The Indian Journal of Animal Reproduction; 25(2): 79-82; December 2004

Research Article

Effect of different capacitating agents on *in vitro* fertilization of goat follicular oocytes*

C.H. PAWSHE[1†] AND S.M. TOTEY[2]

Embryo Biotechnology Laboratory,
National Institute of Immunology, New Delhi

Received : May 30, 2003
Accepted : December 30, 2003

ABSTRACT

The present investigation is an attempt to establish optimal culture conditions for *in vitro* fertilization (IVF) of goat oocytes. Ovaries were obtained from a slaughter house and cumulus oocytes complexes (COCs) were washed three times in TL-HEPES medium and were matured for 24 h in TCM-199 (M-199) media supplemented with 10% fetal calf serum (FCS) and hormones (1 mg/ml 17β estradiol, E_2; 0.5 mg/ml ml follicle stimulating hormones (FSH) and 5.0 mg/ml leutinizing hormone, LH). After 24 hrs. of incubation oocytes were inseminated with percoll separated epididymal spermatozoa in Brackett and Oliphant (BO) or Talp-fertilization medium and treated with either Ca^{++} ionophore (A-23187) (0.1, 0.2 and 0.5 mM/ml + 5mM caffeine / L) or heparin (0,1,10 and 100 mg/ml) + 5mM caffeine / L or heparin (0,1,10 and 100mg/ml) alone in Talp-fertilization medium.

Key words : Goat; oocyte, maturation, fertilization, capacitation

The normal fertilization rates of spermatozoa treated with caffeine and Ca^{++} ionophore at the concentration of 0.1, 0.2 and 0.5 mM/ml were 27.8%, 39.5% and 47.6%, respectively. For spermatozoa treated with caffeine and heparin at the concentration of 1,10 and 100 mg/ml, the normal fertilization rates were 26.4%, 40.6% and 50.4% respectively. Normal fertilization rate was significantly increased when spermatozoa treated with 10 or 100 mg/ml heparin in Talp-fertilization medium (71.4% or 66.4%, respectively). *In vitro* fertilization (IVF) is a complex process, which results in the union of two gametes, the restoration of the somatic chromosome number and initiation of development of a new individual. Successful IVF requires appropriate preparations of both spermatozoa and oocytes, as well as culture conditions that are favourable for the metabolic activity of the male and female gametes. It is also well documented that various chemical substances, such as Ca^{++} ionophore (Byrd, 1981), caffeine (Hanada, 1985) and heparin (Parrish *et al.*, 1985) can capacitate bovine spermatozoa. Comparison of the various recommended protocols of *in vitro* maturation (IVM) and sperm treatments have been recently carried out in bovines (Yang *et al.*, 1993). Although there has been a remarkable increase in the knowledge on the various procedure involved in the IVF of cattle and sheep, information is still far from complete in case of IVF in goat. Therefore, there exists need to develop a similar system in the goat.

MATERIALS AND METHODS

Unless otherwise specified the reagent used in this study were tissue culture tested and obtained from Sigma Chemicals Co. St.Louis Mo , USA.

Isolation and maturation of oocyte : Goat ovaries were collected immediately after slaughter and brought to the laboratory in normal saline at 30°C within 1-2 hr of removal. Cumulus oocyte complexes (COCs) were isolated from the follicles by slicing method as described by Pawshe *et al.* (1994) and washed 3 to 4 times in TL-Hepes medium. Final two washings were given in the maturation medium TCM-199 (M-199) supplemented with 10% fetal calf serum (FCS, Gibco Grand Island NY, USA) and 1mg/ml estradiol (E_2), 0.5 mg/m l ovine follicle stimulating hormone (FSH) (NIDDK, NHPP) and 5.0 mg / ml ovine luteinizing hormone (LH) (NIDDK, NHPP). COCs were cultured for 24 hr. at 38°C under 5% CO_2 in air and 95% humidity.

***In vitro* fertilization** : After 24 hr of the maturation, the oocytes were fertilized by goat epididymal spermatozoa separated by percoll gradient (Totey *et al.*, 1993) and treated by three different methods as follows :

*Part of Ph.D. Thesis submitted to Jamiya Hamdard Univ, New Delhi

[1]Assistant Professor, Department of Gynaecology, Post Graduate Institute of Veterinary and Animal Sciences, Akola-444104.
[2]Senior Scientist, Reliance Life Sciences Pvt. Ltd. , Mumbai-400004.

[†]Corresponding author

Method I : Spermatozoa were washed with BO medium (Brackett & Oliphant, 1975) without BSA containing 10 mM caffeine/1 and suspended in BO medium at a concentration 4 x 10^6 spermatozoa / ml. The matured oocytes were washed and placed into 48 ml droplets of BO medium supplemented with 20 mg/ml BSA (essentially fatty acid free), covered with sterile paraffin oil and equilibrated with 5% CO_2 in air for 2 hr. Shortly before fertilization, the spermatoza were divided into 3 aliquots of 500 ml each, and treated with calcium ionophore (A- 23187) at concentrations of 0.1, 0.2, 0.5 mM/ml for one minute. The procedure for the preparation of calcium ionophore was based on Yang *et al.* (1993). The calcium ionophore of calcium inophore treated sperm suspension was introduced into 48 ml fertilization droplets of the fertilization medium containing oocytes at a final concentration of 2x10^6 spermatozoa/ml.

Method II : Spermatozoa were washed and suspended in BO medium containing 10 mM caffeine/1. The matured oocytes were washed and placed into 48 ml droplets of BO medium supplemented with 20 mg/ml BSA (essentially fatty acid free) and three different concentrations of heparin i.e., 1, 10 and 100 mg / ml. The caffeine treated sperm suspension was introduced into the fertilization droplets at a final concentration of 2 x 10^6 spermatozoa/ml.

Method III : Spermatozoa were washed with sperm-TL medium (Parrish *et al.*, 1989) containing 6 mg/ml BSA. The matured oocytes were washed in TL-Hepes medium followed by Talp-fertilization medium containing 6 mg/ml fatty acid free BSA and 0.25 mM sodium pyruvate (Parrish *et al.*, 1988). Finally, the oocytes were placed into 48 ml droplets of the fertilization medium containing different concentrations of heparin (1, 10, mg/ml). A 2 ml aliquot of the spermatozoa was then added into the fertilization drops at a concentration of 2 x 10^6 spermatozoa/ml.

After insemination, oocytes and spermatozoa were co-incubated for 24 hr at 38ºC under 5% CO_2 in air. At the end of the incubation period, the oocytes were washed, mounted and fixed. They were then stained with 1% aceto-orcein and examined under phase contrast microscope for evidence of fertilization. The oocytes were considered to be fertilized if at least one decondensing sperm head or two pronuclei are visible within the oocytes. The fertilization was considered to be normal, if the oocyte were fertilized by a single spermatozoa; and polyspermic, if fertilized by more than one spermatozoa.

Data analysis : The data of fertilization percentages from 5 replicates were compared within the media and the efficiencies of the various concentrations of capacitating agents with analysis of variance (ANOVA) following arscine transformation (Snedecor and Cochram, 1967) and Tadpole III stastical system (Elsevier-BIOSOFT, Cambridge, UK) was used for data analysis. ANOVA revealed a significant treatments effect, the means were compared by Tukey's hsd test.

RESULTS AND DISCUSSION

***In vitro* fertilization** : A total of 1411 matured oocytes were utilized which were randomly distributed into three different fertilization experiments containing three different capacitating agents.

In method I, fertilization rates were determined by a dose response study with three different concentrations of Calcium inophore (A -23187) i.e., 0.1, 0.2 and 0.5 mM/ml in the presence of 5 mM Caffeine/L. Spermatozo treated with 0.1 mM/ml did not alter the penetration rate over that of control. However, normal fertilization rate was increased significantly ($P<0.05$) when spermatozoa were treated either with 0.2 or 0.5 mM/ml Calcium inophore (A23187) (Table 1). In method II, three different concentrations of heparin (1, 10 and 100 mg/ml) were compared in the presence of 5 mM caffeine/1. The highest rate of fertilization was achieved when spermatozoa were treated with either 10 or 100 mg/ml heparin ($P<0.05$) (Table 2). However, 100 mg/ml heparin resulted in the heighest degree of polyspermic fertilization, whereas in 1 or 10 mg/ml heparin displayed lower polyspermic fertilization rates.

In method III, spermatozoa were treated with three different concentrations of heparin i.e., 1, 10, 100 mg/ml. Higher degree of fertilization rates were observed when spermatozoa were treated with either 10 mg (71.4%) or 100 mg/ml heparin (66.4%) as compared to untreated control (32.0%) or spermatozoa treated with 1mg/ml heparin (50.4%) ($P<0.05$) (Table 3). Polyspermic fertilization remained similar among control, 1 and 10 mg heparin/ ml groups but increased significantly for the 100 mg /ml heparin concentration. Our results show that heparin alone can capacitate goat epididymal spermatozoa more efficiently than Calcium inophore (A- 23187) + caffeine or heparin + caffeine. Heparin capacitated the goat epidymal spermatozoa in a dose dependent manner. 10 mg/ml heparin was found to be the most suitable for achieving an optimum fertilization rate.

Table 1. Effect of media and capacitating agents (Caffein + Calcium inophore) on *in vitro* fertilization of goat oocytes

Media	Capacitation agents	Concentration/ml	Total no. of oocytes	Fertilization (%) Total	Polyspermic	Normal
BO	--	--	120	36(30.0)	6(5.0)	30(25.0)[a,b]
BO	Caffeine+Ca^{++} ionophore	5nM + 0.1 m M	108	34(31.5)	4(3.7)	30 (27.8)[c]
BO	Caffeine+Ca^{++} ionophore	5mM + 0.5 mM	126	52(41.9)	3(2.4)	49(39.5)[aa]
BO	Caffeine+Ca^{++} ionophore	5mM + 0.5 mM	126	64(50.8)	4(3.2)	60(47.6)[bb,cc]

[a,b,c]Means with different corresponding superscripts were significantly different (P<0.05)
BO : Brackett and Oliphant Medium

Table 2. Effect of media and capacitating agents (Caffein + Heparin) *in vitro* fertilization of goat oocytes

Media	Capacitation agents	Concentration/ml	Total no. of oocytes	Fertilization (%) Total	Polyspermic	Normal
BO	--	--	120	36(30.0)	6(5.0)	30(25.0)[a,b]
BO	Caffeine + Heparin	5nM + 0.1 μ g	106	30(28.3)	2(1.9)	28(26.4)[c,d]
BO	Caffeine + Heparin	5mM + 0.5 μg	123	53(43.0)	3(2.4)	50(40.6)[aa,cc,e]
BO	Caffeine + Heparin	5mM + 0.5 μg	125	70(56.0)	7(5.6)	63(50.4)[bb,dd,ee]

[a,b,c,d,e] Means with different corresponding superscripts were significantly different (P<0.05)
BO : Brackett and Oliphant Medium

Table 3. Effect of media and capacitating agents (Heparin) on *in vitro* fertilization of goat oocytes

Media	Capacitation agents	Concentration/ml	Total no. of oocytes	Total	Fertilization (%) Polyspermic	Normal
Talp-Ferti	--	--	106	40(37.7)	6(5.6)	34(32.0)[a,c]
Talp-Ferti	Heparin	1μ g	111	61(54.9)	5(4.5)	56(50.4)[b]
Talp-Ferti	Heparin	10 μg	126	97(76.9)	7(5.6)	90(71.4)[aa,bb]
Talp-Ferti	Heparin	100 μg	116	91(78.4)	14(12.0)	77(66.4)[cc]

[a,b,c]Means with different corresponding superscripts were significantly different (P<0.05).
Talp-Ferti: Talp-fertilization medium

Although there have been several reports of pregnancies resulting from *in vitro* fertilization of *in vitro* matured bovine oocytes (Lu *et al.*, 1987, Xu *et al.*,1987)), very few reports are available on goat pregnancies resulting from this technology (Younis *et al.*, 1991; Smedt *et al.*, 1992). Spermatozoa treated with 10 mg, 100 mg heparin/ ml during goat IVF resulted in an increase in the percentage of oocytes penetrated by spermatozoa than spermatozoa treated with either Calcium inophore or caffeine + heparin. We observed that spermatozoa treated with Calcium inophore resulted in a rapid decrease of motility within 2 hr. of co-incubation. Byrd (1981) and Takashashi and Hanada (1984) have also reported that in bovine spermatozoa, motility rapidly decreased at higher concentrations of Calcium inophore. Decline in motility after the treatment with Calcium inophore may probably due to a high level of intracellular calcium affecting the mitochondrial ATP production (Bradley *et al.*, 1979; Shams-Borham and Harrison, 1981).

Oocyte maturation involves a sequence of cytological and metabolic events allowing an oocyte to be recognized and penetrated by a fertilizing spermatozoa. In all the mammals, nuclear maturation has to proceed in parallel with cytoplasmic and membrane maturation in order to achieve normal fertilization. The 40% of the goat spermatozoa underwent acrosomal reaction treated with Calcium inophore for one minute whereas, 85% acrosome reacted spermatozoa were observed after 6 hr. of heparin treatment (Pawshe, 1994). This shows that the oocytes matured for 24 hr. may not be promptly activated by the fertilizing spermatozoa treated with Calcium inophore. Thus, oocyte reaching metaphase II may require an additional period of maturation. According to Parrish *et al.*, (1989) capacitation by heparin normally takes 4 to 5 hr. Within this time oocytes must be achieving complete competence in the fertilization media in order to get activated by the fertilizing spermatozoa. Therefore, heparin treated spermatozoa resulted in a higher degree of fertilization, whereas, by the time taken by goat oocytes achieved fertilization competence there was a decline in the motility of spermatozoa treated with Calcium inophore, resulting in lower rates of fertilization. Time kinetics of IVM and its effect on subsequent fertilization and time kinetics of sperm penetration still need to be ascertained in order to prove this hypothesis. Spermatozoa treated with heparin in the presence of caffeine did not result in a remarkably high rate of fertilization. Perhaps both the chemicals did not act synergistically to induce capacitation and acrosomal reaction of goat spermatozoa. However, in all the experiments dose dependence effect of Calcium inophore and heparin was observed. Increase in the concentration of Calcium inophore (0.2 mM and 0.5 mM) significantly improved the fertilization rate when compared to 0.1 mM or untreated control. Similarly, increase in concentration of heparin in the presence or absence of caffeine resulted in an increase in the fertilization rate than 1 mg or untreated control. Therefore in the present study show that heparin alone in Talp-fertilization medium can capacitate goat epididymal spermatozoa more efficiently than Calcium inophore or caffeine + heparin. 10 mg/ml heparin in Talp-fertilization medium was found most suitable to achieve optimal fertilization rate in the goat.

REFERENCES

Brackett, B.G. and Oliphant, G. (1975). Capacitation of rabbit spermatozoa *in vitro*. Biol. Reprod., **12**: 260-274.

Bradley, M.P., Van Eerten, M.T.V., Rayns, D.G. and Forrestor, I.J. (1979). Membrane integrity and the regulation of calcium in mammalian spermatozoa. Biol. Reprod., **20**: 61 (Abstr.).

Byrd, W. (1981). *In vitro* capacitation and the chemically induced acrosome reaction in bovine spermatozoa. J. Exp. Zool., **215**: 35-48.

Hanada, A. (1985) *In vitro* fertilization in cattle with particular reference to sperm capacitation with ionophore A 23187. Japanese J. Anim. Reprod., **31**: 56-61.

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

Parrish, J.J., Susko-Parrish, J.L. and First, N.L. (1985). Role of heparin in bovine sperm capacitation. Biol. Reprod., **32**: 211 (Abstr.).

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

Parrish, J.J., Susko-Parrish, J.L. and First, N.L. (1989). Capacitation of bovine sperm by heparin: Inhibitory effect of glucose and role of intracellular pH. Biol. Reprod., **41**: 683-699.

Pawshe, C.H. (1994). *In vitro* maturation and fertilization of goat oocyte. Ph.D. Thesis submitted to Jamia Hamdard University, New Delhi.

Pawshe, C.H., Totey, S.M. and Jain, S.K. (1994). A comparison of three methods of recovery of goat oocytes for *in vitro* maturation and fertilization. Theriogenology, **42**: 117-125.

Shams-Borhan, G. and Harrison, R.A.P. (1981). Production characterization and use of inophore-induced, calcium-dependent acrosome reaction in ram spermatozoa. Gamete Res., **4**: 407-432.

Smedt, V.D., Crozet, N., Ahmed Ali, M., Martino, A. and Cognie, Y. (1992) *In vitro* maturation and fertilization of goat oocytes. Theriogenology, **37**: 1049-1060.

Snedecor, G.W. and Cochran, W.G. (1967). *Statistical Methods*, 6th edn., Iawa state university press. Ames. IA. U.S.A. 593.

Takahashi, Y. and Hanada, A. (1984) Penetration of zona free hasmter eggs *in vitro* ejaculated bull spermatozoa after treatment with ionophore A23187. Japanese J. Anim. Reprod., **30**: 30-39.

Totey, S.M., Pawshe, C.H. and Singh, G.P. (1993) *In vitro* maturation and fertilization of buffalo oocytes (*Bubalus bubalis*): effect of media, hormone and sera. Theriogenology, **39**: 1153-1171.

Xu, K.P., Greve, T., Callasen, H. and Hytel, P. (1987). Pregnancy resulting from cattle oocytes matured and fertilized *in vitro*. J. Reprod. Fertil., **81**: 501-504.

Yang, X., Jiang, S. and Foote, R.H. (1993) Bovine oocyte development following different oocyte maturation and sperm capacitaion procedure. Molecular Reprod. Develop., **34**: 94-100.

Younis, A.I., Zuelke, K.A., Harper, K.M., Oliveria, M.A.L. and Brackett, B.G. (1991). *In vitro* fertilization of goat oocytes. Biol. Reprod., **44**: 1177-1182.