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Optimisation of oocyte maturation media for improving efficiency of *in vitro* embryo production

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Abstract
In vitro maturation is essential technology under assisted reproductive technologies (ARTs) which enables oocytes to achieve maturation and acquire competence for subsequent embryonic division leading to blastocyst formation). This technique is valuable not only because they allow production of large numbers of oocytes, but also because they provide valuable *in vitro* model to study the gene expression at different maturational stages of oocyte. In a typical *in vitro* embryo production technique, either by cloning or by *in vitro* fertilization (IVF). IVM of oocytes constitute the most challenging step because an orchestrated gene expression events during this time determines the efficiency of fertilization and subsequent embryonic division process leading to blastocyst formation and even successful implantation. Optimisation of IVM media composition is important to increase the efficiency of *in vitro* embryo production. Foetal Bovine Serum (FBS) was supplemented to IVM media at 10% and 15% levels along with addition of 5 μgmL^{-1}pFSH, 1 μgmL^{-1} 17β-estradiol. The maturation percentage was found to be significantly higher when 15% FBS was used both with the addition of β-Estradiol and FSH and without the hormones (55.37± 2.61 v 79.31± 1.75 and 77.88± 1.91, respectively). Cleavage percentage was also found to be significantly higher with 15% FBS (68.88± 2.50) than with 10% FBS (55.35± 4.31) (P<0.05). Morula percentages were also found to vary in a similar fashion between three groups of IVM media, 33.97± 1.61 v 41.74± 4.31 v 39.14± 2.31, respectively, but the differences were not significant statistically at significance level of P<0.05. Similar was the case with blastocyst percentages, 4.00± 2.31 v 14.80± 3.92 v 9.08± 3.60, respectively

Keywords: Optimisation, oocyte maturation, embryo production

1. Introduction
In modern livestock farming, assisted reproductive technologies are being used for out of season oestrus induction, enhancement of reproductive performance and genetic improvement. IVEP is one of the most powerful tools in controlling and manipulating mammalian reproduction (Cognie *et al*., 2003, Martinez *et al*., 2006) [1]. *In vitro* embryo production is rendered important in sheep as it has shown the potential of producing sheep embryos, even during the non-breeding season (Pugh *et al*., 1991) [8]. The improved IVEP technologies of *in vitro* maturation (IVM) and *in vitro* culture (IVC) have further led to another generation of reproductive techniques, such as intra cytoplasmic sperm injection (ICSI), the production of transgenic animals and improved efficiency of animal cloning procedures.
Oocyte maturation involves cytoplasmic and nuclear events, including changes in plasma membrane, nuclear and cytoplasmic maturation. IVM is essential technology under assisted reproductive technologies (ARTs) which enables oocytes to achieve maturation and acquire competence for subsequent embryonic division leading to blastocyst formation (Lonargan *et al*. 2003; Somfai *et al*. 2011) [5, 12]. This technique is valuable not only because they allow production of large numbers of oocytes, but also because they provide valuable *in vitro* model to study the gene expression at different maturational stages of oocyte. In a typical *in vitro* embryo production technique, either by cloning or by IVF, IVM of oocytes constitute the most challenging step because an orchestrated gene expression events during this time determines the efficiency of fertilization and subsequent embryonic division process leading to blastocyst formation and even successful implantation (Somfai *et al*. 2011) [12]. The capacity of mature oocytes to support the very earliest stages of life, fertilization, preimplantation and implantation is termed as oocyte developmental competence.

The alteration in basic maturation conditions can significantly affect the oocyte developmental competence as reflected by morula and blastocyst yield after *in vitro* embryo production (IVEP) (Atef *et al*., 2002). Successful and reliable oocyte maturation would dramatically improve efficiency of preimplantation embryonic development as well as fetal development (Sagirkaya *et al*., 2007) [9]. Numerous studies have been carried out to study the ability of mammalian oocytes and embryos to develop *in vitro* using wide variety of culture media (Averty *et al*., 1995; Wani *et al*., 1999; Karache *et al*., 2005, 2006) [14, 4]. Shabankareh *et al*., 2011 [10]. The current study was undertaken with a view to investigate the effect of different serum percentages and effect of hormonal supplementation.

2. Materials and Methods

2.1 Oocyte collection

Abattoir derived ovaries from different breeds of sheep slightly above three years of age were collected in warm isotonic saline (32-37 ˚C) and transported to the laboratory within 2-4 h. After removing the extra tissue around ovaries, they were washed 3-4 times with warm isotonic saline (32-37 ˚C) containing 400 IU/ml penicillin and 500 μg/ml streptomycin. Oocytes from follicles (2 to 8 mm in diameter) were harvested by puncturing with an 18-gauge needle. The collection medium consisted of calcium and magnesium-free Dulbecco's Phosphate Buffered Saline (DPBS) containing 0.3% bovine serum albumin (BSA). Oocytes were washed two to four times with the washing medium which consisted of Hepes buffered tissue culture medium (M-199) supplemented with 10-15% fetal bovine serum (FBS) (Hyclone, Canada), 0.68 mM L-glutamine, 0.8 mM sodium pyruvate and 50 μg/ml gentamicin. The aspirated oocytes were graded according to the following criteria:

Usable quality: Grade-A and Grade-B compact cumulus-oocyte-complexes (COCs) with an unexpanded cumulus mass having ≥2 layers of cumulus cells, and with homogenous, evenly granular ooplasm.

Unusable quality: Grade-C and Grade-D oocytes partially or wholly denuded or with expanded or scattered cumulus cells or with an irregular ooplasm.

Oocytes of only usable quality were used for *in vitro* maturation.

2.2 *In vitro* maturation of oocytes

Oocytes of usable quality were selected and after washing thrice with IVM medium (Hepes buffered M-199 supplemented with FBS, 5 μgmL^{-1}pFSH, 1 μgmL^{-1} 17β-estradiol, 0.8 mM sodium pyruvate and 50 μgmL^{-1} gentamicin and epidermal growth factor (EGF), groups of 15–20 COCs were cultured in 100-μL droplets of IVM medium, overlaid with sterile mineral oil in 35-mm diameter Petri dishes, and cultured for 24 h in 5%CO_2 with maximum humidity at 38.5 ºC.

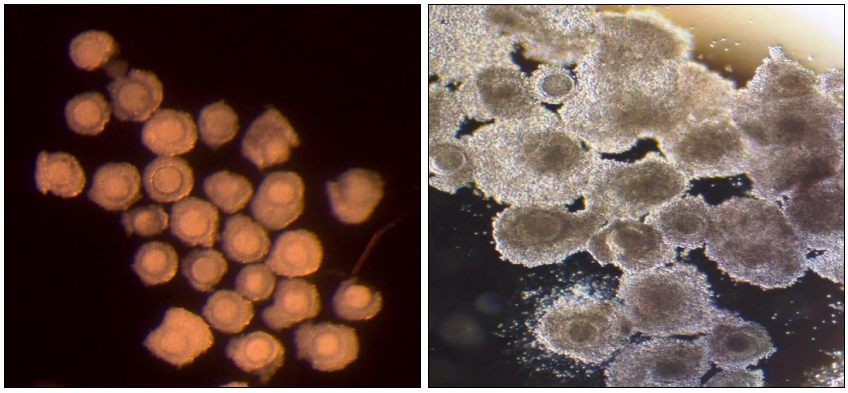
Three different IVM media were used to study the effect of supplementation of IVM media on *in vitro* maturation and *in vitro* development of parthenogenetic sheep embryos. In addition to basal media, Type 1, 2 and 3 contained FBS 10% with hormones, FBS 15% with hormones and FBS 15% without hormones, respectively.

2.3Production of parthenogenetic embryos

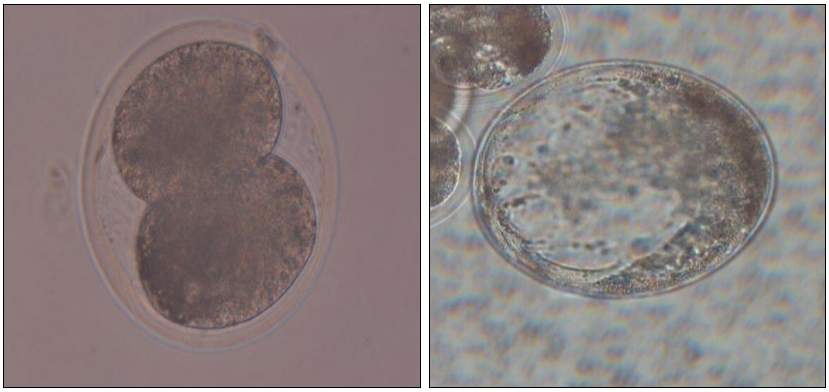
In-vitro matured COCs were cultured in 50-ml drops of maturation medium covered with an overlay of mineral oil and incubated at 38.5 ˚C–39 ˚C in 5% CO_2. After 23–24 h of maturation, the cumulus cells were removed from the matured oocytes by incubating the COC's in warmed 1 mg/ml hyaluronidase and then vortexing the COCs for 2–-3 min in 1 mg/ml hyaluronidase. The denuded oocytes were washed in T20 and kept in 35 mm petri dish containing T20 and incubated for 10 minutes at 38.5 ˚C–39 ˚C in 5% CO_2. The denuded oocytes were then activatedby incubating in T2 containing 5µM calcium ionophore for5min at 38.5 ˚C. After washing thrice with T20 the zona free reconstructed oocytes were individually transferred to droplets of T20 containing 2mM of 6-dimethylaminopurine (6- DMAP) and zona included reconstructs were transferred to 100µL drops of T20 containing 2mM of 6-dimethylaminopurine (6- DMAP) in groups of 20-25 and both were then covered with mineral oil and incubated for 3-6 hours in a CO_2 incubator at 38.5˚C. The activated embryos were then transferred to 500µL of Research Vitro Cleave medium (K-RVCL-50, Cook®, Australia) supplemented with fatty acid free bovine serum albumin (BSA), B27, EGF and cysteamine in 4-well dishes covered with mineral oil and kept undisturbed in a CO2 incubator for 7 days.

3. Results

Overall 822 abattoir derived sheep ovaries in 20 replicates were used for the experiments. 1996 COC's were harvested upon puncturing of immature follicles (size ranging from 2mm - 8mm) on their surface. Thus a harvest ranging from 1.50 to 3.22 with an average of 2.43 COC's per ovary was obtained. 859 COC's (43.04%) were of A grade and 663 (32.22%) COC's were of B grade, while as 318 (15.39%) and 156 (7.81%) COC's were of C and D grade respectively. A total of 1522 (76.26%) COC's were of usable quality, therefore, selected for IVM. C and D grade COC's were not used for IVM.



A. B.



C. D.

Fig 1: *A) Immature oocytes of various grades; B) In vitro matured oocytes; C) Cleavage as seen on Day 2 of in vitro culture of parthenogenetic embryos and D) Blastocyst on Day 7*

For *in vitro* embryo production, immature sheep oocytes are generally matured for 22- 24 hours. In our study also, oocytes were matured for 22-24 hours. Only A and B grade COC's with an unexpanded cumulus of not less than two layers were used for *in vitro* maturation. Mature COC's exhibited a characteristic cytoplasmic granulation and a uniform cumulus expansion. First polar body extrusion was used as indicator of effective maturation.

Different FBS concentrations were used to determine the optimum composition of IVM media for *in vitro* maturation of local sheep oocytes. The effect of addition of β- Estradiol was also simultaneously studied. The results obtained were as under:

Table 1: Comparison of different constituents of IVM media on parthenogenetic embryo production

IVM Media	Constituent in addition to basal medium	No. of COC's	Maturation %
1	FBS 10% with Hormones	235	$55.37^{a} \pm 2.61$
2	FBS 15% with hormones	408	$79.31^{b} \pm 1.75$
3	FBS 15 % without hormones	257	$77.88^{b} \pm 1.91$

Percentages quoted as Mean ± S.E

Values having different superscripts along columns, differ significantly ($P<0.05$)

The maturation percentage as determined by cumulus expansion and polar body extrusion was found to be significantly higher when 15% FBS was used both with the addition of β-Estradiol and without β-Estradiol (55.37± 2.61 v 79.31± 1.75 and 77.88± 1.91, respectively). Higher maturation percentage was observed when β-Estradiol was supplemented to IVM medium (79.31± 1.75 v 77.88± 1.91), although the difference was non-significant ($P<0.05$). (Table-2, Fig.1)

Cleavage percentage was also found to be significantly higher with 15% FBS (68.88± 2.50) than with 10% FBS (55.35± 4.31) ($P<0.05$). Withoutβ-Estradiol, the cleavage percentage obtained was comparable (68.18± 2.80).

Table 2: Comparison of different constituents of IVM media on parthenogenetic embryo production

IVM Medium	Constituent in addition to basal medium	No. Cultured	Cleavage % (n)	Morulae % (n)	Blastocyst % (n)
1	FBS 10% with Hormones	98	$55.35^{a} \pm 4.31$ (53)	$33.97^{a} \pm 1.61$ (18)	$4.00^{a} \pm 2.31$ (2)
2	FBS 15% with hormones	228	$68.88^{b} \pm 2.50$ (156)	$41.74^{a} \pm 4.31$ (62)	$14.80^{a} \pm 3.92$ (26)
3	FBS 15% without hormones	72	$68.18^{b} \pm 2.80$ (50)	$39.14^{a} \pm 2.31$ (20)	$9.08^{a} \pm 3.60$ (4)

Percentages quoted as Mean ± S.E

Morulae and Blastocyst percentages calculated out of total number of cleaved embryos

Values having different superscripts along columns, differ significantly ($P<0.05$

Morula percentages were also found to vary in a similar fashion between three groups of IVM media, 33.97± 1.61 v 41.74± 4.31 v 39.14± 2.31, respectively, but the differences were not significant statistically at significance level of $P<0.05$. Similar was the case with blastocyst percentages, 4.00± 2.31 v 14.80± 3.92 v 9.08± 3.60, respectively.

4. Discussion

In vitro cloned sheep embryo production is an emerging area of research in modern animal biotechnology research centres throughout the world. Despite strenuous research efforts over the last 30 years, results of *in vitro* cloned embryo production are still unpredictable and variable which is an important limitation to its commercial application. Better knowledge of gamete and embryo physiology is needed to use IVEP on a large scale commercial level (Paramio and Izquierdo, 2014) [6] Low live birth rates with cloned embryos being an impediment to success of animal cloning, moreover, *in vitro* developmental rates are low and highly variable between different laboratories. Therefore, further investigations are needed to establish efficiencies of various cloning methodologies and to identify critical points by which *in vitro* developmental rates could be improved. Developing high quality *in vitro* cloned embryos could possibly increase fetal development and live birth rates

Fetal Bovine Serum (FBS) is one of the most commonly used protein supplements in different media supporting *in vitro* cellular development. In our study, two different concentrations of FBS (10% and 15%) were used and their effect on *in vitro* maturation and embryonic development

compared. At the same time, a third group of oocytes was put in IVM medium having 15% FBS but without the addition of β-Estradiol which was part of our basal IVM medium.

The maturation percentage observed with 15% FBS was significantly higher than that with 10 %FBS, our findings are in line with those of Sreenivas *et al*., 2013 who also reported higher maturation with FBS. Similarly Kharche *et al* 2010 also reported higher levels of maturation with higher supplementation of serum in caprine oocytes. The higher maturation rate observed in this study in 15% FBS group could be due to higher concentrations of hormones, trace nutrients and proteins present in serum which were bound to favour nuclear maturation as suggested by Tajik and Esfandabadi, 2003 [13]. Rate of maturation was lower when β-Estradiol and FSH were not added to IVM media, although the difference was not significant, implying the positive role which β-Estradiol plays in maturation of oocyte as reported by Pawshe *et al*., 1996 [7], Izquierdo *et al*., 1999 [2]; Yadav *et al*., 2010.

In vitro embryo development was also higher in terms of proportion of cleaved embryos, morulation and development to blastocyst stage in 15% FBS group than with 10% FBS. The obvious reason for this may be that higher concentrations of nutrients in serum would certainly favour higher development of embryos.

5. Conclusion

A higher maturation percentage was observed when IVM medium was supplemented with 15% FBS than with 10% FBS ($P<0.05$) indicating that FBS can be used to improve efficiency of maturation. Further, Maturation percentage was also higher when IVM was supplemented with hormones β-Estradiol and FSH providing further evidence of their role in *in vitro* maturation of oocyte. Higher cleavage and *in vitro* developmental rates obtained by supplementation of FBS and hormones further stamps their utility. Further investigations are required to optimise the IVM media for efficient *in vitro* embryo production.

6. Acknowledgment

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7. Conflict of Interest

The authors declare that there is no conflict of interest.

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