# Effect of different activators on development of activated *in vitro* matured caprine oocytes

**Sharma, J. R.[1]; Agarwal, S.[2]; Kharche, S. D.[3*]; Goel, A. K.[3]; Jindal, S. K.[3] and Agarwal, S. K.[3]**

[1]MSc, Physiology Reproduction and Shelter Management Division, Central Institute for Research on Goats (CIRG), Makhdoom, Farah-281122, Mathura, Uttar Pradesh, India; [2]Ph.D. Scholar, Physiology Reproduction and Shelter Management Division, Central Institute for Research on Goats (CIRG), Makhdoom, Farah-281122, Mathura, Uttar Pradesh, India; [3]Physiology Reproduction and Shelter Management Division, Central Institute for Research on Goats (CIRG), Makhdoom, Farah-281122, Mathura, Uttar Pradesh, India

[*]***Correspondence:*** *S. D. Kharche, Physiology Reproduction and Shelter Management Division, Central Institute for Research on Goats (CIRG), Makhdoom, Farah-281122, Mathura, Uttar Pradesh, India. E-mail: kharche62@gmail.com*

(Received 21 May 2014; revised version 5 Aug 2014; accepted 27 Oct 2014)

## Summary

This study was designed to compare the effectiveness of different activation treatments for activation of *in vitro* matured oocytes and their developmental potency in $mCR_2aa$ medium so as to obtain maximum number of embryos. A total of 1090 cumulus oocyte complexes (COC's) were collected from 480 ovaries. *In vitro* matured oocytes were randomly divided into four groups. Group 1 *in vitro* matured oocytes (n=226) were exposed to 7% ethanol for 5 min followed by treatment with 2.0 mM DMAP for 4 h in $mCR_2aa$ medium. Group 2 *in vitro* matured oocytes (n=294) were exposed to 7% ethanol for 5 min followed by treatment with 10 µg/ml CHX for 4 h in $mCR_2aa$ medium. Group 3 *in vitro* matured oocytes (n=325) were exposed to 7% ethanol for 5 min followed by treatment with 2.0 mM DMAP and 10 µg/ml CHX for 4 h in $mCR_2aa$ medium. Group 4 *in vitro* matured oocytes (n=108) were cultured for 4 h without any chemical treatment in $mCR_2aa$ medium (control). The cleavage rate in groups 1, 2, 3 and 4 was 54.42%, 44.55%, 51.69% and 0.00%, respectively. The percentage of morula and blastocyst production in group 1, group 2 and group 3 was 26.01%, 29.77% and 29.76% and 2.43%, 1.52% and 1.78%, respectively. These results suggest that the activation of *in vitro* matured oocytes by 7% ethanol for 5 min followed by treatment with 2.0 mM DMAP for 4 h in $mCR_2aa$ is most favorable for parthenogenetic caprine embryos production.

**Key words:** Parthenogenesis, Ethanol activation, Morula, Blastocyst, Caprine

## Introduction

Methods used for parthenogenetic activation of oocytes are electrical pulse method (Kono *et al*., 1989), ethanol (Kharche *et al*., 2013), chemical activation (Mishra *et al*., 2006) or by combined electrical-chemical parthenogenetic activation of oocytes (Hossenni *et al*., 2008). There are several other methods available for the induction of parthenogenetic activation that promote an increase in intracellular free calcium concentrations by the release of calcium from cytoplasmic stores such as: calcium ionophore (Funahashi *et al*., 1994), ethanol (Loi *et al*., 1998), A-23187, calcium ionophore and cycloheximide alone (Nussbaum and Prather, 1995) or combined with a protein phosphorylation inhibitor, 6-dimethyl amino purine (6-DMAP) (Liu and Yang, 1999), electrical shock (Kim *et al*., 1996), $CaCl_2$ (Machaty *et al*., 1996), Ca-EDTA (Zaeand Ryoo, 2007), G protein stimulation (Machaty *et al*., 1996), Ionomycin (Loi *et al*., 1998), ultrasound (Sato *et al*., 2005), strontium (Meo *et al*., 2004) and magnetic field (Max *et al*., 2007). Methods used for parthenogenetic activation could be divided into two groups according to the mechanism used to increase intracellular calcium. One method for obtaining free calcium is by exposure to strontium or ionomycin to release cytoplasmic calcium. A second method is to promote influx of calcium from the extracellular medium by using an electrical stimulus or ethanol exposure (Meo *et al*., 2004).

Currently, combined treatment of two activation stimuli are commonly used to ensure better development of reconstructed oocytes. Limited literature on activation protocols for goat oocytes is available, creating the need for effective oocyte activation protocols that can be used during nuclear transfer in goats. Therefore, in the present study attempts were made to compare different activation protocol on the development competence of parthenogenetic goat embryos. Thus, the present study was designed with the objectives to study the effect of different chemical activation protocols on cleavage rate of *in vitro* matured goat oocytes and to compare the development of parthenogenetic embryos produced from different chemical activation protocols.

## Materials and Methods

### Recovery of oocytes and *in vitro* maturation (IVM)

During the spring season, ovaries from goats, regardless of the stage of estrous cycle, were collected from a local abattoir located at Agra. Goats in this area are not seasonal breeders. A total of 1090 oocytes (Fig.

1A) were recovered by using follicle puncture technique. The collected oocytes were finally graded under the inverted phase contrast as per the method of Kharche *et al*. (2008). The cumulus oocyte complexes (COCs) were matured in maturation media (TCM-199 (Sigma) containing L-glutamine (100 µg/ml), sodium pyruvate (0.25 mmol), gentamycin (50 µg/ml), FSH (5 µg/ml), LH (10 µg/ml), oestradiol-17β (1 µg/ml) supplemented with 10% FBS and 3 mg/ml BSA) for 27 h in humidified 5% $CO_2$ at 38.5°C in $CO_2$ incubator. Out of a total 1090 oocytes recovered, 1036 matured (95.04%).

### Activation of oocytes

A total of 1036 *in vitro* matured oocytes (Fig. 1B) were collected, out of which 953 *in vitro* matured oocytes were selected and randomly divided into the following treatment groups:

Group 1 comprised *in vitro* matured oocytes (n=226) after 27 h of *in vitro* maturation, activated with 7% ethanol for 5 min followed by treatment with 2.0 mM DMAP for 4 h in $mCR_2aa$.

Group 2 comprised *in vitro* matured oocytes (n=294) after 27 h of *in vitro* maturation, activated with 7% ethanol for 5 min followed by treatment with 10 µg/ml CHX for 4 h in $mCR_2aa$.

Group 3 comprised *in vitro* matured oocytes (n=325) after 27 h of *in vitro* maturation, activated with 7% ethanol for 5 min followed by treatment with 2.0 mM DMAP and 10 µg/ml CHX for 4 h in $mCR_2aa$.

Group 4 comprised *in vitro* matured oocytes (n=108) after 27 h of *in vitro* maturation, cultured without any chemical activation treatment for 48 h in $mCR_2aa$.

After parthenogenetic activation of oocytes for 4 h, activated oocytes were washed in embryo development medium ($mCR_2aa$) and transferred in 100 µl embryo culture drops placed in a $CO_2$ incubator at 38.5°C and 5% $CO_2$ in a humidified atmosphere.

The maturation stage of oocytes was calculated as a percentage. Cleavage rates between the different treatment groups were compared 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 *in vitro* maturation

From 480 ovaries, a total of 1090 oocytes were recovered by puncture technique, resulting in an average recovery of 2.27 oocytes per ovary. After 27 h, maturation rate was recorded on the basis of morphological cumulus expansion of oocytes. The maturation rate was found to be 95.04%.

### Parthenogenetic activation of *in vitro* matured oocytes

The cleavage rate of chemically activated *in vitro* matured goat oocytes in group 1 (activation with ethanol + DMAP), group 2 (activation with ethanol + CHX), group 3 (activation with ethanol + DMAP + CHX) and group 4 (no activation), were 54.42%, 44.55%, 51.69% and 0.00%, respectively. Statistically (Chi-square) no significant difference was observed in cleavage rate between group 2 and 3 and group 1 and 3. In group 1 significantly higher ($P<0.05$) cleavage rate was observed compared to group 2 and group 4 (Table 1).

### Developmental potential of parthenogenetic embryos

In control (group 4) no cleavage was observed. Therefore, embryo development was not studied in group 4. The embryo development among ethanol + DMAP (group 1), ethanol + CHX (group 2) and ethanol + DMAP + CHX (group 3) was compared. In group 1 (ethanol + DMAP), the percentage of 2 cell, 4 cell, 8 cell, morula and blastocyst was 23.57%, 24.39%, 23.57%, 26.01% and 2.43%, respectively. In group 2 (ethanol + CHX), the percentage of 2 cell, 4 cell, 8 cell, morula and blastocyst was 7.63%, 17.55%, 43.51%, 29.77% and 1.52%, respectively. In group 3 (ethanol + DMAP + CHX), the percentage of 2 cell, 4 cell, 8 cell, morula and blastocyst was 9.5%, 13.09%, 45.63%, 29.76% and 1.78%, respectively. In group 1 (ethanol + DMAP) maximum blastocyst production was observed with 2.34%. The relevant data for embryo development (Figs. 1C-F) in $mCR_2aa$ medium are shown in Table 2.

## Discussion

In our experiment, a total of 480 ovaries were used for oocyte recovery by follicle puncture technique. Total oocyte recovery calculated was 2.27. Almost similar levels of oocytes were recovered by Das *et al*. (1996) in buffalo and Kharche *et al*. (2007, 2008, 2011) in goats. The variation in oocytes recovery could be due to many factors like age of donor, effect of follicle size or may be due to the estrous cycle stage. We obtained ovaries from slaughtered goats from a slaughter house, so we did not consider these factors.

In our experiment, 95.0% oocytes showed maturation. Our results are superior to many workers (Kharche *et al*., 2007; Yadav *et al*., 2007; Singh *et al*., 2009) in goat. Maturation rate observed in the present study is similar to Younis *et al*. (1991) in goat and Mishra *et al*. (2006) in buffalo. This variation observed

**Table 1:** Cleavage rate of *in vitro* matured goat oocytes activated by different activating agents in $mCR_2aa$ medium

| S. No. | Group | No. of oocytes taken for activation | No. of oocytes Cleaved | Cleavage rate (%) |
|---|---|---|---|---|
| 1 | Group 1 (ethanol + DMAP) | 226 | $123^{b}$ | 54.42 |
| 2 | Group 2 (ethanol + CHX) | 294 | $131^{c}$ | 44.55 |
| 3 | Group 3 (ethanol + DMAP + CHX) | 325 | $168^{bcd}$ | 51.69 |
| 4 | Group 4 (control) | 108 | $0.00^{a}$ | 0.00 |

Values in a column with different superscripts are significantly different ($P<0.05$)

**Table 2:** Effect of different inhibitors on development of activated *in vitro* matured goat oocytes

| S. No. | Group | No. of cleaved oocytes (%) | 2 cell (%) | 4 cell (%) | 8 cell (%) | Morula (%) | Blastocyst (%) |
|---|---|---|---|---|---|---|---|
| 1 | Group 1 (ethanol + DMAP) | 123 (54.42) | $29^{a}$ (23.57) | $30^{a}$ (24.39) | $29^{a}$ (23.57) | $32^{a}$ (26.01) | $3^{a}$ (2.43) |
| 2 | Group 2 (ethanol + CHX) | 131 (44.55) | $10^{b}$ (7.63) | $23^{a}$ (17.55) | $57^{b}$ (43.51) | $39^{a}$ (29.77) | $2^{a}$ (1.52) |
| 3 | Group 3 (ethanol + DMAP + CHX) | 168 (51.69) | $16^{bc}$ (9.5) | $22^{a}$ (13.09) | $77^{bc}$ (45.63) | $50^{a}$ (29.76) | $3^{a}$ (1.78) |

Values in a column with different superscripts are significantly different (P<0.05)

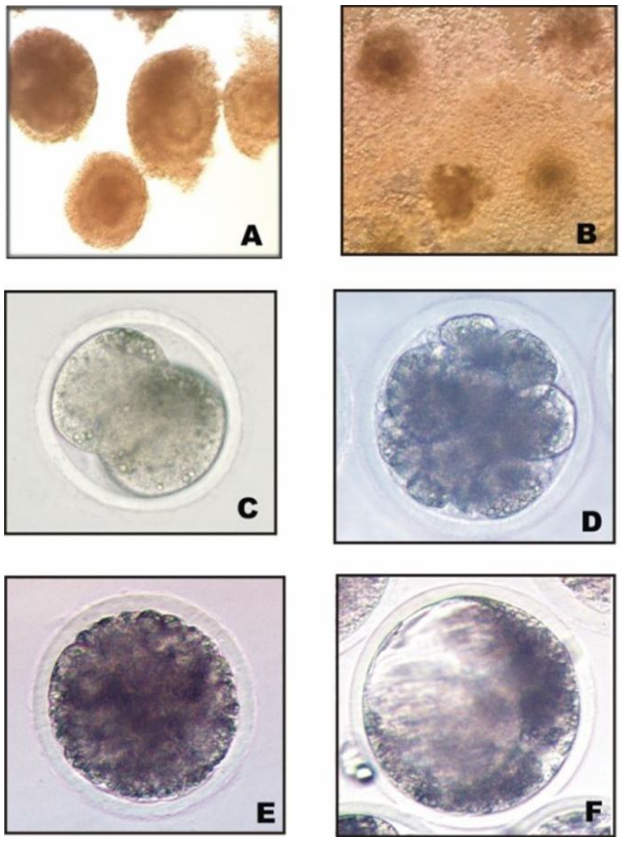


**Fig. 1:** Different stages of oocytes and embryos. (A) Immature oocytes, (B) Mature oocytes, (C) 2-Cell embryo, (D) 8-Cell embryo, (E) Morula, and (F) Blastocyst

in maturation rate could be due to many reasons as maturation rate is influenced by many factors like presence of follicular cells, protein supplementation, hormones, antioxidants and growth factors in maturation media (Kharche *et al*., 2011). The reasons for good maturation rate in our experiment were hormones that were added in maturation media (FSH (5 µg/ml) and LH (10 µg/ml)). However, it is also observed that slaughterhouse oocytes cultured in simplified medium matured significantly faster than laproscopic ovum pick up oocytes at 18 and 22 h (Souza-Fabjan *et al*., 2014).

In this experiment, the cleavage rate following different activation treatments with ethanol + DMAP (54.42%), ethanol + CHX (44.55%) and (51.69%) was significantly higher than the respective control (group 4, no activation (0.00%)). The cleavage rate between the various chemical activation treatments was also significantly different from each other except that no significant difference was observed between group 1 and group 3, and group 2 and group 3. However, the cleavage rate was significantly higher (P<0.05) in group 1 (54.42%) than that of other groups. These results demonstrate the possibility of chemical activation of *in vitro* matured goat oocytes, leading to cleavage and embryo development. A significant improvement in cleavage rate was observed in group 1 (Ethanol + DMAP), suggesting that the *in vitro* matured goat oocytes had acquired more cleavage potential after ethanol + DMAP activation treatment. The activation methods used in this trial have been proven to be effective for activation of bovine oocytes (Mishra *et al*., 2008).

When we compared cleavage rate following different activation treatments with other workers we found that our results regarding cleavage rate are higher than Kumar *et al*. (2014) with Ca + CHX + DMAP (43.2%). Our results of cleavage rate are in agreement with Ongeri *et al*. (2000). He reported cleavage of 57.8% and 58.0% with ionomycin and ethanol, respectively in *in vitro* matured goat oocytes. Furthermore, our results regarding cleavage rate are lower than Tanaka *et al*. (1997) with ethanol (78.0%), Gasparrini *et al*. (2004) with ethanol + DMAP (71.4%), Hosseini *et al*. (2008) with Ca ionophore (85.5%) and Bevacqua *et al*. (2010) with ionomycin + DMAP (71.2%) in *in vitro* matured bovine oocytes and Jena *et al*. (2012) with Ca ionophore + DMAP (75.7% (RVCL), 72.0% (mSOF) and 57.1% (EDM)) in goat oocytes. It is suggested that the difference in the cleavage rate might be due to difference in the species, culture media and activation treatments used for parthenogenetic activation.

In present study, all chemical activation treatments in $mCR_2aa$ media were able to sustain embryo development up to the blastocyst stage. A significant difference was found in 2 cell embryos of group 1 and group 2 (23.57% vs 7.63%) and group 1 and group 3 (23.57% vs 9.5%). However, no significant difference was observed in 2-cell embryos of group 2 and group 3 (7.63% vs 9.5%). In 4 cell embryos, no significant difference was found among the groups (24.39%, 17.55% and 13.09%, respectively). In 8 cell embryos, a significant difference was found in group 1 and group 2 (23.57% vs 43.51%) and of group 1 and group 3 (23.57% vs 45.63%). However, no significant difference was observed in 8 cell embryos of group 2 and group 3 (43.51% vs 45.63%). Also, no significant difference was found in morula production (26.01%, 29.77% and 29.76%, respectively) and blastocyst production (2.43%, 1.52% and 1.78%, respectively) among all the groups. Similarly, the development of blastocysts in group 1 with ethanol + DMAP (2.43%) was higher than group 2 with ethanol + CHX (1.52%) and group 3 with ethanol + DMAP + CHX (1.78%). It might be due to less number of embryos were arrested in 2 cell stage in group 1.

In our experiments the embryo development in terms of morula production following different activation treatments is superior to ethanol + DMAP (6.5%) and inomycin + DMAP (8.3%) in hSOF media (Gasparrini *et al*., 2004) and ionomycin + DMAP (21.2%) and ionomycin + ethanol (20.5%) in SOF media (Bevacqua *et al*., 2010) in buffalo. However, in morula production our results are not in agreement with that of Mishra *et al*. (2008), following activation treatment in mSOF media with ethanol + DMAP (26.4%), ethanol + CHX (35.7%) and ethanol + DMAP + CHX (50.9%) in bubaline oocytes.

Furthermore, when we compared the embryo development in terms of blastocysyt production following different activation treatments with other workers we found that our results are superior to Abdalla *et al*. (2009), who observed blastocyst production of 1% in goat with two different activation treatments of inomycin + CHX and inomycin + DMAP in TCM-199. However, compared to our observations, higher blastocyst production was found in goat by De *et al*. (2011) with Ca ionophore (21.6%) and electrical pulse (23.11%). Furthermore, our results of blastocyst production are not comparable with ethanol + DMAP (49.8%) and ionomycin + DMAP (50.1%) in G1.2 and G2.2 media (Ongeri *et al*., 2001) and ethanol + DMAP (8.8%) and ionomycin + DMAP (7.9%) in hSOF media (Gasparrini *et al*., 2004) in buffalo which could be due to the additional effect produced by ionomycin. Our results of blastocyst production are not comparable with ionomycin + DMAP (14.5%) in SOFaa media, ethanol + DMAP (9.4%), ethanol + CHX (4.8%) and ethanol + DMAP + CHX (30.9%) in mSOF media (Mishra *et al*., 2008), ethanol + DMAP (25.1%) in $CR_1$aa media (Hou *et al*., 2009) and ionomycin + DMAP (18.7%) in SOF media (Bevacqua *et al*., 2010) in bovine. It is suggested that the difference in the blastocyst production might be due to difference in the species, activation treatments and various media used for embryo development.

The results indicated that parthenogenetic activation of caprine oocytes in m$CR_2$aa media can be effectively done by ethanol + DMAP, ethanol + CHX and ethanol + DMAP + CHX combinations, which may enhance better development of parthenogenetic embryos. Considering the fact that the cleavage rate and subsequent development of blastocysts following chemical activation in ethanol + DMAP (54.42% and 2.43%, respectively) was numerically higher, our study indicates that goat oocytes had better inherent developmental competence following ethanol + DMAP chemical activation.

## Acknowledgements

The authors would like to thank the National Director, NAIP, New Delhi for providing the funding and Director, CIRG, Makhdoom, Farah, Mathura, UP, India for providing the facilities needed.

## References

**Abdalla, H; Shimoda, M; Hirabayashi, M and Hochi, S** (2009). A combined treatment of ionomycin with ethanol improves blastocyst development of bovine oocytes harvested from stored ovaries and microinjected with spermatozoa. Theriogenology. 72: 453-460.

**Bevacqua, RJ; Pereyra, BF; Fernandez, MR and Salamone, DF** (2010). High rates of bovine blastocyst development after ICSI-mediated gene transfer assisted by chemical activation. Theriogenology. 74: 922-931.

**Das, GK; Jain, GC; Solanki, VS and Tripathi, VN** (1996). Efficacy of various collection methods for oocyte retrieval in buffalo. Theriogenology. 46: 1403-1411.

**De, AK; Malakar, D; Jena, MK; Dutta, R; Garg, S and Akshey, YS** (2011). Zona-free and with-zonaparthenogenetic embryo production in goat (*Capra hircus*)-effect of activation methods, culture systems and culture media. Livestock Sci., 143: 35-42.

**Funahashi, H; Cantley, T; Stumpf, TT; Terlouw, SL; Rieke, A and Day, BN** (1994). *In vitro* development of *in vitro* matured pig oocytes following chemical activation or *in vitro* fertilization. Biol. Reprod., 50: 1072-1077.

**Gasparrini, B; Boccia, L; Rosa, AD; Palo, RD; Campanile, G and Zicarelli, L** (2004). Chemical activation of buffalo (*Bubalus bubalis*) oocytes by different methods: effects of aging on post-parthenogenetic development. Theriogenology. 62: 1627-1637.

**Hosseini, SM; Hajian, M; Moulavi, F; Shahverdi, AH and Nasr, EMH** (2008). Optimized combined electrical-chemical parthenogenetic activation for *in vitro* matured bovine oocytes. Anim. Reprod. Sci., 1081: 122-133.

**Hou, Y; Liu, Y; Dai, Y; Li, R; Shi, WQ; Wang, H; Wang, Ll; Li, N and Zhu, S** (2009). Improved parthenogenetic development of vitrified-warmed bovine oocytes activated with 9% ethanol plus 6-DMAP. Theriogenology. 72: 643-649.

**Jena, MK; Malakar, D; De, AK; Garg, S; Akshey, YS; Dutta, R; Sahu, S; Mohanty, AK and Kaushik, JK** (2012). Handmade cloned and parthenogenetic goat embryos - a comparison of different culture media and donor cells. Small Ruminant Res., 105: 255-262.

**Kharche, SD; Goel, AK; Jindal, SK; Goel, P and PandJha, BK** (2011). Birth of twin kids following transfer of *in vitro* produced goat embryos. Indian J. Anim. Sci., 81: 1132-1134.

**Kharche, SD; Goel, AK; Jindal, SK; Jha, BK and Goel, P** (2013). Assessment of parthenogenetic embryo production by activation of *in vitro* matured caprine oocytes with different concentrations of ethanol. Small Ruminant Res., 111: 100-103.

**Kharche, SD; Goel, AK; Jindal, SK and Sinha, NK** (2007). Birth of a female kid from *in vitro* matured and fertilized caprine oocytes. Indian J. Anim. Sci., 78: 680-685.

**Kharche, SD; Goel, AK; Jindal, SK; Sinha, NK and Yadav, P** (2008). Effect of somatic cells co-cultures on cleavage and development of *in vitro* fertilized caprine embryos. Indian J. Anim. Sci., 78: 686-692.

**Kim, NH; Simerly, C; Funahashi, H; Schatten, G and Day, BN** (1996). Microtubule organization in porcine oocytes during fertilization and parthenogenesis. Biol. Reprod., 54: 1397-1404.

**Kono, T; Iwasaki, S and Nakahara, T** (1989). Parthenogenetic activation by electric impulse of bovine oocytes matured *in vitro*. Theriogenology. 32: 569-576.

**Kumar, D; Gopalakrishna, R; Singh, AP; Ranjan, R;**

**Pandey, SK and Sarkhel, BC** (2014). Developmental potency of pre-implant parthenogenetic goat embryos: effect of activation protocols and culture media. In Vitro Cell. Dev. Biol. Anim., 50: 1-6.

**Lee, SR; Kim, JW; Kim, BS; Kim, MO; Kim, SH; Yoo, DH; Shin, MJ; Lee, S; Park, YS; Park, YB; Ha, JH and Ryoo, ZY** (2007). The parthenogenetic activation of canine oocytes with Ca-EDTA by various culture periods and concentrations. Theriogenology. 67: 698-703.

**Liu, L and Yang, X** (1999). Interplay of maturation-promoting factor and mitogen-activated protein kinase inactivation during metaphase-to-interphase transition of activated bovine oocytes. Biol. Reprod., 61: 1-7.

**Loi, P; Ledda, S; Fulka, JJ; Cappai, P and Moor, RM** (1998). Development of parthenogenetic and cloned ovine embryos: effect of activation protocols. Biol. Reprod., 58: 1177-1187.

**Machaty, Z; Funahashi, H; Mayes, MA; Day, BN and Prather, RS** (1996). Effects of injecting calcium chloride into *in vitro* matured porcine oocytes. Biol. Reprod., 54: 316-322.

**Max, A; Grabiec, A and Tischner, M** (2007). Parthenogenetic activation of domestic cat oocytes using ethanol, calcium ionophore, cycloheximide and a magnetic field. Theriogenology. 67: 795-800.

**Meo, SC; Leal, CL and Garcia, JM** (2004). Activation and early parthenogenesis of bovine oocytes treated with ethanol and strontium. Anim. Reprod. Sci., 81: 35-46.

**Mishra, V; Mishra, AK and Sharma, R** (2006). Effect of ambient temperature on *in vitro* fertilization of bubaline oocyte. Anim. Reprod. Sci., 100: 379-384.

**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.

**Nussbaum, DJ and Prather, RS** (1995). Differential effects of protein synthesis inhibitors on porcine oocyte activation. Mol. Reprod. Dev., 195: 70-75.

**Ongeri, EM; Bormann, CL; Butler, RE; Melican, D; Gavin, WG; Echelard, Y; Krisher, RL and Behboodi, E** (2001). Development of goat embryos after *in vitro* fertilization and parthenogenetic activation by different methods. Theriogenology. 55: 1933-1945.

**Sato, K; Yoshida, M and Miyoshi, K** (2005). Utility of ultrasound stimulation for activation of pig oocytes matured *in vitro*. Mol. Reprod. Dev., 72: 396-403.

**Singh, KP; Saxena, A; Kharche, SD and Singh, P** (2009). Studies on *in vitro* maturation, fertilization and cleavage rate of prepubertal and pubertal goat oocytes. Indian J. Anim Sci., 79: 550-553.

**Snedecor, GW and Cochran, WG** (1989). *Statistical methods*. 8th Edn. Ames, Iowa State University Press. PP: 120-125.

**Souza-Fabjan, JMG; Locatelli, Y; Nicolas, D; Emilie, C; Jean-Luc, T; Christine, P; François, BJ; Freitas Vicente José, F and Pascal, M** (2014). *In vitro* embryo production in goats: slaughterhouse and laparoscopic ovum pick up-derived oocytes have different kinetics and requirements regarding maturation media. Theriogenology. 81: 1021-1031.

**Tanaka, H and Kanagawa, H** (1997). Influence of combined activation treatments on the success of bovine nuclear transfer using young or aged oocytes. Anim. Reprod. Sci., 49: 113-123.

**Yadav, EN; Kharche, SD; Goel, AK; Jindal, SK and Johri, DK** (2007). Comparative efficacy of different technique for oocyte recovery from prepubertal goat ovaries. Indian J. Anim. Sci., 77: 988-990.

**Younis, AI; Zuelke, KA; Harper, KM; Oliveira, MAL and Brackett, BG** (1992). *In vitro* maturation and fertilization of Toggenburg goat oocytes. Theriogenology. 37: 330.