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EFFECT OF BULL AND SPERM PREPARATION METHOD ON IN VITRO FERTILIZATION OF BUFFALO OOCYTES

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ABSTRACT

The present study was designed to compare fertilization rates following oocyte exposure to spermatozoa from different buffalo bulls, using three sperm preparation methods i.e. percoll density gradient, swim-up (modified Ca^2 free Tyrode's medium and TALP medium) and sodium citrate washing prior to co-incubation with oocytes. Buffalo oocytes were aspirated from ovarian follicles within 1.5 to 2 hours after slaughter. They were matured in TCM-199 supplemented with 20% oestrus buffalo serum drops under mineral oil in CO_2 incubator at 39°C for 24 hours. Matured oocytes were transferred to the fertilization droplets and inseminated with $1x10^6$ capacitated sperms prepared by different experimental methods. Data collected on recovered sperm motility immediately after treatment and 24 hours after incubation (at 37°C) and cleavage rate of co-incubated oocytes were subjected to statistical analysis. The percentage of motile spermatozoa was significantly higher ($P<0.05$) in semen samples prepared by swim-up method in Ca^2 free Tyrode's medium compared to other experimental techniques. Bull wise comparison showed significantly higher ($P<0.05$) motility in bull B1 (50.50 ± 5.92%), followed by bull B2 (46.46 ± 5.99%) and B3 (45.96 ± 5.79%). Fertilization rate was also significantly ($P<0.05$) higher for spermatozoa prepared by Swim-up method in Ca^2 free Tyrode's medium (63.75 ± 2.81%), followed by sodium citrate (26.70 ± 5.08%), swim-up TALP (29.14 ± 3.74%) and Percoll gradient density (23.89 ± 3.88%). Fertilization rate was significantly higher ($P<0.05$) in oocytes inseminated with semen from bull B1 (43.43 ± 8.59%), followed by B2 (33.38 ± 9.95%) and B3 (30.80 ± 9.56%). The results of present study indicate that bulls and sperm preparation methods differ in their contribution to in vitro fertilization rate. Further studies are suggested to ascertain the factors responsible for such specific effects.

Key words: In vitro fertilization, oocytes, bulls, sperm preparation, buffaloes.

INTRODUCTION

Buffalo is the main dairy animal in Pakistan, as it contributes approximately 65% of the total milk and 50% of the beef available in the country (Annonymous, 2005). Reproductive efficiency in this species is hampered by inherent late maturity, silent oestrus, distinct seasonal reproductive pattern and long calving interval (Madan and Raina, 1984; Madan, 1988). Assisted reproductive technologies such as artificial insemination (AI), multiple ovulation and embryo transfer (MOET) and in vitro fertilization (IVF) have been introduced to improve reproductive efficiency in this species (Madan *et al.,* 1994; Nandi *et al.,* 2002). However, appreciable improvement in the buffalo reproductive efficiency has not been observed even with the application of these biotechnologies. This seems to be due to the fact that the methods developed for cattle are applied as such for buffaloes, assuming that reproductive physiology of females of the two species is the same.

In vitro embryo production efficiency in buffalo is still very low and variable compared with cattle (Lu *et al.,* 1987) due to various factors including poor oocyte recovery rate (Totey *et al.,* 1992), lack of standardization of cultural conditions (Madan *et al.,* 1996) and low IVF performance of buffalo bull spermatozoa (Barandi *et al.,* 1993; Chauhan *et al.,* 1998). In vitro fertilization results are also affected by individual bulls (Misra *et al.,* 1999) and sperm preparation methods (Parrish *et al.,* 1995). Sperm preparation methods currently available to remove the undesirable spermatozoa, debris and other factors and to increase sperm qualitative characteristics include washing/centrifugation (Fukuda *et al.,* 1990), swim-up (Parrish *et al.,* 1986), Percoll density gradient (Arora and Jain, 1988) and glass wool filtration (Stubbings and Wasik, 1991).

For buffalo IVF programme, little information is available on the effect of bulls and sperm preparation methods. The objective of the present study was to investigate the effect of bulls and sperm preparation methods on IVF of buffalo oocytes. The data obtained from such basic studies may then be used to develop and test models for enhancing reproductive efficiency in buffaloes through in-vitro embryo production programme.

MATERIALS AND METHODS

Collection of oocytes

A total of 92 ovaries from sexually mature buffaloes were collected within 30 minutes after slaughter from a local abattoir and transported to the laboratory in a vacuum flask containing sterilized phosphate buffered saline (PBS, pH 7.35) supplemented with 100 iu penicillin G and 100 ug/ml of streptomycin at the 29°C (Totey *et al.,* 1992). In the laboratory, extraneous tissue was removed and ovaries were washed with 70% ethanol, followed by three rinses of PBS. Oocytes were collected by scoring method (Suss and Madison, 1983) and those possessing a full cumulus mass, unfragmated cytoplasm and intact zona were selected for further processing (Hasler *et al.*, 1995).

In vitro maturation

Tissue Culture Medium (TCM-199, Sigma St. Louise, USA) supplemented with 20% estrus buffalo serum (EBS) and 10 µg/ml gentamycin (Sigma, USA) at pH 7.4 was used for in vitro maturation (IVM). Selected oocytes were washed twice in maturation medium and 10-15 oocytes were incubated in 200 µl drops of TCM-199 covered with sterile mineral oil (Sigma) and placed in a CO_2 incubator at 39°C and 5% CO_2 in air for 24 hours. Then these oocytes were examined under stereomicroscope and those having well expanded cumulus cells with extrusion of the polar body were considered matured (Jainudeen *et al.*, 1993).

Sperm preparation

For preparation of sperms, fresh semen was collected through artificial vagina from three buffalo bulls B_1, B_2 and B_3 (12 ejaculates/bull) maintained at the Semen Production Unit, Department of Animal Reproduction, University of Agriculture, Faisalabad, Pakistan. The ejaculates having at least 70% motile spermatozoa were selected and subjected to one of the following experimental sperm preparation procedures:

Percoll density gradient method

As per procedure of Rosenkrans *et al.* (1993), 1.5 ml of 90% Percoll was mixed with 1.5 ml of sperm-Tyrode's-Lactate (Sp-TL) stock to obtain 3 ml of 45% Percoll in a 15 ml tube. With a transfer pipette, 90% Percoll (3 ml) was carefully deposited under 45% Percoll in the bottom of the tube. Semen (0.25 ml) was deposited on the top of the Percoll gradient. This was centrifuged at 400G for 30 minutes at room temperature (25°C). Sperm pellet formed at the bottom was removed and mixed with Sp-TL to make final volume of 5 ml which was again centrifuged for 10 minutes. After discarding supernatant, the pellet was examined for sperm concentration and diluted with Tyrode-albumin-lactate-pyruvate (TALP: NaCL 99.0 mM, KCl 3.1 mM, $NaHCO_3$ 25.0 mM, NaH_2PO_4 0.35 mM, bovine serum albumin 6.0 mg/ml, sodium pyruvate 1.0 mM, DL-lactic acid 21.6 mM, Hepes 10 mM) medium to have final concentration of $1x10^6$/ml of TALP.

Swim up method

Swim-up method (Parrish *et al.*, 1985) was performed with two types of media separately; i) Modified Ca^{2+} free Tyrode's medium and ii) Tyrode-albumin-lactate-pyruvate (TALP) medium. Each medium (pH 7.3-7.4) was incubated in an atmosphere of 5% CO_2 in air at 38.5°C for 2 hours prior to use. A 0.25 ml of fresh semen was deposited at the bottom of 1.5 ml of each medium separately. The tubes were incubated in an atmosphere of 5% CO_2 in air at 38.5°C for 30 minutes and supernatant from same media tubes was pooled separately in sterile conical tube and centrifuged at 100G for 10 minutes. The supernatant was discorded, saving 100 ul sperm suspension at the bottom of each tube. This sperm suspension in each tube was diluted with 1 ml of respective medium. This preparation was equilibrated at room temperature for 5 minutes. After adding 5 ml of more medium, it was again centrifuged for 10 minutes at 100 G. The supernatant was again discarded and remaining 100 ul of sperm suspension in each tube was diluted with same medium containing heparin (21.87 iu/ml) and incubated finally for 15 minutes in CO_2 incubator at 38.5°C.

Sodium citrate washing

In sodium citrate washing method (Samad *et al.*, 1998), 0.25 ml of fresh semen was mixed with 2.9% sodium citrate to make final volume of 5 ml. The suspension was centrifuged at 300G for 10 minutes. The supernatant was discarded and the pellet containing sperms was dissolved in 3 ml of 2.9% sodium citrate and centrifuged again for 10 minutes. After discarding supernatant, the concentration was determined in sperm pellet and sufficient Tyrode solution was added to obtain final sperm concentration of $1x10^6$/ml of medium.

In vitro fertilization

For in vitro fertilization, 10-15 IVM oocytes were washed with IVF media and shifted to fertilization drops (200 ul) made in separate petri dishes for separate IVF media. Oocytes were inseminated with $1x10^6$ spermatozoa obtained from three bulls prepared by different experimental methods. The oocytes were co-incubated with spermatozoa for 24 hours at 39°C, after which cleavage rates were recorded.

Statistical analysis

The data collected on effect of sperm preparation methods and bulls on motility percentage and fertilization rate were subjected to completely randomized design (Steel and Torrie, 1980). Means were compared by Duncan's Multiple Range Test (Duncan, 1955).

RESULTS

The comparative values of motility percentage (mean ± SE) for three bulls and four sperm preparation methods are given in Table 1. The post treatment motility was significantly higher ($P<0.05$) in Swim-up method with Ca^2 free Tyrode's medium compared with other sperm preparation techniques. All other methods differed non significantly among themselves. Sperm incubation at 37°C for 24 hours showed significantly lower motility percentage compared with that recorded immediately after treatment. This was true for all sperm preparation methods.

Bull wise comparison showed that the recovery of motile spermatozoa was significantly higher ($P<0.05$) in bull B1 (50.50 ± 5.92%) than B2 (46.46 ± 5.99%) and B3 (45.46 ± 5.79%). The latter two bulls differed non significantly from each other (Table 1).

The data on effect of bulls and sperm preparation methods on fertilization rate have been shown in Table 2. The sperms prepared by swim-up method in Ca^2 free Tyrode's medium showed highest fertilization rate (63.75 ± 2.81%), followed by swim-up in TALP (29.14 ± 3.74%), sodium citrate (26.70 ± 5.08%) and Percoll density gradient (23.89 ± 3.88%), the difference was significant ($P<0.05$). The fertilization rate was also significantly higher ($P<0.05$) for semen samples collected from bull B1 (43.43 ± 8.59%) than bull B2 (33.38 ± 9.95%) and B3 (30.80 ± 9.56%). The latter two bulls differed non significantly from each other.

DISCUSSION

Sperm preparation for IVF procedures not only remove seminal plasma, but also harvest the best spermatozoa from ejaculated or preserved semen. The present study revealed that swim-up in modified Ca^2 free Tyrode's medium gave significantly higher %age of motile spermatozoa immediately following treatment and also 24 hrs incubation at 37°C. Several IVF techniques have been used successfully to capacitate fresh or frozen thawed bull semen (Parrish *et al.,* 1988; Ijaz and Hunter, 1989; Ayoub and Hunter, 1993). Techniques of sperm washing involve the removal of seminal coating proteins adsorbed on surface membrane of the sperm (Yanagimachi, 1988). Ejaculated or frozen sperm have been washed free of seminal plasma components using percoll density gradient (Utsumi *et al.,* 1991), centrifugation (Parrish *et al.,* 1988) or swim-up procedure (Choi *et al.,* 1991).

The present study shows that buffalo bull spermatozoa can be successfully capacitated in Ca^2 free Tyrode's medium, as has been reported earlier by Behnke *et al*. (1987) for the cow bull. Working and Meizel (1983) reported that the addition of calcium had no effect, but an alkaline medium was necessary for the sperm cells to capacitate and for acrosome reaction to occur. In the present study, alkaline pH of the medium appears to be sufficient to capacitate spermatozoa over calcium requirement that was reported to be necessary for mammalian spermatozoa (Yanagamachi and Usui, 1986; Behnke *et al.,* 1987). Buffalo bull spermatozoa in calcium free medium behaved similar to that of cow bull spermatozoa. In the buffalo IVF programme, the motile spermatozoa have been obtained by swim-up (Boni *et. al.,* 1994), percoll density gradient (Totey *et al.,* 1993; Jainudeen *et al.,* 1993) and sodium citrate washing (Samad *et al.,* 1998) methods. Modified Ca^2 free Tyrode's medium for the preparation of buffalo

Table 1: Effect of bulls and sperm preparation methods on recovery of motile spermatozoa immediately post treatment (Ime) and after 24 hrs incubation (Inc) at 37°C

Sperm preparation method		Recovery of motile spermatozoa (%)			Overall
		B 1	B 2	B 3	
Percoll density gradient	Ime	73.67 ± 2.96^{ab}	71.67 ± 1.67^{bc}	67.67 ± 1.45^{bcd}	70.12 ± 3.62^{b}
	Inc	16.00 ± 3.06^{f}	11.67 ± 1.67^{fgh}	11.00 ± 2.08^{fgh}	12.89 ± 1.78^{d}
Swim up – TALP	Ime	74.00 ± 2.08^{ab}	67.67 ± 1.45^{bcd}	70.00 ± 2.89^{bc}	70.56 ± 4.25^{b}
	Inc	15.67 ± 1.45^{fg}	8.00 ± 1.25^{h}	11.00 ± 2.08^{fgh}	11.56 ± 2.31^{d}
Swim up Ca^2 free	Ime	78.67 ± 0.67^{a}	73.33 ± 1.67^{ab}	71.67 ± 1.67^{bc}	74.34 ± 5.41^{a}
	Inc	67.67 ± 1.45^{bcd}	62.33 ± 1.45^{dc}	61.00 ± 2.08^{c}	63.67 ± 1.85^{c}
Sodium Citrate	Ime	72.33 ± 1.45^{ab}	67.67 ± 1.45^{bcd}	65.67 ± 3.48^{cdf}	68.56 ± 4.68^{b}
	Inc	14.00 ± 1.00^{fgh}	9.33 ± 0.67^{gh}	9.67 ± 2.73^{fgh}	11.00 ± 5.84^{d}
Overall		50.50 ± 5.92^{a}	46.46 ± 5.99^{b}	45.46 ± 5.79^{b}	

Values sharing similar letters in a column or a row differed non significantly ($P<0.05$).

Table 2: Effect of bulls and sperm preparation methods on fertilization rate of buffalo follicular oocytes

Sperm preparation method	Fertilization rate (%)			Overall
	B1	B2	B3	
Percoll media	31.57 ± 0.93	21.05 ± 0.80	19.04 ± 1.18	23.89 ± 3.88[c]
Swim up-TALP	36.36 ± 0.73	27.27 ± 1.62	23.80 ± 0.68	29.14 ± 3.74[b]
Swimup-Ca^2 free	68.96 ± 1.15	62.96 ± 0.85	59.32 ± 0.91	63.75 ± 2.81[a]
Sodium citrate	36.84 ± 1.49	22.22 ± 0.99	21.05 ± 1.61	26.70 ± 5.08[bc]
Overall	43.43 ± 8.59[a]	33.38 ± 9.95[b]	30.80 ± 9.56[b]	

Values sharing similar letters in a column or a row differed non significantly (P<0.05).

bull sperms has not been documented in the literature earlier. This study concluded that modified Ca^2 free Tyrode's medium in swim-up method gives significantly higher motile spermatozoa recovery compared to other experimental media.

In the present study, spermatozoa prepared in modified Ca^2 free Tyrode's medium with swim-up gave higher fertilization rate than spermatozoa treated with other sperm preparation media. This might have been due to better capacity of Ca^2 free medium in terms of sperm capacitation and its penetration in to the ovum than other IVF media. Earlier authors have reported different methods of sperm preparation and in vitro penetration, pronucleus formation and cleavage rates (Polomo *et al.*, 1994; Dode *et al.*, 2002). Parrish *et al.* (1995) reported higher penetration and cleavage rates of bovine oocytes by swim-up method compared to percoll treatment. Mendes *et al.* (2003) reported that sperms obtained from percoll centrifugation resulted in higher cleavage rates and embryo production compared to sperms obtained from washing or centrifugation. However, Avery and Greve (1995) and Samad *et al.* (1998) reported that sodium citrate washing was better than Percoll density gradient for the preparation of buffalo sperms for IVF with no detrimental effect on cleavage rates.

Bulls differ in their ability to produce offsprings, both in natural and artificial breeding systems (Saacke *et al.*, 1994). It is well known that sperm from different bulls differ in their ability to fertilize the oocyte in vitro, thus kinetics of early cleavage can be used to differentiate bulls of high and low fertility (Ward, 2002).

In the present study, the results of fertilization/cleavage varied among the spermatozoa used from three buffalo bulls. Lambert *et al.* (1984) used spermatozoa from five different bulls and recorded fertilization rates varying from 14 to 46%. These workers regarded individual bull variation as one of the most important factors influencing in vitro fertilization rate. Similar results have been recorded in the studies of Hanada (1985) and Aoyagi *et al.* (1988). Shi *et al.* (1990) suggested that the variation arising from different bulls is greater than that arising from same bull's ejaculates in their contribution to fertilization and embryonic development. The differences between bulls appear to be dependent on their individual characteristics, rather than the results of a random error in semen sampling. Bulls may influence events as a result of the mechanism by which the sperm activates the oocyte and triggers synthesis of compounds needed for the cell cycle. The sperm cell itself contributes something that is required for regulating the zygotic cell cycle.

In conclusion, swim-up in modified Ca^2 free Tyrode's medium was found to be the best method for preparation of buffalo bull spermatozoa, as determined by significantly higher recovery of motile spermatozoa immediately post treatment and after 24 hours incubation of treated spermatozoa at 37°C. Spermatozoa obtained with this method also gave higher fertilization rate of IVM oocytes compared to spermatozoa treated with other techniques. Additionally, specific effect of bulls on the fertilization rate of IVM oocytes was also positive.

REFERENCES

Annonymous, 2005. Economic Survey of Pakistan. Finance Division, Economic Advisory Wing, Islamabad.

Aoyagi, Y., K. Fujii, Y. Iwazumi, M. Furudate, Y. Fukeii and H. Ono, 1988. Effect of two treatments on semen from different bulls on in vitro fertilization results of bovine oocytes. Theriogenology, 30: 973-985.

Arora, N. and M. C. Jain, 1988. Sephadex G-200 column chromatography studies on bubaline semen fractions. Cell Mol. Biol., 34: 239-246.

Avery, B. and T. Greve, 1995. Impact of Percoll on bovine spermatozoa used for in vitro insemination. Theriogenology, 44: 871-878.

Ayoub, M. and A. G. Hunter, 1993. Parthenogenetic activation of in vitro matured bovine oocytes. J. Dairy Sci., 74: 421 (Abstract).

Barandi, Z., I. Solti, S. Cseh, Z. S. Varga, Z. Machaty and G. Vajta, 1993. Comparison of in vitro fertilizing ability of sperm from endangered Hungarian Grey bulls. Anim. Reprod. Sci., 31: 13-19.

Behnke, E. J., A. P. Byers, A. G. Hunter, E. F. Bostwick and R. J. Orts, 1987. A bovine sperm epitope whose expression changes with in vitro capacitation conditions. 20th Meeting Society for the Study of Reproduction, Urbana-Illinois, USA. P. 74 (Abstract). .

Boni, R., S. Rovielo, V. Barbieri and L. Zicarelli, 1994. In vitro embryo production in buffalo species. Proc. 48th Cong. Italian Soc. Vet. Sci., (S.I.S. Vet): 307-311.

Chauhan, M. S., S. K. Singla, P. Palta, R. S. Manik and M. L. Madan, 1998. In vitro maturation and fertilization and subsequent development of buffalo (*Bubalus bubalis*) embryos: Effects of oocytes quality and type of serum. Reprod. Fertil. Develop., 10: 173-177.

Choi, Y. H., Y. Fukui and H. Ono, 1991. Effects of media and the presence of bovine epithelial cells during in vitro fertilization on fertilizability and developmental capacity of bovine oocytes. Theriogenology, 36: 863-873.

Dode, M. A. N., N. C. Rodovalho, V. T. Ueno and C. E. Fernandes, 2002. The effect of sperm preparation and co-incubation time on in vitro fertilization of *Bos indicus* oocytes. Anim. Reprod. Sci., 69: 15-23.

Duncan, D. B., 1955. Multiple range and multiple F-test. Biometrics, 11: 1-42.

Fukuda, Y., M. Iehikawa, K. Naito and Y. Toyoda, 1990. Birth of normal calves resulting from bovine oocytes matured, fertilized and cultured with cumulus cells in vitro upto the blastocyst stage. Biol. Reprod., 42: 114-119.

Hanada, A., 1985. In vitro fertilization in cattle with particular reference to sperm capacitation by ionophore A-23187. Japan's J. Anim. Reprod., 31: 56-61.

Hasler, J. F., W. B. Henderson, P. J. Hurtgen, Z. Q. Jin, A. D. McCauley, S. A. Mowes, B. Neely, L. S. Shuey, J. E. Stokes and S. A. Trinmer, 1995. Production, freezing and transfer of bovine IVF embryos and subsequent calving results. Theriogenology, 43: 141-152.

Ijaz, A. and A. G. Hunter, 1989. Effect of washing and capacitating media pH on bull sperm motility, acrosome integrity and ability to penetrate zona-free hamster oocytes. J. Dairy Sci., 72: 2691-2699.

Jainudeen, M. R., Y. Takahashi, M. Nihayah and H. Kanagawa, 1993. In vitro maturation and fertilization of swamp buffalo (*Bubalus bubalis*) oocytes. Anim. Reprod. Sci., 31: 205-212.

Lambert, R. D., M. A. Sirard, C. Bernard and R. Beland, 1984. The fertilization performance of bovine and rabbit spermatozoa capacitated in vitro. Proc. 10th Intern. Cong. Anim. Reprod. Artif. Insem., Illinois, USA, 3: 292.

Lu, K. H., I. Gordon, M. Gallagher and H. McGovern, 1987. Pregnancy established in cattle by transfer of embryos derived from in vitro fertilization of oocytes matured in vitro. Vet. Rec., 121: 259-260.

Madan, M. L. and V. S. Raina, 1984. Fertility and performance of buffaloes under tropical conditions. In: 10th Intern. Cong. Anim. Reprod. Artif. Insem., Illinois, USA, 2: 142.1-142.4.

Madan, M. L., 1988. Status of reproduction in female buffalo. 2nd World Buffalo Congress, New Dehli, Compendium of Latest Research, 89-100.

Madan, M. L., S. K. Singla, M. B. Chauhan and R. S. Manik, 1994. In vitro production and transfer of embryos in buffaloes. Theriogenology, 41: 139-143.

Madan, M. L., S. K. Das and P. Palta, 1996. Application of reproductive technology to buffaloes. Anim. Reprod. Sci., 42: 299-306.

Mendes, J. U. B., Jr. P. D. Burns, J. F. De LaTorre-Sanchez and G. E. Seidel, Jr., 2003. Effect of heparin cleavage rates and embryo production with four bovine sperm preparation protocols. Theriogenology, 60: 331-340.

Misra, A. K., M. M. Rao, R. Kasiraj, N. S. R. Reddy and H. C. Pant, 1999. Bull specific effect on fertilization rate and viable embryo recovery in the superovulated buffalo (*Bubalus bubalis*). Theriogenology, 52: 701-707.

Nandi, S., H. M. Raghu, B. M. Ravindranatha and M. S. Chauhan, 2002. Production of buffalo (*Bubalus bubalis*) embryos in vitro: Premises and promises. Reprod. Dom. Anim, 37: 65-74.

Parrish, J. J., J. L. Susko-Parrish and N. L. First, 1985. Effect of heparin and chondroitin sulfate on acrosome reaction and fertility of bovine sperm in vitro. Theriogenology, 24: 557-549.

Parrish, J. J., J. L. Susko-Parrish, M. L. Leibfried-Rutledge, E. S. Crister, W. H. Eyestone and N. L. First, 1986. Bovine in vitro fertilization with frozen thawed semen. Theriogenology, 25: 591-600.

Parrish, J. J., J. L. Susko-Parrish and N. L. First, 1988. Capacitation of bovine sperm by heparin. Biol. Reprod., 38: 1171-1180.

Parrish, J. J., J. L. A. Krogenaes and J. L. Susko-Parrish, 1995. Effect of bovine sperm separation by either swim-up or Percoll method on success of in vitro fertilization and early embryonic development. Theriogenology, 44: 859-869.

Polomo, M. J., D. Izquierdo, T. Moges and M. T. Paramino, 1994. Effect of semen preparation on IVF of prepubertal goat oocytes. Theriogenology, 41: 969-980.

Rosenkrans, C. F., G. Q. Zeng, G. T. MeNamara, P. K. Schoff and N. L. First, 1993. Development of bovine embryos in vitro as affected by energy substrate. Biol. Reprod., 49: 459-462.

Saacke, R. G., S. Nadir and R. L. Nebel, 1994. Relationship of semen quality to sperm transport, fertilization and embryo quality in ruminants. Theriogenology, 41: 45-50.

Samad, H. A., I. Q. Khan, N. U. Rehman and N. Ahmad, 1998. The recovery, in vitro maturation and fertilization of Nili-Ravi buffalo follicular oocytes. Asian Austr. J. Anim. Sci., 11: 491-497.

Shi, D. S., K. H. Lu and I. Gordon, 1990. Effects of bulls on fertilization of bovine oocytes and their subsequent development in vitro. Theriogenology, 33: 324 (Abstract).

Steel, R. G. D. and J. H. Torrie, 1980. Principles and Procedures of Statistics. A Biometrical Approach. 2nd Ed., McGraw Hill, Tokyo, Japan.

Stubbings, R. B. and C. P. Wasik, 1991. Glass wool versus swim up separation of bovine spermatozoa for in vitro fertilization. Theriogenology, 35: 276 (Abstract).

Suss, U. and V. Madison, 1983. Morphology and meiotic development of bovine oocytes cultured in vitro. Arch. Androl., 11: 217-218.

Totey, S. M., G. Singh, M. Taneja, C. H. Pawshe and G. P. Talwar, 1992. In vitro maturation, fertilization and development of follicular oocytes from buffalo (*Bubalus bubalis*). J. Reprod. Fertil., 95: 597-607.

Totey, S. M., C. H. Pawshe and G. P. Singh, 1993. Effects of bull, heparin and sperm concentrations on in vitro fertilization of buffalo (*Bubalus bubalis*) oocytes matured in vitro. Theriogenology, 39: 887-898.

Utsumi, K., H. Kato and A. Iritani, 1991. Full-term development of bovine follicular oocytes matured in culture and fertilized in vitro. Theriogenology, 35: 28-33.

Ward, F., E. Brian, R. Dimitrios, B. Maurice and L. Patrick, 2002. Optimization of in vitro bovine embryo production: effect of duration of moderation, length of gamete co-incubation, sperm concentration and sire. Theriogenology, 57: 2105-2117.

Working, P. K. and S. Meizel, 1983. Correlation of increased intra-acrosomal pH with the hamster sperm acrosome reaction. J. Exp. Zool., 227: 97-107.

Yanagimachi, R., 1988. Mammalian fertilization. In: The Physiology of Reproduction. E. Knobil and J. Neill (eds). Raven Press, New York NY, USA. pp: 1-135.

Yanagamachi, R. and N. Usui, 1986. Calcium dependence of the acrosome reaction and activation of guinea pig spermatozoa. Exp. Cell. Res., 89: 161-174.