Original Research Article

Effect of Two Different Culture Media on Developmental Rate of Bovine Embryos in vitro

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Abstract

In the present study, effect of two different culture media on the developmental competence of bovine oocytes was studied. For the study a total of 1110 oocytes were collected using ovum pick-up (OPU) technique and the average oocyte yield was 8.34 ± 0.23 per OPU session per animal. The oocytes were graded as A, B, C and D grades based on the layers of compact cumulus cells surrounding the zona pellucida. Among this only A, B and C grade oocytes were used for in vitro maturation. The oocytes were matured in TCM 199 media supplemented with 10 per cent FBS, 1 µg/ml Folltropin (Bioniche, Canada), 0.02 IU/ml LH, 1 µg/ml estradiol with addition of 100 µM cysteamine, 10 ng/ml EGF, 100 ng/ml IGF, 10 µg/ml insulin, 5.5 µg/ml transferrin and 5 µg/ml selenium and the overall maturation rate was 91.30 ± 1.27 per cent. The presumptive zygotes were cultured in two different culture media viz. i) two step sequential synthetic oviduct fluid (SOF) media and ii). single step potassium simplex optimised medium (KSOM). The mean (± SE) cleavage, 4 cell, 8 cell and morula development rate was 73.04 ± 1.50 and 79.01 ± 1.34 per cent, 54.88 ± 1.76 and 60.69 ± 1.89 per cent, 35.89 ± 1.57 and 44.67 ± 1.62 per cent and 19.18 ± 1.10 and 25.36 ± 1.37 per cent in SOF media and KSOM, respectively. Based on the cleavage and embryo development observed, it was concluded that KSOM could be better than SOF media for in vitro production of bovine embryos.

Keywords
Ovum pick-up, SOF media, KSOM, bovine embryo, in vitro culture

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Introduction

Assisted Reproductive Technology (ART) is a biotechnological tool which has been employed to improve the reproductive efficiency like infertility in human beings and production of high genetic merit animals. In vitro fertilization is one of the assisted reproductive technologies used for the multiplication of genetically superior animals and the preservation of genetics (Lonergan, 2007).

The successful application of ART and
related technologies are critically dependent on basic techniques like in vitro maturation of oocytes, in vitro fertilization and in vitro culture of embryos. Without significant improvements of these ART techniques, application of developments in cloning and the production of transgenic farm animals will remain limited and extremely costly (Galli et al., 2014).

After introduction of ultrasonic guided aspiration of bovine follicular oocyte (Callesen et al., 1987 and Pieterse et al., 1991), a significant improvement was observed in terms of recovered oocytes and in vitro embryo production. Oocyte quality determines the developmental competence of an embryo and it depends on the oocytes nuclear and cytoplasmic maturation (Sirard et al., 2006).

In vitro culture systems play a vital role in supporting in vitro development of pre implantation embryos (Thompson et al., 2007). The use of defined culture media is necessary to acquire a better comprehension of metabolism and biochemical requirements for in vitro embryo production (IVEP). It is well established that embryo metabolism, cleavage, and pregnancy rates are affected by the media composition, which may lead to a diminished embryonic and fetal developmental capacity (Camargo et al., 2006).

With the above background, the present study was conducted to investigate the effect of two different in vitro culture systems on the developmental competence and quality of bovine embryos.

Materials and Methods

All reagents used for the preparation of media were from Sigma (Sigma–Aldrich), unless stated otherwise. Freshly made media were stored at 4°C and were used up to 1 month.

Oocyte collection by ovum pick-up

For the study, bovine oocytes were collected from live animals using ovum pick-up (OPU) technique using a real-time B mode ultrasound scanner (SA-600V; Kretztechnik AG, BCF Technology, Scotland) equipped with a 6.5 MHz curved array (convex) transvaginal probe (VE5-8/20R, 6.5 MHz/20R/86D, BCF Technologies, Scotland). It was used for scanning of ovaries and subsequent follicular aspiration.

Twelve physiologically normal crossbred cows were used as oocyte donors in this experiment. The cows were regularly cyclic and in optimal nutritional status. The oocyte collection was carried out on every Monday, Wednesday and Friday. On a particular day/session, four animals were used for collection. Before the start of OPU, the animals were restrained in the trevis and the perineal area was cleaned with running tap water and with 70% alcohol. The epidural anesthesia was given with 5 ml of 2% lignocaine hydrochloride. The ovaries were manipulated per rectum and either the right or left ovary was positioned between the fingers and the needle was inserted in the guide and advanced through the fornix vagina and into the follicle antrum. The follicles of >2 mm dia. were aspirated through the transvaginal probe using pre-equilibrated (38.5°C) oocyte collection medium (modified HEPES – buffered tyrodes medium, pH 7.2-7.4) supplemented with 0.3 per cent BSA, 20 IU/ml heparin, (Beparine®, Biological E Ltd.,) and 1 per cent penicillin - streptomycin. After aspiration, the collection tube with contents of aspirated follicles was transported to the laboratory within 5 min. of collection and kept undisturbed for 5 min. The follicular fluid was observed under a zoom stereo microscope (Nikon, Japan) for the presence of cumulus oocyte complexes (COCs). The COCs were transferred into another petridish containing oocyte collection medium. The
collected immature COCs were categorized into A, B, C, and D grades as described by Cetica et al., (1999). Among the four grades only grade A (COCs with more than 5 layers of compact cumulus cells surrounding the zona pellucida), grade B (COCs with 3-5 layers of cumulus cells surrounding the zona pellucida) and grade C (COCs with ≤3 layers of cumulus cells surrounding the zona pellucida) oocytes were subjected to in vitro maturation. Grade D oocytes (completely devoid of cumulus mass and having irregular and dark ooplasm) were discarded.

**Experimental design**

**Study 1**

Studied the effect of different grades of COCs on in vitro maturation of bovine oocytes.

**Study 2**

Evaluated the effects of two different culture media on in vitro developmental competence of bovine embryos.

*In vitro* culture of presumptive zygotes in two step sequential synthetic oviduct fluid (SOF) media

*In vitro* culture of presumptive zygotes in single step potassium simplex optimised medium (KSOM)

**In vitro maturation**

The *in vitro* maturation of the selected oocytes was carried out in TCM 199 medium supplemented with 10 per cent FBS, 1 µg/ml Follitropin (Bioniche, Canada), 0.02 IU/ml LH, 1 µg/ml estradiol with addition of 100 µM cysteamine, 10 ng/ml EGF, 100 ng/ml IGF, 10 µg/ml insulin, 5.5 µg/ml transferrin and 5 µg/ml selenium. Grade A, B and C immature oocytes were transferred into each droplet (10 COCs per droplet) and incubated in a CO₂ incubator maintained in 5 per cent CO₂, 38.5°C with 90-95 per cent relative humidity for 24h. After 24h of incubation in the maturation media, the degrees of cumulus expansion of the oocytes were ascertained under zoom stereo microscope and the in vitro maturation was assessed based on cumulus cell expansion (Figure 1). Oocytes with full cumulus cell expansion (degree 2) and moderate cumulus expansion (degree 1) were considered as matured and oocytes with slight or no expansion of cumulus cell mass (degree 0) were considered as not matured (Kobayashi et al., 1994).

**In vitro fertilization**

Frozen semen from single bull was used for the entire study. For each trial, four frozen semen straws (0.25 ml French mini) were thawed in a water bath containing water at 37°C for 30 seconds. The straws were removed from water bath, wiped with 70 per cent alcohol and the contents were collected in a sterile glass test tube. The progressively motile sperms for in vitro fertilization were separated by swim up technique as described by Parrish et al., (1986). In brief, the contents of semen straws were diluted with 5 ml of pre-equilibrated sperm TALP (SpTALP) (Totey et al., 1993) by centrifugation at 350 g for 5 min. at room temperature. The supernatant was removed and fresh SpTALP was added and the above procedure was repeated twice. Finally 100 µl of the sperm pellet was layered under 1 ml of SpTALP medium in three sugar tubes and incubated for swim up in CO₂ incubator maintained at 5 per cent CO₂, 38.5°C with 90-95 per cent relative humidity for one hour. At the end of incubation the superficial layer of 0.5 ml of the medium containing the motile fraction was removed from each tube and pooled in a 15 ml centrifuge tube, and washed with 5 ml SpTALP by centrifugation at 350 g for 5 min.
at room temperature. Concentration of the final sperm pellet was determined with a haemocytometer and the sample was diluted with SpTALP to yield a concentration of 1-2 ×10^6 sperms/ml.

After 24h of maturation the degree 2 and degree 1 oocytes were washed thrice in oocyte collection media to remove any extraneous surrounding cumulus layers, followed by final washing in IVF TALP medium. Then about 8-10 matured oocytes were placed in each IVF droplets and incubated to 18-20h in CO₂ incubator.

**In vitro culture**

The cumulus cells from the fertilized oocytes were removed by manual repeated pipetting. The presumptive zygotes were washed three times in in vitro culture medium to remove the spermatozoa, cellular debris and chemical residues and transferred randomly into pre-equilibrated 50µl IVC droplets (10-15 presumptive zygotes/droplet). In each trial one batch of presumptive zygotes were cultured in two step SOF media and another batch in single step KSOM at 38.5°C in 5 per cent CO₂ in air. In two step SOF media, the presumptive zygotes were cultured in early-stage medium (SOF supplemented with 0.5mM glucose) for the 72h and then transferred to later-stage media (SOF without glucose). In the single step KSOM, the presumptive zygotes were cultured in pre-equilibrated KSOM. The cleavage rate and developmental competence of early embryos to morula/blasto cyst (Figure 2 and 3) were assessed once in 24 hours. The collected data were analyzed statistically.

**Results and Discussion**

In the present study, a total of 1110 oocytes were retrieved by OPU. The average oocyte yield was 8.34 ± 0.23 per OPU session per animal. The average yield (Mean ± SE) of A, B and C grade oocytes were 3.49 ± 0.11, 2.51 ± 0.17 and 1.21 ± 0.06 per OPU session per animal, respectively giving a total of 7.21 ± 0.20 usable oocytes per OPU session per animal. The yield of D grade oocytes per OPU session per animal was 0.53 ± 0.45.

In accordance to this study, Looney et al., (1994) and Karadjole et al., (2010) collected an average of 6.3 and 6.5 oocytes, respectively per aspiration in cows. When compared to this study, Garcia and Salaheddine (1998) and Manik et al., (2003) obtained a lower recovery rate of 5.6±1.18 and 4.0±0.5 oocytes in Holstein Friesian and Karan Fries cows, respectively. Merton et al., (2003) reported that oocyte recovery rate through trans-vaginal follicular aspiration has been variable among laboratories and experience of the operator and his team. Oliveira et al., (2016) reported that genetics of the donor, vacuum pressure while performing aspiration, type of needle used for OPU, and number of follicles present on the ovary was some of the factors affecting the recovery rate of oocytes. These factors may influence the quality of oocytes collected, success of in vitro maturation, in vitro fertilization and subsequent in vitro embryo development.

In order to obtain more oocytes from the donor, it was necessary to have more follicles on the cow ovary (Ward et al., 2000). Gimenes et al., (2015) reported that the number of follicles on the ovary was influenced by breed of cattle, nutritional status of cow and climatic conditions. Wolfenson et al., (2000) reported that the temperature played a key role on bovine follicle formation and development, oocyte quality and embryo development and they also stated that heat stress suppressed follicular dominance, resulting in a number of changes in follicular growth. The number
oocytes matured and maturation rate obtained in the present study is presented in table 1. In vitro maturation of different grades of COCs was carried out in TCM 199 media supplemented with 10 per cent FBS, 1 µg/ml Folltropin (Bioniche, Canada), 0.02 IU/ml LH, 1 µg/ml estradiol with addition of 100 µM cysteamine, 10 ng/ml EGF, 100 ng/ml IGF, 10 µg/ml insulin, 5.5 µg/ml transferrin and 5 µg/ml selenium. TCM 199 is a standard medium for in vitro maturation of bovine oocytes either with serum supplementation (Do et al., 2016) or serum replacer (Moore et al., 2007). Only A, B, C grade bovine oocytes were utilized for in vitro maturation and maturation rate was assessed based on the cumulus expansion. Out of 1034 oocytes cultured, 523 showed degree 2 cumulus expansion with a mean maturation rate of 50.57 ± 1.04 per cent, 421 showed degree 1 cumulus expansion with a mean maturation rate of 40.74 ± 1.17 per cent and 90 showed degree 0 cumulus expansion with a mean maturation rate of 8.70 ± 0.98 per cent. The overall maturation rate obtained in the present study was 91.30± 1.27 per cent (944 oocytes matured out of 1034 oocytes used). In accordance to this study, Lonergon et al., (1996) reported 91 per cent of maturation rate in bovine oocytes cultured in vitro with EGF (10 ng/ml) supplemented media. The overall oocyte maturation rate observed in the present study in TCM199 media was higher than the maturation rates reported by other researchers viz., 70.20 per cent (Lonergon et al., 1994) and 75.53 per cent (Pontes et al., 2011) from OPU derived COCs.

The higher maturation rate obtained in this study might be due to supplementation of in vitro maturation medium with EGF and IGF which showed positive influence on cumulus expansion (Kelly et al., 2008). The developmental rate of bovine presumptive zygotes cultured under SOF media, 344 cleaved into 2 cell stage with a cleavage rate of 73.04 ± 1.50 per cent. In KSOM, 473 presumptive zygotes were cultured and 374 were cleaved with a cleavage rate of 79.01 ± 1.34 per cent. Statistical analysis revealed a significantly higher (P<0.05) cleavage rate in KSOM when compared to SOF media. In contrast to this study, Zicarelli et al., (2003) reported that there was no significant difference between SOF and KSOM with regard to cleavage rate in buffaloes. Kim et al., (2014) reported a lower cleavage rate of 65 and 41 per cent in KSOM and SOF media, respectively when bovine embryos cultured in vitro. When compared to this study, the cleavage rate of 81 and 82 per cent was reported by Reis et al., (2002) and Rizos et al., (2002) in SOF media and 89 per cent by Felmer et al., (2011) in KSOM. The percentage of embryos (mean ± SE) that progressed to 4 cell stage was significantly (P<0.05) higher in KSOM (60.69 ± 1.89) when compared to SOF (54.88 ± 1.76). The mean percentage of embryos that developed to 8 cell stage was 35.89 ± 1.57 and 44.67 ± 1.62 in embryos cultured under SOF media and KSOM, respectively. The progression to 8 cell stage was significantly (P<0.05) higher in KSOM than in SOF media. The percentage of embryos (mean ± SE) that progressed to morula stage was 19.18 ± 1.10 and 25.36 ± 1.37 per cent in embryos cultured under SOF media and KSOM, respectively. The progression to morula stage was significantly (P<0.05) higher in embryos cultured in KSOM than in SOF media. Kim et al., (2014) reported 41 and 30 per cent morula development in KSOM and SOF media, respectively when bovine embryos cultured in vitro which was higher than the results obtained in this study. Nedambale et al., (2004) obtained morula percentage of 38 in KSOM and 40 in SOF media.
Table 1 No. of oocytes matured and maturation rate (per cent) of bovine oocytes cultured in TCM 199 medium

<table>
<thead>
<tr>
<th>No. of oocytes used for maturation</th>
<th>No. of oocytes matured (per cent)</th>
<th>Total No. of oocytes matured (per cent)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Full cumulus cell expansion (Degree 2)</td>
<td>Moderate cumulus cell expansion (Degree 1)</td>
</tr>
<tr>
<td>1034</td>
<td>523 (50.57±1.04)</td>
<td>421 (40.74±1.17)</td>
</tr>
</tbody>
</table>

Table 2 Developmental rate of bovine oocytes in SOF media and KSOM

<table>
<thead>
<tr>
<th>Culture media</th>
<th>No. of presumptive zygotes cultured</th>
<th>Percentage of presumptive zygotes developed to</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2 cell</td>
</tr>
<tr>
<td>SOF</td>
<td>471</td>
<td>73.04±1.50&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>KSOM</td>
<td>473</td>
<td>79.01±1.34&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values bearing different superscripts (a, b) within the same column differ significantly (p< 0.05)

Figure 1 Matured oocytes showing cumulus cell expansion

Figure 2 cell embryo
Figure. 3 Morula stage embryo

Tervit et al., (1972) had first described the successful in vitro culture eight-cell cattle embryos to morula or blastocyst stages in SOF media. This medium was based on the biochemical composition of sheep oviductal fluid. Mouse embryos cultured in KSOM were closer to in vivo embryos in terms of gene expression profiling (Doherty et al., 2000). Felmer et al., (2011) observe higher blastocyst formation rate (32 per cent) in KSOM supplemented with BSA. The composition of SOF and KSOM differs slightly. The KSOM had glutamine whereas SOF did not have it. Glutamine has been reported to have an important role in embryo metabolism and blastocyst formation. Summers (2013) reported that glutamine gives an advantage to KSOM.

Since KSOM contained EDTA and a lower concentration of sodium chloride (95 mM) compared to mSOF (EDTA-free and 105 mM sodium chloride), KSOM as basal medium was hypothesized to support better pre-implantation development of bovine embryos than SOF media (Bhuiyan et al., 2004).

The higher cleavage rate and embryo development obtained in the present study also indicated the superiority of the KSOM than the SOF media in the in vitro bovine embryo production as described by Bhuiyan et al., (2004) and Summers (2013).

Ovum pick-up technology can be used effectively to obtain cattle oocytes in large scale.

In vitro embryo production is an efficient procedure for producing embryos from post-puberty heifers and from adult cattle in the laboratory through ovum pick-up and subsequent maturation, fertilization and culture in vitro.

The developmental competence of presumptive zygotes is affected by media composition.

The cleavage rate and morula development was higher in KSOM than SOF media.

The KSOM is better due to its composition in terms of presence of EDTA, glutamine and lactic acid in triple concentration when compared to SOF media.

References


Reis, A., M. E. Staines, R. G. Watt, D. F. Dolman and McEvoy, T.G. 2002. Embryo production using defined oocyte maturation and zygote culture media following repeated ovum pick-up (OPU) from FSH-stimulated simmental

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