium lactate (21.6 mM) and Human Tubal Fluid (HTF), (Cat. MR-070-D, Merck Millipore, MA, USA) was used for semen processing.

Animals and semen collection

Two cross breed (Karan Fries, KF) and two Indian native (Sahiwal, SW) Zebu bulls of proven fertile of 3-5 years of age were used for different media optimization. Bulls were available at the Animal Breeding Research Complex (ABRC) at ICAR-NDRI, Karnal, India under uniform nutritional condition. Each bull semen was collected twice a week using artificial vagina (IMV, L'Aigle cedex, France) maintained at 41°C. Semen volume, sperm mass activity

and individual motility were evaluated immediately after semen collection. Ejaculates showing a score of +3 or above mass activity and more than 80% progressive motility were further kept for use in this study.

Semen processing

Five hundred microlitre of freshly ejaculated semen was taken into three 15-mL polypropylene tubes and diluted by 3 mL of all three different medium separately and washed twice using centrifugation at 1100 rpm for 7 min. After washing, each tube was further split into three aliquots with a final concentration of 30×10^6 cells/mL using all three different media separately and kept all tubes at 37°C for 1hr to assess viability for optimization of media.

Fluorescent staining

The membrane impermeable stain propidium iodide (PI) is the most frequently used viability stain. It is easy to use, rapid and can be excited with the 488-nm laser that is installed in most cytometers. PI penetrates only cells with a damaged plasma-membrane, emitting red fluorescence (PI: 636 nm) when bound to nucleic acids and reveals only dead cells. The PI (Cat. P4170, Sigma Aldrich, USA) was dissolved in Tyrode's salt solution at 2 mg/mL. Each Aliquot (1mL) of diluted semen in 3 different medium with 30×10^6 cells/mL were stained with PI at the final concentration of 4µg/mL at 36°C for 2-3 min.

Flow cytometric analysis

Quantitative data of the fluorescently stained samples were acquired through use of MoFlo XDP (Beckman Coulter, USA) sorter equipped with a 100 mW argon laser. FSC (Forward Scatter) and SSC (Side Scatter) light were analysed in linear mode and all doublets are excluded from analysis. Stained samples were excited by 488 nm argon laser and emission was detected through 635-nm band-pass filter. A total of 10,000 sperm per sample were analyzed for the log of their fluorescence for each sample.

Statistical analysis

All the experiments were carried out in four replicates i.e. two semen ejaculates from two individual bulls. Results are expressed as the Means \pm Standard Error Mean (SEM).

Data were analysed by two-way analysis of variance (ANOVA) using GraphPad Prism version 7.01 (GraphPad Prism software, CA, USA) at 95% of confidence limit. A value of $p \leq 0.05$ was considered statistically significant. Flow cytometric data were analysed using Summit Software 5.1 (Beckman Coulter Inc. USA).

RESULTS

Flow cytometric analysis

Dot-plot cytograms was generated for each semen sample (10,000 sperm) obtained from KF and SW bulls in all three different media. Unstained samples from all three media were used as controls to analyze auto-fluorescence of cells in PI channel. Gates were applied for the forward and side scatter light parameters so that only those cells possessing the light scatter characteristics of sperm were analyzed for fluorescence intensity (Fig. 1).

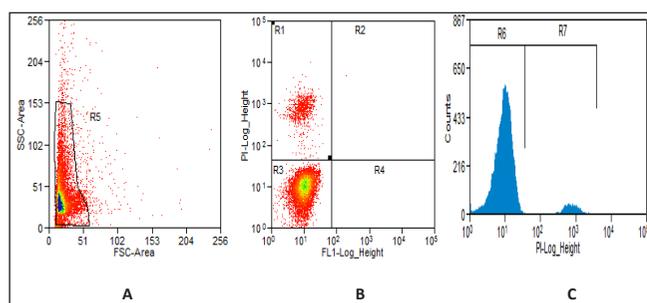


Fig. 1: Viability assessment using flow cytometer. (A) Selection of sperm cells on the basis of scattering, only sperm cells were selected and gated (R5), (B) Cytogram of PI stained dead cells (R1) and non-stained live cells (R3), (C) Histogram of PI stained dead cells (R7) and non-stained live cells (R6)

Comparative efficiency of semen processing medium

The sperm viability of fresh semen was assessed with three different sample preparation medium, and results are shown in Table 1. The statistical analysis of data of comparative study revealed that TRIS media is more suitable for semen processing as $92.09 \pm 1.93\%$ live and intact sperms (PI impermeable cells) were observed in semen of cross breed (KF) bull which was significantly higher ($P \leq 0.001$ for KF) than other two media used for study. Although, for SW semen, no major difference in survivability was observed in TRIS ($80.03 \pm 2.29\%$) and TALP ($77.18 \pm 1.61\%$) media

($P > 0.05$). The obtained result also indicates that semen from KF is more stable in TRIS as compared to that of SW in same media. After preparation with all three media, sperm viability significantly decreased ($P \leq 0.05$) in TALP ($82.61 \pm 2.71\%$) and HTF medium (79.78 ± 2.94) ($P \leq 0.05$) for KF breed. Similar pattern of low survivability was observed for SW semen in HTF media (71.32 ± 2.51).

Table 1: Effect of three different sample preparation medium on sperm viability (%) (Mean \pm SE) of cross breed (KF) and Indian breed (SW) bovine bulls

Media	Viability	Bull KF	Bull SW
TRIS	Live %	92.09 ± 1.93^{aA}	80.03 ± 2.29^{aB}
	Dead %	7.91 ± 1.93	19.92 ± 2.29
TALP	Live %	82.61 ± 2.71^{bA}	77.18 ± 1.61^{aB}
	Dead %	17.38 ± 2.71	22.76 ± 1.61
HTF	Live %	79.78 ± 2.94^{bA}	71.32 ± 2.51^{bB}
	Dead %	20.20 ± 2.94	28.61 ± 2.53

All data represented as mean \pm SEM where $n = 4$, $P \leq 0.05$, confidence of limit = 95%; *Down the column: values with different superscripts (a, b) represent significant difference; ** Along the row: values with different superscript (A, B) represent significant difference.

Beyond type of media used, the bull breed also has its significant effect on sperm viability. Semen obtained from KF breed of bulls were shown more survivability than that of SW irrespective of media used. With all three media viability of sperm was significantly higher ($P \leq 0.05$) in KF in compare to SW (Fig. 2).

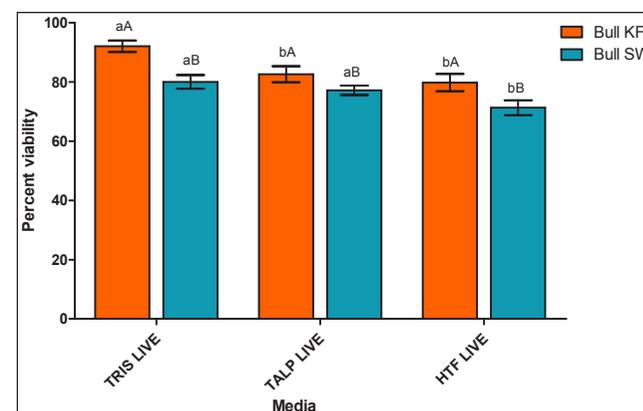


Fig. 2: Comparative Efficiency of Semen Processing Medium in Cross breed (KF) and Indian breed (SW)

DISCUSSION

Acquisition of high-quality precise data derived from flow cytometric analysis are very much depends on the use of proper semen preparation medium or diluter in which sperm cells are suspended, that ensure optimum binding of dye with target molecule and contribute to produce accurate figures. The medium formulation must also meet the metabolic requirements for the spermatozoa during the incubation period (Ferre *et al.*, 2015). Numbers of reports are available for different media that are used as extenders for storage of fresh liquid bovine semen (Vishwanath *et al.*, 2000) and as fertilization medium (Brackett and Oliphant, 1975; Parrish *et al.* 1988; Choi *et al.*, 1991; Gandhi *et al.*, 2000; Nedambale *et al.*, 2006). Many reports proposed for use of Tris-extenders or TRIS based medium (Lapointe *et al.*, 1998; Schenk *et al.*, 1999; Taşdemir *et al.*, 2013), TALP medium (Parrish *et al.*, 1988; Parrish *et al.*, 1989; Galantino-Homer *et al.*, 1997; Pauw de *et al.*, 2003) and HTF medium (Zavos and Centola, 1992; Osheroff *et al.*, 1999; Riel *et al.*, 2011) for cell and embryo culture medium but still there was a complete lack of information about any medium that can improve flow cytometric analysis and maintain sperm survivability simultaneously.

To our knowledge, this is the first study that evaluated sperm viability after dilution with defined medium to improve flow cytometric analysis. All three medium evaluated in this study didn't contain animal origin proteins, antibiotics, cryoprotectant or any other preservative. The results of the present study demonstrated that Tris medium with energy source like fructose showed greatest survivability of bovine sperm cell after semen processing and incubation. TRIS medium thus protect semen against external stress factors and prolonged semen survivability significantly in both cases including cross breed and Indian native bovine bulls.

Beyond the advantages of being cost effective and easily available commercially, TRIS media also didn't contains any insoluble particles or debris or antibiotics (only required for long term preservation) thus making it a preferred media for use efficiently as a semen preparation medium in all flow cytometric assessment. Due to these properties TRIS buffered medium can also serve the purpose of sheath fluid in all flow cytometers that will work as electrolytes which can assist to provide charge during sorting process.

In conclusion, TRIS preparation yielded the best sperm viability with the highest percentage of plasma-membrane intact sperm and a lowest percentage of damaged sperm in both species. Based on present finding we propose TRIS medium as an alternative source for semen preparation medium throughout the flow cytometric assessment.

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