Original Research

Effect of Media for *in- vitro* Capacitation of Swamp Buffalo Spermatozoa

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Abstract

Freshly ejaculated spermatozoa of eight Swamp buffalo bulls were capacitated by incubating in TALP, MCM and KRB media at a sperm concentration of 6×10^9 spermatozoa/ml at 37ºC for six hours. The hyperactivated motility, acrosome membrane integrity, total HOST reacted spermatozoa, activity of ALT and AST, sperm membrane protein and cholesterol content were estimated from 40 ejaculates, 5 ejaculates from each bull at one hour interval starting from zero hour to six hours. The results revealed that the highest hyperactivation of spermatozoa was recorded on 4 hours of incubation in TALP than MCM and KRB media. The hyperactivated motility of the spermatozoa significantly (P<0.01) increased irrespective of media upto 4 hours then it decreased upto 6 hours. The significantly (P<0.01) highest live acrosome reacted spermatozoa were recorded on 4 hours of incubation in TALP than MCM and KRB media. The total HOST reacted spermatozoa were significantly (P<0.01) decreased under increased incubation period in all three media. The ALT and AST activity also increased significantly (P< 0.01) under increased incubation period in all three media. The sperm membrane protein and cholesterol levels decreased significantly (P< 0.01) at each period of incubation i.e. 2, 4, 6 hrs in all three media. In conclusion, though TALP is the best media, however MCM and KRB are also comparable.

Key words: *In- vitro* Capacitation, Media, Spermatozoa, Swamp Buffalo

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Introduction

The Swamp buffalo (*Bubalus carabensis*) is an irreplaceable producer of both energy and protein in north-eastern region of India (Talukdar *et al.,* 2017). Presently, the best tool to augment the maternal contribution to genetic improvement is ovum pick-up (OPU) and *in vitro* embryo production (IVEP) and in this technique many factors are known to influence the total efficiency, such as the sperm quality, the bull, the

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environment, the appropriate time of insemination, as well as an appropriate capacitation of either fresh or frozen-thawed sperm. Indeed, sperm need to undergo capacitation to acquire fertilizing ability.

Capacitation process, which occurs *in vivo* within the female genital tract, must be induced *in vitro*. The *in vitro* capacitation is possible in the absence of reproductive tract fluids and several compounds viz. heparin, bicarbonate, calcium, serum albumin, pyruvate and lactate are known to induce in vitro capacitation and successful fertilization (De Lamirande *et al*., 1997; Visconti, 2009; Talukdar *et al.,* 2015a). Heparin and other glycosaminoglycans are known to enhance this process they have a positive effect on *in vitro* capacitation (Parrish *et al*., 1985; Talukdar *et al.,* 2015b) and fertilization of bovine spermatozoa (Saeki *et al*., 1995). The process of sperm capacitation, as well as the acrosome reaction, depends on the increase in intracellular Ca^{++} levels and it may be speculated that the promoting effect on capacitation is mediated by its ability to influence the oscillations of this ion (Jaconi *et al*., 1991). It had been reported that different integrin receptors can trigger the increase in intracellular Ca^{++} (Haque *et al.,* 2019). Meizel (1985) stated that oviductal and follicular fluid contained serum albumin which stimulated capacitation of spermatozoa *in vitro* by removing fatty acids and cholesterol from sperm membrane. Parrish *et al.* (1985) mentioned that the *in vitro* fertilization frequency of bull spermatozoa was 31 per cent without glycosaminoglycans, 34 per cent with chondroitin sulfate A and 67 per cent with heparin in the medium. Consequently, the rate of capacitation depends on the chemical composition of the media and its concentration (Bansal, 2010; Talukdar *et al.,* 2015b). Although limited research has been done with *in vitro* capacitation of swamp buffalo spermatozoa by using such compounds. Therefore, the present study was designed to study the different changes during *in vitro* capacitation by using three different media.

Materials and Methods

A total of 40 ejaculates, 5 ejaculates from each bull (n=8) were collected by artificial vagina method from buffalo bulls aged 5 to 8 years maintained at "ICAR- Network Project on Swamp Buffalo" College of Veterinary Science, Khanapara, Assam, India. Each ejaculate was evaluated for volume, mass activity, and initial motility immediately after collection. Samples having volume 1.0 ml or more, mass activity 3 + or more and initial sperm motility 70 per cent or more were used for *in vitro* capacitation by Tyrode Albumin Lactate Pyruvate (TALP) Medium (Rogers and Yanangimachi, 1975), Modified Mininal Culture Medium (MCM) (Barros, 1974) and Modified Krebs- Ringer bicarbonate buffered (KRB) Medium (Kaul *et al.,* 1997) at 37°C for 6 hours.

The fresh semen samples were washed using 2ml of phosphate buffered saline (PBS) (pH 7.4) by centrifugation at 3000 rpm for 20 minutes. The supernatant was discarded and the pellet was washed again by the same procedure after adding 2 ml of PBS and resuspended in PBS to make desired concentration of sperm depending upon the experiment (Lone *et al*., 2018). Washed fresh spermatozoa were suspended in

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three media such as TALP, MCM and KRB media at the concentration of 6×10^9 spermatozoa/ml of medium. Spermatozoa, suspended in all three media were incubated at 37˚C for 6 h. Each sample were evaluated at one-hour interval starting from 0 h for hyperactivated motility, acrosomal status, hypo-osmotic swelling test, activity of Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST), quantitative estimation of sperm membrane protein and sperm cholesterol level.

The *in vitro* capacitated spermatozoa samples were incubated starting from 0 to 6 hours and hyperactivated motility was evaluated at every hour. One drop of sperm suspension from each was taken on a preheated slide and covered with a coverslip. The slide was then placed on the warm stage of the biotherm and the sperm samples were examined under phase contrast optics at a magnification of 40X and the percentage of spermatozoa with hyperactivated and non-activated motility was recorded. A spermatozoa was considered to be hyperactivated when it swim in a vigorous figure-eight pattern as described by Marquez and Susan (2004). For evaluation of acrosomal status, the Eosin- Nigrosin- Giemsa staining was done as per the method described by Tamuli and Watson (1994). One drop of semen was placed on a warm (35˚C) glass slide and stained with two drops of pre- warmed (35˚C) Eosin-Nigrosin stain on mixing and left for 30 seconds. A thin uniform smear out of the mixture was made on a clean grease-free glass slide and dried. The dried smear was fixed in a tartrate phosphate buffer [Potassium sodium tartrate (77mM)- 2.179 g, Sodium dihydrogen orthophosphate (50mM)- 0.700 g, Potassium dihydrogen orthophosphate (25mM)- 0.340 g and distilled water up to 100 ml, P^H 7.0] with 10% formaldehyde solution for 10 minutes. The fixed smear was washed under slow running tap water for 10 minutes and then rinsed with distilled water. The smear was then stained with Giemsa working solution for 60 minutes, rinsed with distilled water and dried in air. The stained slide was examined under oil immersion lens of the microscope at 1000X magnification. A minimum of 200 spermatozoa were counted and four categories of spermatozoa *viz*., Live acrosome intact, Live acrosome reacted, Dead acrosome intact and Dead acrosome reacted were identified and percentage of each category of spermatozoa was determined.

The functional integrity of the sperm membrane was studied using Hypo-Osmotic Solution maintained at 150 mOsm /L (Jeyendran *et al*., 1984). A total of 200 spermatozoa were examined in different fields at a magnification of 400X using a phase contrast microscope and total type of swellings were recorded and calculated in percentage. The *in vitro* capacitated semen samples from each bull were evaluated for ALT and AST activity as per the recommendation of manufacturer (Siemens Ltd., 589, Sayajipura, Ajwa Road, Vadodara-390 019, Gujarat, India) in a Systronics Spectrophotometer 106 and expressed in unit/ 10^8 spermatozoa. Protein extraction was done as per the method described by Cheema *et al.* (2011). Aliquots of *in vitro* capacitated semen were washed two times with PBS (pH 7.4) by centrifugation at 4000 rpm for 10 minutes. Sperm membrane proteins were extracted by incubating 1.0×10^9 spermatozoa in 1.0 ml of 1% deoxycholate (DOC) in 0.02 M Tris-HCl buffer (pH 6.8) in boiling water bath (5 min). Sperm suspension

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was centrifuged at 6000 rpm for 30 minutes at room temperature. To get sperm membrane extract, 5 % mercapto-ethanol was added to the supernatant, kept in boiling water bath for 5 min and again centrifuged at 6000 rpm for 30 min. The pellet was discarded and supernatant was stored at -20°C for protein analysis. Estimation was done by Burette method using Siemens kit (Siemens Ltd., 589, Sayajipura, Ajwa Road, Vadodara-390 019, Gujarat, India) in a Systronics Spectrophotometer 106 and expressed in mg/10^9 spermatozoa

Cholesterol was estimated according to the method described by Srivastava *et al.* (2013). Aliquots of *in vitro* capacitated semen were washed three times with PBS (P^H 7.4) by centrifugation at 800 rpm for 10 minutes. The pellet of approximately 100 million washed spermatozoa was taken in a 10 ml vial (Srivastava *et al.*, 2013). The sperm pellet was extracted with 20 volume of chloroform: methanol (1:1, v/v) solution and vortexed for 20 seconds (Srivastava *et al.*, 2013). Subsequently, it was centrifuged at 800 rpm for 5 minutes 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 enzymatic method using cholesterol assay kit (Siemens Ltd., 589, Sayajipura, Ajwa Road, Vadodara-390 019, Gujarat, India) in a Systronics Spectrophotometer 106 and expressed in µg/10^8 spermatozoa. The statistical analysis of the data was done using SAS Enterprise Guide 4.2 version. Data were expressed in mean ± SE. Analysis of variance between the media and between the hours of capacitation of means at 10% significance level by Duncan's multiple range test (DMRT) was carried out. Results were considered significant at $P<0.01$.

Results and Discussion

The mean ± SE of different parameters studied in the swamp buffalo spermatozoa at different hours of incubation in different capacitation media are presented in the Table 1.

Hyperactivated Motility

The hyperactivated sperm motility is characterized by high-amplitude and asymmetrical flagellar beating that assists sperm in penetrating the oocyte zona pellucida (Marquez and Susan, 2004). In the present study, significantly higher hyperactivated motility of spermatozoa was recorded on 4 hours of incubation in TALP than MCM and KRB media with an overall mean of 64.75±1.10 per cent (Table 1). The present finding is in agreement with Bansal (2010). The hyperactivated motility of the spermatozoa irrespective of media significantly ($P<0.01$) increases upto 4 hours then it decreases upto 6 hours, which is in agreement with the findings of Agarwal and Venha- Perttula (1987) and Bansal (2010). These might be due to molecular changes related to the sperm capacitation begin after 1 hour of incubation and further, a significant ($P < 0.01$) decrease in per cent hyperactivity from 4 to 6 hours showed the occurrence of acrosome reaction during which many metabolic and ionic changes occurred in sperm membrane leading to the decreased in

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per cent hyperactivity (Talukdar *et al.,* 2015b). In the present study, the maximum hyperactivated motility of the spermatozoa was recorded in TALP media than the MCM and KRB media which might be due changes in the chemical composition of the media. The calcium is required for the capacitation of spermatozoa (Kaul *et al.*, 2001; Talukdar *et al.,* 2015a) which is more in TALP media. Miyamoto and Chang (1973) stated that the serum albumin and metabolic intermediates alone or with sodium lactate and sodium pyruvate induced capacitation of spermatozoa and fertilize the eggs *in vitro*. The content of such chemicals are more in TALP media than MCM and KRB media.

Live Acrosome Reacted Spermatozoa

Acrosome of the spermatozoa is necessary to protect and releases the enzymatic contents at the right time and place for the effective fertilization. The enzymes stored between inner and outer membrane of acrosome when released at the time of acrosomal reaction act sequentially and specifically on cumulus, corona radiate and zona pellucida of the ovum. Further the plasma membrane undergoes capacitational changes and acrosomal reaction in the uterine environment, which is a prerequisite for successful fertilization (Esteves *et al*., 2007; Goodman, 2009). The significantly (P<0.01) highest live acrosome reacted spermatozoa was recorded on 4 hours of incubation in TALP than MCM and KRB media (Table 1) with an overall on 3 hours (49.44±1.55%). The mean incidence of live acrosome reacted spermatozoa recorded in the present study is higher than that reported by Bansal (2010).

Total HOST Reacted Spermatozoa

The ability of spermatozoa to swell in the presence of hypo osmotic medium reflects normal water transport across the sperm plasma membrane, which is a sign of normal membrane integrity and functional activity (Jayendran *et al.,* 1984) and the presence or absence of sperm tail swelling might be indicative of sperm head membranes would react during capacitation and acrosome reaction (Talukdar *et al.,* 2016). Yanagimachi (1994) reported that the plasma membrane integrity of sperm is of crucial importance for optimal sperm function and only a sperm with an intact plasma membrane can undergo a series of complex changes in the female reproductive tract and can acquire the ability to fertilize an oocyte. Thundathil *et al.* (2002) reported that there was a statistically significant positive correlation between the percentage of uncapacitated spermatozoa and the percentage of HOST positive spermatozoa and also similarly positive correlation was obtained between the proportion of spermatozoa with a negative HOST response and the proportion of acrosome reacted spermatozoa. In the present study, the total HOST reacted spermatozoa were significantly (P<0.01) decreased while incubation period increased in all three media (Table 1). The percentage of total HOST reacted spermatozoa between the media were significant.

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Table 1: Different activity of swamp buffalo spermatozoa at different hours of incubation in different capacitation media

Criteria	Hours / Media	0	1	2	3	4	5	6	Effect
Hyperactivated Motility (%)	TALP	14.00^{a} ±0.80	35.75^{b}±1.12	52.87^{c}±1.38	60.25^{e}±1.05	74.50^{f}±1.78	47.50^{g}±1.11	41.25^{d}±1.30	P < 0.01
	MCM	13.12^{a}±0.75	36.00^{b}±1.04	36.87^{b}±0.97	42.62^{d}±1.10	59.50^{e}±1.47	34.87^{b}±1.19	28.75^{h}±1.34	
	KRB	13.12^{a}±0.77	36.87^{b}±1.37	41.12^{d}±1.05	42.75^{d}±1.18	60.25^{e}±1.45	35.00^{b}±0.87	27.00^{h}±1.34	
Live acrosome reacted sperm (%)	TALP	8.62^{j}±0.33	45.57^{cde}±2.97	52.42^{ab}±2.49	50.92^{abc}±2.39	56.92^{a}±1.88	54.27^{ab}±1.83	49.37^{bcd}±2.59	P < 0.01
	MCM	7.60^{j}±0.44	29.00^{i}±2.35	39.17^{fg}±2.96	43.47^{def}±2.82	44.05^{def}±1.98	46.07^{cde}±2.43	42.17^{efg}±2.57	
	KRB	6.40^{j}±0.43	32.22^{hi}±1.79	39.20^{fg}±2.07	53.92^{ab}±2.62	44.67^{def}±2.22	36.65^{gh}±2.52	28.72^{i}±2.15	
Total HOST reacted Sperm (%)	TALP	82.55^{a}±1.00	78.02^{abc}±1.26	77.22^{bc}±1.12	75.55^{cd}±1.21	71.70^{de}±1.49	69.25^{ef}±1.61	61.57^{gh}±2.20	P < 0.01
	MCM	81.77^{ab}±1.03	58.20^{h}±2.75	52.17^{i}±1.94	51.17^{ij}±2.11	48.90^{ijk}±1.87	46.50^{jkl}±1.79	42.70^{l}±1.84	
	KRB	82.07^{ab}±0.97	71.12^{ed}±1.75	69.35^{ef}±1.61	65.22^{fg}±2.29	60.25^{gh}±2.57	52.70^{i}±2.82	45.05^{kl}±2.60	
AST(unit/ 10^{8} sperm)	TALP	12.63±1.14	31.28±1.11	39.67±4.52	53.96±1.37	70.39±1.53	88.98±2.28	90.40±2.87	
	MCM	10.02±0.49	34.47±1.73	37.52±1.17	56.64±1.78	77.23±2.19	87.69±2.19	94.34±3.27	P < 0.01
	KRB	13.36±1.05	36.71±1.56	40.93±2.07	61.02±3.04	73.53±3.37	84.68±2.05	97.49±2.83	
ALT (unit/ 10^{8} sperm)	TALP	0.89^{l}±0.11	1.77^{k}±0.15	3.65^{ij}±0.18	4.91^{gh}±0.12	6.17^{f}±0.20	7.64^{d}±0.31	13.20^{a}±0.40	P < 0.01
	MCM	0.94^{l}±0.10	1.71^{k}±0.15	4.23^{hi}±0.19	5.32^{g}±0.27	6.90^{e}±0.34	7.45^{de}±0.28	11.06^{b}±0.43	
	KRB	0.94^{l}±0.10	2.02^{k}±0.15	3.35^{j}±0.25	5.07^{g}±0.19	5.02^{g}±0.13	8.55^{c}±0.37	10.74^{b}±0.47	
SMP (mg/ 10^{9} sperm)	TALP	5.13±0.12	3.92±0.13	3.25±0.12	2.64±0.11	2.55±0.13	1.82±0.06	1.45±0.08	P < 0.01
	MCM	5.13±0.12	3.69±0.18	3.44±0.10	3.10±0.13	2.74±0.14	2.29±0.13	1.85±0.13	
	KRB	5.13±0.12	4.08±0.19	3.28±0.15	2.95±0.15	2.45±0.14	2.20±0.13	1.95±0.09	
Cholesterol (µg/ 10^{8} sperm)	TALP	21.95±0.44	19.08±0.51	14.65±0.51	12.94±0.47	11.07±0.59	8.45±0.54	5.64±0.46	
	MCM	22.44±0.43	20.56±0.50	17.52±0.69	13.76±0.51	11.70±0.58	9.61±0.57	6.10±0.42	P < 0.01
	KRB	22.01±0.43	20.23±0.41	15.71±0.54	14.34±0.55	11.38±0.58	10.46±0.78	7.90±0.72	

Means bearing different superscripts in a row and column differs significantly.

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ALT and AST Activity

In the present study, it was observed that the ALT and AST activity increased significantly (P< 0.01) while incubation period increased in all three media. Similar trend, as reported by Nath *et al.* (1996), which might be due to acrosome reaction in changes occurs in mitochondrial sheath with loss of protein from mid piece (Graham *et al.*, 1974; Talukdar *et al.,* 2016) and increase in cell membrane permeability with or without rupture of cell membrane (Roychoudhary *et al*., 1974).

Sperm Membrane Protein

The sperm surface proteins during capacitation, are modified or removed and an range of proteins have been shown to undergo tyrosine phosphorylation in different species (Luconi *et al*., 1996; Galantino *et al*., 1997; Talukdar *et al.,* 2015b). In fertilization, these mammalian sperm membrane proteins are also involved in the penetration of cumulus matrix, recognition of zona pellucida and fusion with the oocyte plasma membrane (Myles and Primakoff, 1997). In the present study, it was observed that the sperm membrane protein levels decreased significantly (P< 0.01) while incubation period increased in all three media (Table 1), and it was more in TALP in comparison to other media which was in agreement with Dhanju *et al*. (2006) who reported that the protein content of capacitated spermatozoa decreased significantly (P<0.05) as capacitation time increased and it showed a correlation with the rate of acrosome reaction. The present observations suggested that the rate of capacitation and acrosome reaction can be predicted from the leakage of proteins from the spermatozoa and this leakage possibly essential for increasing membrane fluidity foremost acrosome reaction (Talukdar *et al.,* 2016).

Cholesterol Content of the Spermatozoa

During capacitation, there is inactivation of spermatozoan enzymes, which ultimately causes efflux of the cholesterol and influx of Ca^{2+} ion through the plasma membrane and outer acrosomal membrane and thus, resulting into acrosomal reaction (Langlais and Roberts, 1985). In the present study, it was observed that the cholesterol levels decreased significantly (P<0.01) while incubation period increased in all three media and it was more in TALP in comparison to other media (Table 1). Similar findings, as reported by Sharma *et al.* (1998) that there was a significant (P<0.01) fall in the cholesterol level of the spermatozoa after capacitation. Present finding also corroborate with the findings of Visconti *et al*. (1999) that less of cholesterol initiate the signal transduction pathway which promotes capacitation by altering the sperm membrane permeability. These membrane alterations increased permeability to ions such as Ca^{2+} and HCO_3^-, which enters the cytoplasm and stimulate adenylyl cyclase to promote cAMP production leading to the stimulation of PKA and ultimately to initiate capacitation associated with hyperactivation (Talukdar *et al.,* 2017).

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Conclusion

In conclusion, the study showed that the highest hyperactivation and live acrosome reacted spermatozoa was recorded on 4 hours of incubation in where maximum capacitation of the spermatozoa occurs. The AST and ALT activity increased significantly while incubation period increased. The total HOST reacted spermatozoa, sperm membrane protein and cholesterol levels decreased significantly at each period of incubation which is related with rate of capacitation and acrosome reaction. So, the process of sperm capacitation is associated with membrane protein and cholesterol depletion. For *in vitro* capacitation, though TALP is the best media, however MCM and KRB are also comparable.

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