

# A comparative study of parthenogenetic activation and *in vitro* fertilization of *in vitro* matured caprine oocytes

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## Summary

The aim of the study was to compare the parthenogenetic activation and *in vitro* fertilization (IVF) of *in vitro* matured caprine oocytes. A total of 881 cumulus-oocyte complexes (COC's) were collected from 243 ovaries. Oocytes were matured in TCM-199 medium containing eCG (20 IU/ml), hCG (20 IU $\mu$ g/ml), oestradiol-17 $\beta$  (1  $\mu$ g/ml), BSA embryo tested (3 mg/ml) supplemented with 10% fetal bovine serum at 38.5°C and 5% CO<sub>2</sub> in an incubator under humidified air for 27 h. Based on cumulus expansion, the maturation rate was 86.86%. Morphological matured oocytes (n=749) were selected, denuded and randomly divided into two groups. Group 1 (n=223) *in vitro* matured oocytes activated with 5  $\mu$ m calcium ionophore for 5 min and cultured in mCR<sub>2</sub>aa medium containing 5 mM DMAP for 4 h. After 4 h of DMAP treatment, the presumptive zygotes were washed and cultured in the embryo culture medium. Group 2 (n=526) *in vitro* matured oocytes processed for IVF in mTALP using fresh semen of a fertile pure bred adult Sirohi buck and *in vitro* culture in mCR<sub>2</sub>aa medium. Development of putative zygotes was observed every 24 h till day 9 post activation or fertilization under inverted phase contrast microscope. The cleavage rate, morula and blastocyst percentage in groups 1 and 2 were 67.36%, 23.07% and 9.23%, and 30.99%, 19.63% and 9.82%, respectively. The results indicated that the cleavage rate was comparatively higher following parthenogenetic activation with ionomycin/6-DMAP than IVF.

**Key words:** Goat, Maturation, Oocytes, IVF, Parthenotes

## Introduction

Recent advances in knowledge of oocyte maturation and research on different culture conditions have led to substantial progress in *in vitro* embryo production (IVEP) systems (Freitas and Melo, 2010) for small ruminants. The process of IVEP consists of 3 main steps: 1) Oocyte collection and *in vitro* maturation (IVM) of cumulus-oocyte complexes (COCs), 2) *In vitro* fertilization (IVF) and 3) *in vitro* culture of embryos (Garcia-Garcia *et al.*, 2007). In the provision of *in vitro* produced embryos, the processes of IVM of oocytes and IVF are almost inextricably linked (Kane, 2003). Natural activation of oocytes by sperm following fertilization and artificial activation of recipient oocytes following somatic cell nuclear transfer is necessary for the further development of oocyte to embryo. Before fertilization, the first step is to select the most motile and viable spermatozoa from the whole fresh ejaculate or the frozen-thawed sperm. The principal techniques used to select spermatozoa are swim-up and centrifugation in Percoll or Ficoll density gradient. Cognie *et al.* (2003) reported capacitation of frozen-thawed sperm using 10% (v/v) estrus sheep serum in SOF medium and 0.5 g/ml of heparin during 1 h. Paramio (2010) after fresh sperm selection by swim-up, the supernatant recovered and capacitated in mDM with 50 g/ml heparin for 45 min. After oocyte maturation and sperm capacitation, oocytes are transferred to microdrops of modified Tyrode's

medium (TALP) as described by Parrish *et al.* (1986), supplemented with hypotaurine and glutathione. Different fertilization media have been used by different authors: BO (Ongeri *et al.*, 2001), SOF (Rho *et al.*, 2001) and TALP-fert medium (Katska-Ksiazkiewicz *et al.*, 2004). Mammalian oocytes of most species arrest at the metaphase II (MII) stage immediately prior to ovulation from ovarian follicles. Resumption of meiosis of an MII oocyte is inhibited by maturation promoting factor (MPF), which is abundant in the mature oocyte (Shen *et al.*, 2008). When a spermatozoa penetrates the zona pellucid and attaches to the vitelline membrane of the MII oocyte, oscillations of intracellular calcium (Ca<sup>2+</sup>) occur (Whitaker and Patel, 1990). The elevation of Ca<sup>2+</sup> triggered by sperm penetration inhibits dephosphorylation of P<sup>34cdc2</sup> kinase, which in turn reduces MPF activity and activates the oocytes (Shen *et al.*, 2008).

Although sperm supply the natural stimulus for oocyte activation, oocytes may also be activated parthenogenetically, without any contribution from sperm (Mishra *et al.*, 2008). In traditional method for production of cloned goat embryos, electric pulses were applied for activation of reconstructed oocytes (Melican *et al.*, 2005; Shen *et al.*, 2006). Oocyte activation by electrical pulse is initiated by an elevation of intracellular Ca<sup>2+</sup>. Immediately after electrical stimulation, there is an influx of extracellular Ca<sup>2+</sup> which in turn triggers an increase of intracellular Ca<sup>2+</sup> (Cheong *et al.*, 2002). Chemical treatment can also induce oocyte activation via

elevation of ooplasmic  $\text{Ca}^{2+}$  concentration to down-regulate MPF activity and activate oocytes parthenogenetically. Various chemicals like Ca ionophore, ethanol and strontium have been used for activation of oocytes (Varga *et al.*, 2008; Pathak *et al.*, 2013).

To our knowledge, standard IVF protocol compared to *in vitro* parthenogenetic activation of oocytes matured with PMSG and hCG has not been previously investigated in detail. The present study was designed to activate *in vitro* matured caprine oocytes parthenogenetically and compared them with the standard IVF protocol for embryo developmental rates.

## Materials and Methods

### Chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), except where otherwise indicated.

### Harvest of oocytes

Ovaries (n=243) were obtained from various breed of goats (non descript, Sirohi, Boer, Barbari) at Agra slaughterhouse from June to September during the non breeding season and transported in 3-4 h to the laboratory in a thermos containing physiological saline solution (0.9% w/v NaCl) supplemented with 100 IU penicillin-G and 100 µg streptomycin sulphate per ml at 30 to 35°C. Oocytes were harvested by slicing (Pawshé *et al.*, 1994) with a surgical blade and the oocyte collection medium consisted of Dulbecco's phosphate-buffered saline (D-5773) with 3 mg/ml BSA. Only oocytes (n=881) surrounded by compact, dense cumulus cell layers, grade A and B (Kharche *et al.*, 2008) were selected for the maturation under stereomicroscope.

### *In vitro* maturation

Selected COCs (n=881) for *in vitro* maturation were washed 10-14 times in oocyte holding medium (OHM) containing TCM-199 (M-7528), Sodium pyruvate (0.25 mM), gentamycin (50 µg/ml), glutamine (100 µg/ml), BSA embryotested (3 mg/ml). The medium used for maturation of COCs was OHM supplemented with 20 IU/ml eCG Folligon® from Intervet, International B. V., Boxmeer-Holland, 20 IU/ml hCG Pregnyl® (Kouamo and Kharche, 2014) from Nile Co. for Pharmaceutical and Chemical Industries A.R.E., 1 µg/ml estradiol 17-β, and 10% FBS (fetal bovine serum, Cat N° A15-104 from PAA Laboratories GmbH, The Cell Culture Company Austria) under 400 µL of mineral oil. The pH was adjusted to 7.4 and the osmolality was adjusted to 280 mOsm/kg. Oocytes were incubated for 27 h at 38.5°C in 5% CO<sub>2</sub> of humidified air in a CO<sub>2</sub> incubator. Assessment of maturation was done by the degree of expansion of cumulus cell mass. During IVM process, 132 oocytes were lost.

### Parthenogenetic activation

Cumulus cells were removed from oocytes (n=223) after *in vitro* maturation (27 h) using 50 µg hyaluronidase (H-4272) and repeated pipetting. The

oocytes were washed 5 times in a mCR<sub>2</sub>aa medium. The unfertilized oocytes were exposed to 5 µM Ca ionophore in mCR<sub>2</sub>aa for 5 min at room temperature followed by 5 mM 6-DMAP for 4 h (Kharche *et al.*, 2012). Activated oocytes (n=193) were then washed three times in mCR<sub>2</sub>aa and were transferred to the drops with the culture medium (10-15 oocytes per drop) and incubated for 9 days at 38.5°C in 5% CO<sub>2</sub> of humidified air.

### *In vitro* fertilization

After 27 h of culture, matured oocytes (n=526) with expanded cumulus cells were separated from the cumulus cells by treating the complex with 0.1% hyaluronidase enzyme and passing it repeatedly through a fine pipette. Fresh semen samples were collected using the artificial vagina from a fertile pure bred adult Sirohi buck. The first and second seminal ejaculates were examined for volume, colour, consistency and gross sperm and progressive motility. The sperm concentration was counted by hemocytometer and checked for acceptable motility (at least 80% progressively motile). A sample of 100 µl fresh semen was diluted with 5 ml of sperm-TALP medium (100 mM NaCl, 3.1 mM KCl, 25 mM NaHCO<sub>3</sub>, 0.3 mM NaH<sub>2</sub>PO<sub>4</sub>, 21.6 mM Lactate, 2 mM CaCl<sub>2</sub>, 0.4 mM MgCl<sub>2</sub>, 10 mM HEPES, 1 mM pyruvate, 50 mg ml<sup>-1</sup> Gentamycin, 10 µg ml<sup>-1</sup> Heparin and 10% FBS) for the respective treatment groups 1 and 2 and washed by centrifugation at 1200 × g for 5 min. The supernatant was discarded and the pellet was diluted with 5 ml of medium and kept for capacitation in a CO<sub>2</sub> incubator at 38.5°C for 1 h (Cognie *et al.*, 2003). After incubation, the sperm suspension was centrifuged and the supernatant again discarded and 100 µl of the sperm pellet was diluted in 750 µl Fert-TALP medium (Sperm-TALP medium without HEPES + 4 mg ml<sup>-1</sup> BSA Embryo tested). An insemination drop containing the oocytes was inseminated with 25-50 µl of the final diluted semen so as to obtain a sperm concentration of 1 × 10<sup>6</sup> sperm/ml. After *in vitro* insemination, the oocytes and sperm were co-incubated for 18 h at 38.5°C in 5% CO<sub>2</sub> incubator in humidified air.

### *In vitro* culture

The culture of parthenogenetic and IVF embryos was performed with 100 µL mCR<sub>2</sub>aa medium culture droplets (10-15 embryo/drop) under mineral oil. The culture environment consisted of 5% CO<sub>2</sub>, 5% O<sub>2</sub> and 90% N<sub>2</sub> in humidified air at 38.5°C. Cleavage, morula and blastocyst development rates were evaluated from the presumptive zygotes on nine days after each 24 h after parthenogenetic activation or IVF under an inverted phase contrast microscope.

### Statistical analysis

Cleavage rate, morula, blastocyst stages in parthenogenetic activation and IVF groups were analyzed using the Chi-square test. The level of significance was recorded at the 5% level of confidence (Snedecor and Cochran, 1989).

## Results

### Recovery of oocytes and maturation rate

The overall COCs recovered per ovary was  $3.57 \pm 0.21$  and 86.86% were matured, done by the expansion of cumulus cell mass.

### *In vitro* fertilization and parthenogenetic activation of *in vitro* matured oocytes

Data on cleavage, morula and blastocyst/cleavage rate of parthenogenetically activated and IVF embryos are presented in the Table 1. Figs. 1A, B, C, D, E and F show different stages of embryos from parthenogenic activation and IVF. The cleavage rate is comparatively higher following parthenogenetic activation. Parthenogenetically activated embryos also cleaved earlier than IVF-derived embryos.

## Discussion

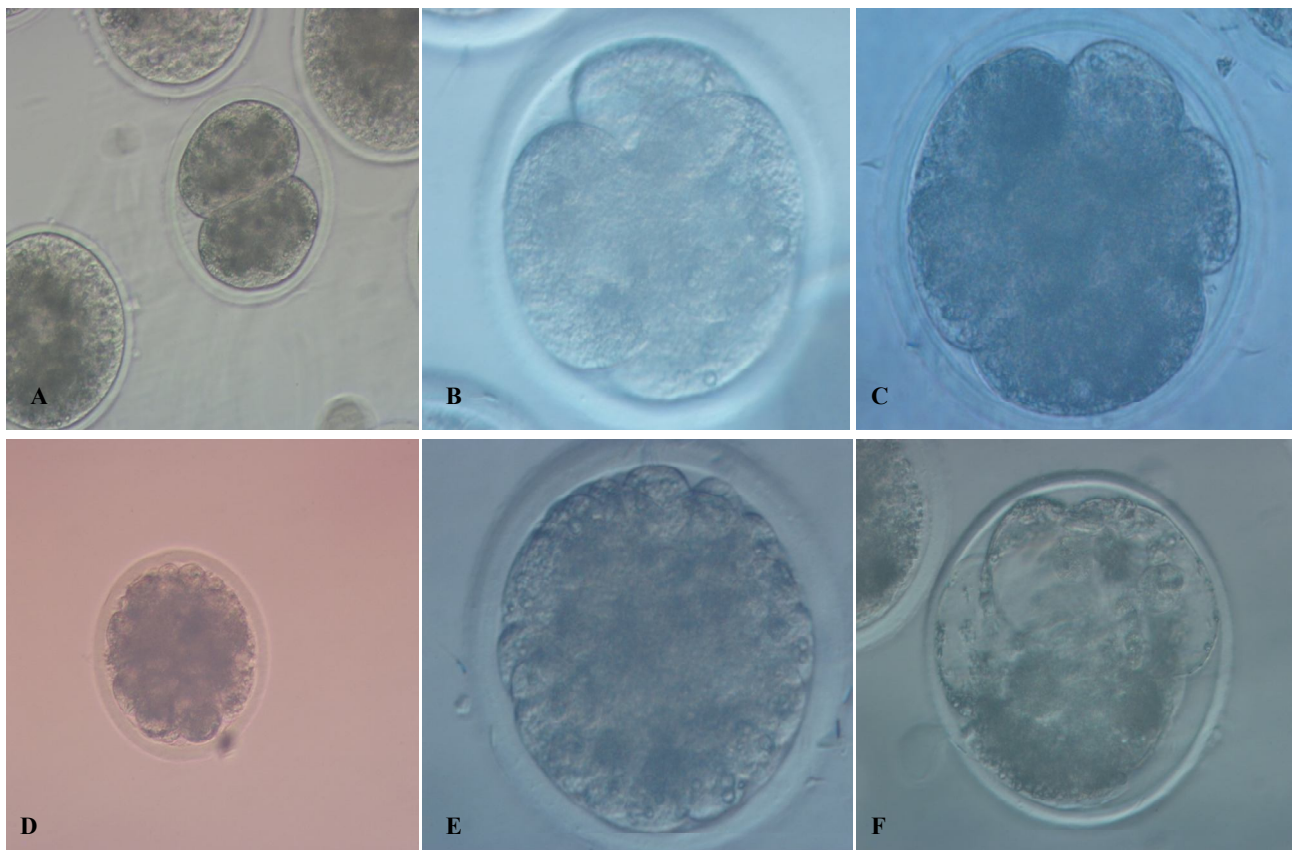
The activation of oocyte is induced by an increase in the oocyte's intracellular free calcium concentration (De

*et al.*, 2012). In normal fertilization, sperms trigger release of calcium from the endoplasmic reticulum and this act as a signal for oocyte activation (Whitaker, 2006). Combined treatments with different chemicals for parthenogenetic activation have been widely used for reconstructed oocytes (sheep: Schnieke *et al.*, 1997; cattle: Cibelli *et al.*, 1998; goat: Keefer *et al.*, 2001, 2002) to increase intracellular  $Ca^{2+}$  concentration (such as EP, ethanol, calcium ionophore or ionomycin) and inhibit protein synthesis (e.g., CHX) or MPF activity (e.g., 6-DMAP). In this study, cleavage rates following parthenogenetic activation (67.36) and IVF (30.99) revealed significant difference ( $P=0.00$ , Table 1). This is in accordance with Pablo *et al.* (2008). The latter hypothesized that because all oocytes used for parthenogenetic activation were denuded prior to activation, which allowed for selection based on the presence of a polar body and evenly granulated cytoplasm, a higher proportion of developmentally competent oocytes might have been selected for these procedures. The combination of calcium ionophores with 6-DMAP induces high rates of activation, pronucleus

**Table 1:** Embryo development following IVF and parthenogenetic activation of caprine oocytes matured *in vitro*

Experiments	No. of treated oocytes	Total cleaved (%)	Embryo development (%)					
			2C (%)	4C (%)	8C (%)	16C (%)	Morula (%)	Blastocyst (%)
Parthenogenetic activation	193	130 (67.36) <sup>b</sup>	22 (16.92)	19 (14.62)	35 (26.92)	12 (9.23)	30 (23.07) <sup>a</sup>	12 (9.23) <sup>a</sup>
IVF	526	163 (30.99) <sup>a</sup>	9 (5.52)	41 (25.15)	43 (26.38)	22 (13.49)	32 (19.63) <sup>a</sup>	16 (9.82) <sup>a</sup>

<sup>a, b</sup> Values in the same column with different letter are different at  $P<0.05$ . C: Cells, and No.: Number



**Fig. 1:** Different stages of embryos. (A) 2 cells, (B) 4 cells, (C) 8 cells, (D) 16 cells, (E) Morula, and (F) Blastocyst

formation and development to blastocyst stage like in sheep (Loi *et al.*, 1998) and cattle (Liu *et al.*, 1998). In contrast, Onger *et al.* (2001) and Çevik *et al.* (2009) found that cleavage rate had no significant difference following the two treatments. The cleavage rate of activated oocytes and their potential for further embryonic development depends on several factors, like species, source and quality of oocytes, IVM conditions, type and composition of culture media and activating agent (Çevik *et al.*, 2009).

The efficiency of the culture system may be expressed in terms of the percentage of embryos that cleaved and reached more than the 8-cell stage (Keskin-tepe and Brackett, 1996). In our study, the blastocyst development rates revealed no significant difference between the two treatments. In cattle, where the IVF system is better developed, percent blastocyst development obtained from oocytes that were activated with a calcium ionophore followed by 6-DMAP was similar to that of IVF (Liu *et al.*, 1998). So, all competent goat oocytes activated or coincubated with spermatozoa could potentially develop to blastocyst. In both cases, the blastocyst rates were similar to those reported by Kharche *et al.* (2012) and lower than those reported by Dinnyés *et al.* (2000), Arat *et al.* (2006) and Çevik *et al.* (2009). The difference might be due to factors such as culture system (Çevik *et al.*, 2009), media, quality of oocytes and quality of sperm or capacitation. The low percentage of blastocysts developing from IVF may also be attributed in part to the polyspermy (De Smedt *et al.*, 1992; Onger *et al.*, 2001). In this study, embryos activated with ionomycin/DMAP cleaved earlier than IVF-derived embryos. With the parthenogenetic procedures, the time of activation is synchronized by ionomycin treatment while sperm entry during IVF takes place within a period of 6 h (Pablo *et al.*, 2008).

The present study indicated that matured caprine oocytes activated with ionomycin/6-DMAP exhibit higher cleavage rate than IVF procedure, however, the developmental competence of embryos or parthenotes to blastocyst stage grow similarly.

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## References

Arat, S; Bağış, H; Taş, A and Akkoç, T (2006). Effect of several parameters on parthenogenetic bovine embryo

development *in vitro*. *Reprod. Fert. Develop.*, 18: 119 (Abst.). doi: 10.1071/RDv18n2Ab21.

Çevik, M; Taş, A; Akkoç, T; Bağış, H and Arat, S (2009). A comparative study of parthenogenetic activation and *in vitro* fertilization of *in vitro* matured bovine oocytes. *Turk. J. Vet. Anim. Sci.*, 33: 393-399.

Cheong, HT; Park, KW; Im, GS; Lai, L; Sun, QI; Day, BN and Prather, RS (2002). Effect of elevated Ca<sup>++</sup> concentration in fusion/activation medium on the fusion and development of porcine fetal fibroblast nuclear transfer embryos. *Mol. Reprod. Dev.*, 61: 488-492.

Cibelli, JB; Stice, SL; Golueke, PJ; Kane, JJ; Jerry, J; Blackwell, C; Ponce de Leon, FAP and Robl, JM (1998). Cloned transgenic calves produced from non quiescent fetal fibroblasts. *Science*. 280: 1256-1258.

Cognie, Y; Baril, G; Poulin, N and Mermillod, P (2003). Current status of embryo technologies in sheep and goat. *Theriogenology*. 59: 171-188.

De, AK; Malakar, D; Jena, MK; Dutta, R; Garg, S and Akshey, ZS (2012). Zona-free and with-zona parthenogenetic embryo production in goat (*Capra hircus*) - effect of activation methods, culture systems and culture media. *Livestock Sci.*, 143: 35-42.

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

Dinnyés, A; Dai, Y; Jiang, S and Yang, X (2000). High developmental rates of vitrified bovine oocytes following parthenogenetic activation, *in vitro* fertilization, and somatic cell nuclear transfer. *Biol. Reprod.*, 63: 513-518.

Freitas, JFV and Melo, ML (2010). *In vitro* embryo production in small ruminants. *R. Bras. Zootec.*, 39: 409-413 (Supl. especial).

Garcia-Garcia, R; Ward, F; Fair, S; O'Meara, C and Wade, M (2007). Development and quality of sheep embryos cultured in commercial G1.3/G2. 3 sequential media. *Anim. Reprod. Sci.*, 98: 233-240.

Kane, MT (2003). A review of *in vitro* gamete maturation and embryo culture and potential impact on future animal biotechnology. *Anim. Reprod. Sci.*, 79: 171-190.

Katska-Ksiazkiewicz, L; Rynska, B; Gajda, B and Smorag, Z (2004). Effect of donor stimulation, frozen semen and heparin treatment on the efficiency of *in vitro* embryo production in goats. *Theriogenology*. 62: 576-586.

Keefer, CL; Baldassarre, H; Keyston, R; Wang, B; Bhatia, B; Bilodeau, AS; Zhou, JF; Le duc, M; Downey, BR; Lazaris, A and Karatzas, CN (2001). Generation of dwarf goat (*Capra hircus*) clones following nuclear transfer with transfected and non transfected fetal fibroblasts and *in vitro*-matured oocytes. *Biol. Reprod.*, 64: 849-856.

Keefer, CL; Keyston, R; Lazaris, A; Bhatia, B; Begin, I; Bilodeau, AS; Zhou, FJ; Kafidi, N; Wang, B; Baldassarre, H and Karatzas, CN (2002). Production of cloned goats after nuclear transfer using adult somatic cells. *Biol. Reprod.*, 66: 199-203.

Keskin-tepe, L and Brackett, BG (1996). *In vitro* developmental competence of *in vitro* matured bovine oocytes fertilized and cultured in completely defined media. *Biol. Reprod.* 55: 333-339.

Kharche, SD; Goel, AK; Jindal, SK; Goel, P; Kouamo, J and Sarswat, S (2012). Dose dependent effect of protein synthesis inhibitor on the developmental potency of parthenogenetic goat embryos. *J. Biotechnol. Biomater.*, 2: 6. <http://dx.doi.org/10.4172/2155-952X.S1.002>.

Kharche, SD; Goel, AK; Jindal, SK; Sinha, NK and Yadav, P (2008). Effect of somatic cells co-culture on cleavage and development of *in vitro* fertilized embryos. *Indian J. Anim.*

- Sci., 78: 686-692.
- Kouamo, J and Kharche, SD** (2014). Dose dependent effect of pregnant mare serum gonadotropin and human chorionic gonadotropin on *in vitro* maturation of goat oocytes. *Indian J. Anim. Sci.*, 84: 410-414.
- Liu, L; Ju, J and Yang, X** (1998). Parthenogenetic development and protein patterns of newly matured bovine oocytes after chemical activation. *Mol. Reprod. Dev.*, 49: 298-307.
- Loi, P; Ledda, S; Fulka, J; Cappai, P and Moor, RM** (1998). Development of parthenogenetic and cloned ovine embryos: effect of activation protocols. *Biol. Reprod.*, 58: 1177-1187.
- Melican, D; Butler, R; Howkins, N; Chen, LH; Hayden, E; Destrempe, M; Williams, J; Lewis, T; Behboodi, E; Ziomek, C; Meade, H; Echelard, Y and Gavin, W** (2005). Effect of serum concentration, method of trypsinization and fusion/activation utilizing transfected fetal cells to generate transgenic dairy goats by somatic cell nuclear transfer. *Theriogenology*. 63: 1549-1563.
- Mishra, V; Misra, AK and Sharma, R** (2008). A comparative study of parthenogenic activation and *in vitro* fertilization of bubaline oocytes. *Anim. Reprod. Sci.*, 103: 249-259.
- Ongeri, EM; Bormann, CL; Butler, RE; Melican, D; Gavin, WG and Echelard, Y** (2001). Development of goat embryos after *in vitro* fertilization and parthenogenetic activation by different methods. *Theriogenology*. 55: 1933-1945.
- Paramio, MT** (2010). *In vivo* and *in vitro* embryo production in goats. *Small Rumin. Res.*, 89: 144-148.
- Parrish, JJ; Susko-Parrish, JL; Leibfried-Rutledge, M; Crister, E; Eyeston, W and First, NL** (1986). Bovine *in vitro* fertilization with frozen thawed semen. *Theriogenology*. 25: 591-600.
- Pathak, J; Kharche, SD; Goel, AK and Jindal, SK** (2013). A comparative study on parthenogenetic activation and embryo production from *in vitro* matured caprine oocytes. *Small Rumin. Res.*, 113: 136-140.
- Pawshe, CH; Totey, SM and Jain, SK** (1994). A comparison of 3 methods of recovery of goat oocytes for *in vitro* maturation and fertilization. *Theriogenology*. 42: 117-125.
- Rho, GJ; Hahnel, AC and Betteridge, KJ** (2001). Comparisons of oocyte maturation times and three methods of sperm preparation for their effects on the production of goat embryos *in vitro*. *Theriogenology*. 56: 503-516.
- Schnieke, AE; Kind, AJ; Ritchie, WA; Mycock, K; Scott, AR; Ritchie, M; Wilmut, I; Colman, A and Campbell, HS** (1997). Human factor IX transgenic sheep produced by transfer of nuclei from transfected fetal fibroblasts. *Science*. 278: 2130-2133.
- Shen, PC; Lee, SN; Liu, BT; Chu, FH; Wang, CH; Wu, JS; Lin, HH and Cheng, WTK** (2008). The effect of activation treatments on the development of reconstructed bovine oocytes. *Anim. Reprod. Sci.*, 106: 1-12.
- Shen, PC; Lee, SN; Wu, JS; Huang, JC; Chu, FH; Chang, CC; Kung, JC; Lin, HH; Chen, LR; Shiau, JW; Yen, NT and Cheng, WTK** (2006). The effect of electrical field strength on activation and development of cloned caprine embryos. *Anim. Reprod. Sci.*, 92: 310-320.
- Snedecor, GW and Cochran, WG** (1989). *Statistical methods*. 8th Edn., Ames, Iowa, USA, Iowa State University Press. PP: 125-128.
- Varga, E; Pataki, R; Lorincz, Z; Koltai, J and Papp, AB** (2008). Parthenogenetic development of *in vitro* matured porcine oocytes treated with chemical agents. *Anim. Reprod. Sci.*, 105: 226-233.
- Whitaker, M** (2006). Calcium at fertilization and in early development. *Physiol. Rev.*, 86: 25-88.
- Whitaker, M and Patel, R** (1990). Calcium and cell cycle control. *Development*. 108: 525-542.