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The Effect of Culture Methods and Serum Supplementation on Developmental Competence of Bovine Embryos Cultured *In vitro*

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Abstract: The objective of this study, was to compare the developmental competence of bovine *in vitro* fertilized embryos in three different culture methods; microdrop method (50 μL of medium under mineral oil in petri dishes) compared to tube methods (1 mL of medium in tubes) with or without oil overlay and to examine the influence of Fetal Bovine Serum (FBS) in culture methods. They were randomly allocated to one of following culture conditions: (Group 1) microdrop method supplemented with 10% (v:v) FBS at day 5 post-insemination (pi); (Group 2) microdrop method without FBS; (Group 3) tube method with oil overlay and FBS added at day 5 pi; (Group 4) tube method with oil overlay; (Group 5) tube method without oil overlay but FBS added at day 5 pi; (Group 6) tube method without oil overlay and FBS. There were no differences ($p>0.05$) in cleavage rates among the culture methods (78.3, 75.6 and 74.9% for microdrop, tube with oil overlay and tube method without overlay, respectively). However, regardless of serum addition, blastocyst rates in the microdrop method (30.5%) were significantly higher ($p<0.05$) than those in the tube method without oil overlay (9.8%). There was no difference ($p>0.05$) between with or without serum in blastocyst rate regardless culture methods. Numerically, the highest blastocyst rate was observed in the microdrop method with the FBS supplementation (Group 1; 36.6%). Overall, the microdrop method was the optimum culture method among the culture methods; however, serum supplementation did not significantly affect the blatocyst rate.

Key words: Bovine preimplantation embryos, culture methods, serum supplementation

INTRODUCTION

Bovine embryo *in vitro* Production (IVP) has become an important tool for basic science research and for applications in animal biotechnologies (Lohuis, 1995; Galli *et al*., 2003). In support of this, a diverse array of *in vitro* culture methods (e.g., microdrop, tube and four well methods) have been developed and studied among different laboratories (Rombauts and Wood, 2000). In these component based IVP systems, bovine preimplantation embryos have been cultured from the zygote to blastocyst stages in various volumes of culture medium, and in the presence or absence of oil overlay with different culture methods and serum has been frequently added to culture medium as a protein source for *in vitro* Culture (IVC) (Bavister, 1995). Since, Brinster first developed the microdrop culture method (Brinster, 1963), this culture method has become the most commonly used culture method among IVF laboratories (Boatman, 1987; Nagy *et al*., 2003). The microdrop method is achieved by culturing a group of embryos in a small volume (10-100 μL) of culture medium covered with liquid oil (e.g. mineral, paraffin, or silicon oil) that prevents the microdrop of medium from rapid evaporation and controls the pH changes of the culture medium (Brinster, 1968; Miller *et al*., 1994b; Tae *et al*., 2006). Nevertheless, some studies have shown that oil overlay of culture medium results in interactions with steroid hormones such as oestradiol and progesterone present in the medium causing alterations in medium composition (Miller and Pursel, 1987; Shimada *et al*., 2002). Additionally, some batches or lots of oil in this method can be a source of toxic contaminants in embryo culture (Erbach *et al*., 1995; Provo and Herr, 1998; Van Soom *et al*., 2001; Otsuki *et al*., 2007). In order to avoid oil use, a group of embryos could be cultured in a relatively large volume (1-3 mL) of culture medium in either culture tubes or 4-well dishes. Culturing embryos in a large volume of medium, however, potentially has an adverse effect. It may dilute embryo-derived growth factors which may have beneficial effects on their development (Canseco *et al*., 1992; O'Neill, 1998).

Additionally, serum supplementation of the culture medium during the later stage (morulae or blastocyst stage) of embryo development is known to increase

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blastocyst and hatching rates and cell numbers of bovine embryos (Pinyopummintr and Bavister, 1991; Lim *et al*., 1994; Van Langendonckt *et al*., 1997; Yoshioka *et al*., 1997). However, the composition of serum is highly variable from different suppliers and even between batches or lots (Maurer, 1992). Moreover, some batches of serum contain non-defined molecules that could be detrimental to embryo development (Bavister, 1995).

These components of *in vitro* culture systems used for IVP influence the developmental competence of mammalian embryos (Bavister, 1995; Galli *et al*., 2003; Gardner and Lane, 2004). For this reason, success rates of the embryo culture vary among different protocols and the efficiency of culture methods also differs between laboratories and studies. Furthermore, a recent review by Gardner (2008) emphasized the necessity of prescreening batches and lots of serum in order to optimize the efficacy of culture systems. It is therefore, important to examine these components and culture methods affecting the development of *in vitro* derived embryos when applying routine IVP to new studies. In this study, we designed a simple and comparative experiment evaluating different culture methods and effects of serum on bovine embryo development. The developmental competence of bovine embryos were compared between the microdrop method with 50 μL of culture medium covered with mineral oil and the tube methods with 1 mL culture medium (with or without mineral oil overlay), which contained an equivalent number of embryos and to examine the influence of serum supplementation on embryos in *in vitro* culture.

MATERIALS AND METHODS

All the chemicals utilized in the present investigation were purchased from Sigma Chemicals (St. Louis, MO) unless otherwise indicated. Bovine Cumulus-Oocyte Complexes (COCs) were purchased from a commercial supplier (BOMED, INC., Madison, WI). Each week, approximately 600 bovine oocytes were shipped in 2 mL of the maturation medium in tightly closed tubes and were over-night shipped in a 39°C portable incubator. On arrival at the laboratory, COCs were removed from the shipping unit and were incubated at 39°C and 5% CO_2 in air for 24 h.

Sperm preparation and *In Vitro* Fertilization (IVF): The sperm preparation for IVF used the BoviPure™ gradient and was performed according to the protocol provided by the manufacturer as follows (Nidacon International AB, Gothenburg, Sweden). All the media used for sperm preparation were warmed at 39°C before use. In a 15 mL conical centrifuge tube, 2 mL of BoviPure™ Bottom Layer was placed and then carefully layered with 2 mL of BoviPure™ Top Layer on the top of the BoviPure™ Bottom Layer to make a gradient. Two straws of frozen semen from a single Angus bull were thawed in a 37°C water bath for 120 sec. The thawed semen were gently mixed with BoviPure™ Extender and brought to a 2 mL volume. The 2 mL of the prepared semen was then gently layered on top of the BoviPure™ gradient and centrifuged for 20 min at 500 × g. After the centrifugation, the fluid above the sperm pellet was carefully removed with a sterile Pasteur pipette. The pellet was resuspended with 5 mL of BoviPure™ Wash and centrifuged for 5 min at 300 × g. This pellet was resuspended again in 5 mL of warmed IVF-TL (Tyrode's Albumine Lactate Pyruvate) medium (Millipore, Billerica, MA) and centrifuged for 5 min at 300 × g. IVF-TL medium was supplemented with pyruvate (0.2 mm), fatty-acid-free Bovine Serum Albumin (BSA-FAF, 6 mg mL^{-1}), penicillin (100 U mL^{-1}), streptomycin (100 μg mL^{-1}), heparine 10 μg mL^{-1}, hypotaurine 0.5 μg mL and epinephrine 0.5 μg mL^{-1} (Miller *et al*., 1994a). The number of sperm was counted using a hemocytometer and the sperm pellet was diluted to a concentration of 50×10^6 spermatozoa mL^{-1}. To make the 2×10^6 final concentration, 2 μL of diluted sperm were added to each 50 μL drop of the fertilization medium (IVF-TL) containing 10 matured oocytes that had been washed previously in the fertilization medium (IVF-TL). Sperm and oocytes were co-incubated at 39°C and with 5% CO_2 in air for 18 h.

***In vitro* culture:** Following fertilization, presumptive zygotes were denuded from cumulus cells by vortexing for 2 min and washed 3 times with HEPES-TL wash medium (Bioniche, Pullman, WA). They were cultured in Synthetic Oviductal Fluid (SOF) culture medium (Millipore, Billerica, MA) supplemented with pyruvate (0.4 mm), 100 × MEM (20 μ mL^{-1}), 50 × BME (10 μL mL^{-1}), BSA-FAF (8 mg mL^{-1}), penicillin (100 U mL^{-1}) and streptomycin (100 μg mL^{-1}) at 39°C and with 5% CO_2 in air (Edwards *et al*., 1997). All the culture medium was equilibrated at 39°C and with 5% CO_2 in air overnight either in the 35×10 mm sterile polystyrene disposable Petri dishes (Fisher Scientific, Pittsburgh, PA) or 12×75 mm sterile polystyrene disposable tissue culture tubes (Fisher Scientific, Pittsburgh, PA) before use. The sterile mineral oil (Cat. No. M5310; Sigma) was used for the culture and it was stored in the dark to avoid the production of embryo-toxic compounds (Provo and Herr, 1998). The 10% (v:v) of FBS (Fetal Bovine Serum, Gibco/Invitrogen, Carlsbad, CA) was added to selected treatment groups in the culture medium at day 5 pi (post insemination) in the

culture (Lim *et al*., 1994). All the FBS and mineral oil that was used in the present study were from one batch (lot).

Experimental designs: To evaluate the effects of culture methods and serum supplementation in bovine *in vitro* culture, three different culture methods (microdrop method, tube method with oil overlay and tube method without oil overlay) and with and without serum supplementation were used in a 3×2 factorial experiment. A total of 1,696 presumptive zygotes were randomly distributed to the following six groups. A group of approximately 50-70 presumptive zygotes were cultured in Group 1: microdrop method (50 μL microdrops of culture medium covered with mineral oil in the petri dish) and with serum supplementation (10% (v:v) FBS added at day 5 pi); Group 2: microdrop method without serum supplementation; Group 3: tube method (1 mL of culture medium in the test tube) with mineral oil and serum supplementation; Group 4: tube method with oil overlay but no FBS supplementation; Group 5: test tube method without oil overlay and FBS added at day 5 pi; Group 6: tube method without oil overlay and no serum supplementation. The cleavage rate of the embryos was assessed and the culture medium in all groups was replaced with the fresh culture medium at day 2 pi. The blastocyst development was assessed at day 9 pi.

Statistical analysis: Experiments were repeated three times and data were analyzed by two-way ANOVA (culture methods and serum supplementation and their interation). All percentage data were subjected to arcsine transformation before statistical analysis. Data are expressed as mean±SEM. A probability of $p<0.05$ was considered to be statistically significant. When ANOVA revealed a significant effect, values were compared by Fisher's PLSD post hoc test. All the analysis was conducted with the STATVIEW program (Abacus Concepts, Inc., Berkeley, CA). The percentages of blastocysts at day 9 pi were based on the total number of cleaved embryos determined at day 2 pi.

RESULTS AND DISCUSSION

The effects of the bovine embryo different culture methods (microdrop, tube with oil overlay and tube without oil overlay) and serum supplementation on the proportions of the cleaved embryos and blastocysts are shown in Table 1. While there were no significant differences ($p>0.05$) culture methods and serum supplementation interaction and serum supplementation in cleavage and blastocyst rates, means of bovine embryo developmental rate were averaged only in each culture methods for pair wise comparisons (Table 2). Regardless of serum supplementation, there were no significant differences ($p>0.05$) among culture methods in cleavage rates at day 2 pi (78.3±3.6, 75.6±2.5 and 74.9±2.1% for microdrop, tube-oil and tube methods, respectively). However, the microdrop method was superior ($p<0.05$) to the tube method without oil overlay for blastocyst formation (30.5±5.4 and 9.8±3.1%, respectively). There was also a trend ($p<0.10$) of increasing blastocyst rates when embryos were cultured in the microdrop method (30.5±5.4%) compared to the tube method with oil overlay (18.7±3.0). Additionally, the blastocyst rates of the embryos cultured in the tube method with oil overlay (18.7±3.0) tended to be higher ($p<0.10$) than those cultured in the tube method without oil overlay (9.8±3.1%). The Table 1 shows that the supplementation of the culture medium with 10% (v:v) serum at day 5 pi had no significant ($p>0.05$) effect on the blastocyst rates regardless of the culture methods. Numerically, the highest development rate of blastocysts was observed in the microdrop method with the serum supplementation (36.6±8.5%) and culturing embryos in the tube method without oil overlay and serum supplementation (6.3±3.5%) exhibited the lowest blastocyst rate (Table 1).

The current study, was conducted to evaluate bovine embryo culture conditions as affected by culture methods and serum supplementation. The microdrop and tube methods are generally used among IVF laboratories (Rombauts and Wood, 2000; Nagy *et al*., 2003). Major differences of these methods are the volume of culture medium and use of oil overlay. The microdrop with oil is the most commonly used embryo culture system because progress of embryo development is easily observed under the microscope and a small volume of culture medium appears to be a beneficial factor for embryo development. Many studies reported that the rates of blastocyst formation and cell numbers were increased when embryos were cultured in the microdrop method where a group of embryo is cultured in a small volume of medium in the mouse (Canseco *et al*., 1992; Gardner *et al*., 1994; Salahuddin *et al*., 1995; Gardner, 2008), sheep (Gardner and Lane, 2004) and bovine (Keefer *et al*., 1994; Palasz and Thundathil, 1998; de Oliveira *et al*., 2005). Paria and Dey (1990) interpreted that embryos secrete growth factors that may act in an autocrine or paracrine manner. Thus, the effect of culturing a group of embryos using the microdrop method is likely to be due to the minimization of the diffusion of growth factors secreted from the embryo themselves. The possible evidence of this effect has been demonstrated by culturing bovine embryos in conditioned medium derived from a roup of other embryos, which enhances the blastocyst formation

Table 1: Effect of culture methods and serum supplementation on bovine embryo developmental rates

Experimental group (culture method-serum supplementation)	Replicates	No. of presumptive zygotes	Cleavage rates (Mean±SEM)	Blastocyst rates[4] (Mean±SEM)
Microdrop[1]-serum[2]	3	295	78.7±4.9	36.6±8.5
Microdrop-no serum	3	266	77.8±6.2	24.3±5.9
Tube[3] oil-serum	3	286	73.2±2.4	22.2±3.5
Tube oil-no serum	3	273	78.1±4.5	15.2±4.6
Tube-serum	3	280	75.0±0.5	13.2±5.0
Tube-no serum	3	296	74.8±4.7	6.3±3.5
p-value (two-way ANOVA)				
Culture method (Microdrop vs. Tube)			$p>0.05$	$p<0.01$
Serum supplementation (serum vs. no serum)			$p>0.05$	$p>0.05$
Culture method×serum supplementation			$p>0.05$	$p>0.05$

[1]Microdrop method: 50 μL of culture medium in petri dishes. [2]Serum: 10% (v:v) fetal bovine serum was added at day 5 post insemination. [3]Tube method: 1 mL of culture medium in test tubes. [4]Number of total cleaved embryos developing to blastocyst (%)

Table 2: Effect of culture methods on bovine embryo developmental rates

Culture methods	Replicates	No. oocytes	Cleavage rates (Mean±SEM)	Blastocyst rates[3] (Mean±SEM)
Microdrop[1]	3	561	78.3±3.6	30.5±5.4[a]
Tube[2] oil	3	559	75.6±2.5	18.7±3.0[b]
Tube	3	576	74.9±2.1	9.8±3.1[c]

[1]Microdrop method: 50 μL of culture medium in petri dishes. [2]Tube method: 1 mL of culture medium in test tubes. [3]Number of total cleaved embryos developing to blastocyst. Superscripts differ: [a,b](p = 0.08); [a,c](p = 0.001); and [b,c](p = 0.07)

rate of single cultured embryos (Fujita *et al*., 2006). Another assumption is that increasing embryo density (ratio of embryos to volume of culture medium) may deplete embryo-toxic substances in the medium or reduce the concentrations of naturally occurring inhibitors like glucose or ammonium (Bavister, 1995). Similar to these previous reports, the present results show that regardless of FBS supplementation, the microdrop method was superior to the tube method with the large volume of medium without oil overlay for blastocyst formation (Table 2). In addition, there was a trend of increasing blastocyst rates when embryos were cultured in the microdrop method compared to tube method with oil overlay. Fukui *et al*. (1996) similarly reported that the microdrop method resulted in a larger proportion of blastocyst formation than the 4 well method, where 500 μL of medium is covered with the oil in the well dish. Moreover, their study has shown that the renewal of culture medium at 48 h intervals in the microdrop lowered the rate of bovine blastocyst formation compared with non-renewal of medium. Thus, the large volume of culture medium and renewal of culture medium causes a decrease in the blastocyst rates presumably by diluting and removing embryo-derived growth factors from the culture (Canseco *et al*., 1992).

In the present study shown in Table 2, the blastocyst rates of the embryos cultured in the large volume of medium with oil overlay tended to be higher than those cultured in the large volume of medium without oil overlay. The primary function of using oil overlay in the embryo culture is that it prevents liquid evaporation which facilitates the maintenance of the appropriate pH and osmotic pressure of culture medium (Brinster, 1968; Boatman, 1987; Miller *et al*., 1994b; Gardner and Lane, 2000; Tae *et al*., 2006). Culturing embryos without the oil overlay can lead to low blastocyst formation even though they were cultured in a high humidity environment. Nevertheless, there were no significant differences in the cleavage rate at day 2 pi between the tube method with and without oil overlay groups (Table 2). Thus, the components in the media may have been concentrated during the 9 days of culture resulting in increased osmolarity. Also, Tae *et al.* (2006) have shown that sterilized-good quality oil absorbs the accumulated toxic components in the medium and resulted in an increased rate of development to the blastocyst formation. Nevertheless, other investigators have shown that unidentified toxic components from some particular batches of oil could be transferred into culture medium (Miller *et al*., 1994b; Erbach *et al*., 1995; Provo and Herr, 1998; Otsuki *et al*., 2007), or oil could absorb substances present in the medium such as oestradiol and progesterone (Miller and Pursel, 1987; Shimada *et al*., 2002). Thus, oil overlay could cause the alteration of medium composition. In this experiment, sterile-filtered, mouse embryo tested and light mineral oil (M5310; Sigma) was used for the embryo culture and apparently the mineral oil did not have either significant inhibitory or stimulatory effects on bovine embryo development in the present experiment.

We also investigated, the effects of serum supplementation in the culture medium on the developmental ability of the bovine embryos produced *in vitro*. The supplementation of the culture medium with FBS had no significant effect on the blastocyst rates. Serum contains a variety of known and unknown substances which may stimulate or inhibit embryo development *in vitro* (Maurer, 1992; Bavister, 1995). Studies have shown that serum supplementation during the early cleavage stages of culture can inhibit the first cleavage but enhances the formation of morulae and

blastocyst when serum is added during the later stage of embryo development (Pinyopummintr and Bavister, 1991; Lim *et al*., 1994). Many investigators have reported the improved embryo development with serum-supplemented media (Pinyopummintr and Bavister, 1991; Yoshioka *et al*., 1997; Khurana and Niemann, 2000; Kim *et al*., 2004); yet Caro and Trounson (1984) did not find any improvement of the embryo development with the serum-supplemented medium vs. non-supplemented medium in the mouse. Serum is a pathological fluid formed by blood clotting, which may induce chemical alterations with possible harmful effects for embryo culture (Maurer, 1992). It has been reported that some batches of serum decrease the blastocyst rate and cell number and increase apoptotic cells on a particular culture system (Van Langendonckt *et al*., 1997). Fukui *et al*. (1991) reported that even the effectiveness of serum supplementation in the culture medium varies by different components of culture conditions used *in vitro*. Under these experimental conditions, supplementation with FBS, which was supplemented in either the microdrop or the tube methods, did not statistically improve the rate of blastocyst formation; although embryos derived from culture medium with FBS supplementation exhibited a numerical increase in apparent developmental competence (Table 1).

CONCLUSION

Numerically, the highest blastocyst rates were obtained by the microdrop method with FBS supplementation and overall, the microdrop method was the optimum culture system among the culture methods (microdrop, tube with oil overlay, tube methods). However, FBS supplementation did not significantly affect bovine embryo development *in vitro*. In the present study, we examined the interactions among culture methods with a constant number of embryos with presence or absence of oil overlay and FBS supplementation in bovine embryo culture. While there are many studies demonstrating positive or negative effects of these components, comparing studies and protocols has been a difficult process and it is necessary to examine exact culture methods and batches and lots of serum to be used for *in vitro* culture at the same time as has been described here relative to their impacts on bovine *in vitro* embryo development.

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REFERENCES

Bavister, B.D., 1995. Culture of preimplantation embryos: facts and artifacts. Hum. Reprod. Update, 1: 91-148. DOI: 10.1093/humupd/1.2.91. PMID: 15726768. http://humupd.oxfordjournals.org/cgi/content/abstract/1/2/91.

Boatman, D.E., 1987. *In vitro* Growth of Non-Human Primate Pre- and Peri-Implantation Embryos. In: Bavister, B.D. (Eds.). The Mammalian Preimplantation Embryo: Regulation of Growth and Differentiation *In vitro*. Plenum Press, New York, pp: 273-303. ISBN: 03 06425955.

Brinster, R.L., 1963. A method for *in vitro* cultivation of mouse ova from two-cell to blastocyst. Exp. Cell Res., 32: 205-208. DOI: 10.1016/0014-4827(63)90093-4. PMID: 14094038. http://www.sciencedirect.com/science?_ob=ArticleURL&_udi=B6WFC-4DXT4CN25&_user=1512607&_rdoc=1&_fmt=&_orig=search&_sort=d&view=c&_acct=C000053407&_version=1&_urlVersion=0&_userid=1512607&md5=910c1d16b1e7329df35fc099569f42ef.

Brinster, R.L., 1968. *In vitro* culture of mammalian embryos. J. Anim. Sci., 27 (Suppl 1): 1-14. PMID: 5212490. http://jas.fass.org/cgi/content/abstract/27/Supplement_1/1.

Canseco, R.S., A.E. Sparks, R.E. Pearson and F.C. Gwazdauskas, 1992. Embryo density and medium volume effects on early murine embryo development. J. Assist. Reprod. Genet., 9: 454-457. DOI: 10.1007/BF01204051. PMID: 1482840. http://www.springerlink.com/content/t7u782tr51148884.

Caro, C.M. and A. Trounson, 1984. The effect of protein on preimplantation mouse embryo development *in vitro*. J. *in vitro* Fert. Embryo. Transf., 1: 183-187. DOI: 10.1007/BF01139212. PubMed: 6336099. http://www.springerlink.com/content/l8337057013261n2.

de Oliveira, A.T., R.F. Lopes and J.L. Rodrigues, 2005. Gene expression and developmental competence of bovine embryos produced *in vitro* under varying embryo density conditions. Theriogenol., 64: 1559-1572. DOI: 10.1016/j.theriogenology.2005.03.019. PMID: 15878193. http://www.sciencedirect.com/science?_ob=ArticleURL&_udi=B6TCM-4G3CWMY-1&_user=1512607&_rdoc=1&_fmt=&_orig=search&_sort=d&view=c&_version=1&_urlVersion=0&_userid=1512607&md5=7d2647669abe26872081946769b9c49b.

Edwards, L.J., P.A. Batt, F. Gandolfi and D.K. Gardner, 1997. Modifications made to culture medium by bovine oviduct epithelial cells: Changes to carbohydrates stimulate bovine embryo development. Mol. Reprod. Dev., 46: 146-154. DOI: 10.1002/(SICI)1098-2795(199702)46:2<146::AID-MRD5>3.0.CO;2-Q. PMID: 9021746 http://www3.interscience.wiley.com/journal/54649/abstract?CRETRY=1&SRETRY=0.

Erbach, G.T., P. Bhatnagar, J.M. Baltz and J.D. Biggers, 1995. Zinc is a possible toxic contaminant of silicone oil in microdrop cultures of preimplantation mouse embryos. Hum. Reprod., 10: 3248-3254. PMID: 8822453. http://humrep.oxfordjournals.org/cgi/content/abstract/10/12/3248.

Fujita, T., H. Umeki, H. Shimura, K. Kugumiya and K. Shiga, 2006. Effect of group culture and embryo-culture conditioned medium on development of bovine embryos. J. Reprod. Dev., 52: 137-142. DOI: 10.1262/jrd.16084. PMID: 16293943. http://www.jstage.jst.go.jp/article/jrd/52/1/52_137/_article.

Fukui, Y., E.S. Lee and N. Araki, 1996. Effect of medium renewal during culture in two different culture systems on development to blastocysts from *in vitro* produced early bovine embryos. J. Anim. Sci., 74: 2752-2758. PMID: 8923190. http://jas.fass.org/cgi/reprint/74/11/2752.

Fukui, Y., L.T. McGowan, R.W. James, P.A. Pugh and H.R. Tervit, 1991. Factors affecting the *in vitro* development to blastocysts of bovine oocytes matured and fertilized *in vitro*. J. Reprod. Fertil., 92: 125-131. PMID: 1905351.

Galli, C., R. Duchi, G. Crotti, P. Turini, N. Ponderato, S. Colleoni, I. Lagutina and G. Lazzari, 2003. Bovine embryo technologies. Theriogenol., 59: 599-616. DOI: 10.1016/S0093-691X(02)01243-8. PMID: 12499007 http://www.sciencedirect.com/science?_ob =ArticleURL&_udi=B6TCM-4783PWR-9&_user=1512607&_rdoc=1&_fmt=&_orig=search&_sort=d&view=c&_version=1&_urlVersion=0&_userid=1512607&md5=2fe928866f24c4bb6c5a52a519801d61.

Gardner, D.K., 2008. Dissection of culture media for embryos: The most important and less important components and characteristics. Reprod. Fertil. Dev., 20: 9-18. DOI: 10.1071/RD07160. PMID: 18154693. http://www.publish.csiro.au/nid/44/paper/RD07160.htm.

Gardner, D.K. and M. Lane, 2000. Embryo Culture Systems. In: Trounson, A.O. and D.K. Gardner (Eds.). In handbook of *in vitro* fertilization. CRC Press, Boca. Raton., pp: 205-264. ISBN: 9780849340024.

Gardner, D.K. and M. Lane, 2004. Culture of the Mammalian Preimplantation Embryo. In: Gardner, D.K., M. Lane and A.J. Watson (Eds.). A Laboratory Guide to the Mammalian Embryo. Oxford University Press, New York, pp: 41-61. ISBN: 0195142268. http://www.oup.com/us/catalog/general/subject/LifeSciences/MolecularCellBiology/DevelopmentalBiology/?view=usa&ci=9780195142266.

Gardner, D.K., M. Lane, A. Spitzer and P.A. Batt, 1994. Enhanced rates of cleavage and development for sheep zygotes cultured to the blastocyst stage *in vitro* in the absence of serum and somatic cells: Amino acids, vitamins and culturing embryos in groups stimulate development. Biol. Reprod., 50: 390-400. PMID: 8142556. http://www.biolreprod.org/cgi/content/abstract/50/2/390.

Keefer, C.L., S.L. Stice, A.M. Paprocki and P. Golueke, 1994. *In vitro* culture of bovine IVM-IVF embryos: Cooperative interaction among embryos and the role of growth factors. Theriogenol., 41: 1323-1331. DOI: 10.1016/0093-691X(94)90491-Z. PMID: 16727487 http: //www.sciencedirect.com/science?_ob=ArticleURL&_udi=B6TCM-49S8492-J&_user=1512607&_rdoc =1&_fmt=&_orig=search&_sort=d&view=c&_version=1&_urlVersion=0&_userid=1512607&md5=2bd5a05b636c475c6a73885c7c6e65e3.

Khurana, N.K. and H. Niemann, 2000. Effects of oocyte quality, oxygen tension, embryo density, cumulus cells and energy substrates on cleavage and morula/blastocyst formation of bovine embryos. Theriogenol., 54: 741-756. DOI: 10.1016/S0093-691X(00)00387-3. PMID: 11101035. http://www.sciencedirect.com/science?_ob=ArticleURL&_udi=B6TCM-42MGB94-8&_user=1512607&_rdoc=1&_fmt=&_orig=search&_sort=d&view=c&_version=1&_urlVersion=0&_userid=1512607&md5=5722f1fa80925055d760fb95dc3d66d2.

Kim, H.S., G.S. Lee, S.H. Hyun, S.H. Lee, D.H. Nam, Y.W. Jeong, S. Kim, S.K. Kang, B.C. Lee and W.S. Hwang, 2004. Improved *in vitro* development of porcine embryos with different energy substrates and serum. Theriogenol., 61: 1381-1393. DOI: 10.1016/j.theriogenology.2003.08.012. PMID: 15036970. http://www.sciencedirect.com/science?_ob=ArticleURL&_udi=B6TCM-4B42FM4-1&_user=1512607&_rdoc=1&_fmt=&_orig=search&_sort=d&view=c&_version=1&_urlVersion=0&_userid=1512607&md5=307a3971df88b05909a002aa39cbba4e.

Lim, J.M., O. Okitsu, K. Okuda and K. Niwa, 1994. Effects of fetal calf serum in culture medium on development of bovine oocytes matured and fertilized *in vitro*. Theriogenol., 41: 1091-1098. DOI: 10.1016/S0093-691X(05)80032-9. PMID: 16727462.

Lohuis, M.M., 1995. Potential benefits of bovine embryo-manipulation technologies to genetic improvement programs. Theriogenol., 43: 51-60. DOI: 10.1016/0093-691X(94)00016-N. http://www.sciencedirect.com/science/article/B6TCM-3YS9133-7X/2/5a697663c92d94cc3421529d43ba9983.

Maurer, H.R., 1992. Towards Serum-Free, Chemically Defined Media for Mammalian Cell Culture. In: Freshney, R.I. (Eds.). Animal Cell Culture: A Practical Approach IRL Press, New York, pp: 15-46. ISBN: 019 963212X.

Miller, G.F., D.W. Gliedt, J.M. Rakes and R.W. Rorie, 1994a. Addition of Penicillamine, Hypotaurine and Epinephrine (PHE) or Bovine Oviductal Epithelial Cells (BOEC) alone or in combination to bovine *in vitro* fertilization medium increases the subsequent embryo cleavage rate. Theriogenol., 41: 689-696. DOI: 10.1016/0093-691X(94)90178-L. PMID: 16727423. http://www.sciencedirect.com/science/article/B6TCM-49NY58V-124/1/408824f4c5457b58b604e354df9676a8.

Miller, K.F., J.M. Goldberg and R.L. Collins, 1994b. Covering embryo cultures with mineral oil alters embryo growth by acting as a sink for an embryotoxic substance. J. Assist. Reprod. Genet., 11: 342-345. DOI: 10.1007/BF02214139. PMID: 7795366. http://www.springerlink.com/content/y107540016451564.

Miller, K.F. and V.G. Pursel, 1987. Absorption of compounds in medium by the oil covering microdrop cultures. Gamete Res., 17: 57-61. DOI: 10.1002/mrd.1120170107. PMID: 3148538. http://www3.interscience.wiley.com/journal/109927069/abstract.

Nagy, A., M. Gertsenstein, K. Vintersten and R. Behringer, 2003. Recovery and *in vitro* Culture of Preimplantation-stage Embryos Manipulating the Mouse Embryo. 3rd Edn. CSHL Press, New York. pp: 161-208. ISBN: 9780879695743. http://www.cshlpress.com/default.tpl?action=full&--eqskudatarq=431.

O'Neill, C., 1998. Autocrine mediators are required to act on the embryo by the 2-cell stage to promote normal development and survival of mouse preimplantation embryos *in vitro*. Biol. Reprod., 58: 1303-1309. PMID: 9603268. http://www.biolreprod.org/cgi/reprint/58/ 5/1303.

Otsuki, J., Y. Nagai and K. Chiba, 2007. Peroxidation of mineral oil used in droplet culture is detrimental to fertilization and embryo development. Fertil. Steril., 88: 741-743. DOI: 10.1016/j.fertnstert.2006.11.144. PMID: 17292894. http://www.sciencedirect.com/science?_ob=ArticleURL&_udi=B6T6K-4N1SJY1-P&_user=10&_rdoc=1&_fmt=&_orig=search&_sort=d&view=c&_version=1&_urlVersion=0&_userid=10&md5=e274419fe37a0852a3bb0a15ee01457b.

Palasz, A.T. and J. Thundathil, 1998. The effect of volume of culture medium and embryo density on *in vitro* development of bovine embryos. Theriogenol., 49: 212 (Abst.). DOI: 10.1016/S0093-691X(98)905659. http://www.sciencedirect.com/ science?_ob=ArticleURL&_udi=B6TCM-49TSCH9-2G&_user=1512607&_rdoc=1&_fmt=&_orig=search &_sort=d&view=c&_version=1&_urlVersion=0&_userid=1512607&md5=dcd601fe5d2cbf10dd32852fc63f200a.

Paria, B.C. and S.K. Dey, 1990. Preimplantation embryo development *in vitro*: Cooperative interactions among embryos and role of growth factors. Proc. Natl. Acad. Sci. USA., 87: 4756-4760. PMID: 2352946. http://www.pubmedcentral.nih.gov/articlerender.fcgi?tool=pubmed&pubmedid=2352946.

Pinyopummintr, T. and B.D. Bavister. 1991. *In vitro*-matured *in vitro*-fertilized bovine oocytes can develop into morulae/blastocysts in chemically defined, protein-free culture media. Biol. Reprod., 45: 736-742. PMID: 1756211. http://www.biolreprod.org/cgi/reprint/45/5/736.

Provo, M.B. and C. Herr, 1998. Washed paraffin oil becomes toxic to mouse embryos upon exposure to sunlight. Theriogenol., 49: 214 (Abst.). DOI: 10.1016/S0093-691X(98)90567-2. http://www.sciencedirect.com/science?_ob=ArticleURL&_udi=B6TCM-49TSCH9-2J&_user=1512607&_rdoc=1&_fmt=&_orig=search&_sort=d&view=c&_version=1&_urlVersion=0&_userid=1512607&md5=86d814bb5f6daa1d9249df0a441211b2.

Rombauts, K. and C. Wood, 2000. Oocyte Retrieval. In: Trounson, A.O. and D.K. Gardner (Eds.). In Handbook of *in vitro* Fertilization. CRC Press, Boca. Raton., pp: 67-84. ISBN: 9780849340024.

Salahuddin, S., S. Ookutsu, K. Goto, Y. Nakanishi and Y. Nagata, 1995. Effects of embryo density and co-culture of unfertilized oocytes on embryonic development of *in vitro* fertilized mouse embryos. Hum. Reprod., 10: 2382-2385. PMID: 8530670. http://humrep.oxfordjournals.org/cgi/content/abstract/10/9/2382.

Shimada, M., N. Kawano and T. Terada, 2002. Delay of nuclear maturation and reduction in developmental competence of pig oocytes after mineral oil overlay of *in vitro* maturation media. Reproduction, 124: 557-564. DOI: 10.1530/rep.0.1240557. PMID: 12361474. http://www.reproduction-online.org/cgi/reprint/124/4/557.

Tae, J.C., E.Y. Kim, W.D. Lee, S.P. Park and J.H. Lim, 2006. Sterile filtered paraffin oil supports *in vitro* developmental competence in bovine embryos comparable to co-culture. J. Assist. Reprod. Genet., 23: 121-127. DOI: 10.1007/s10815-006-9024-6. PMID: 16758343. http://www.springerlink.com/content/t616366450hw187n.

Van Langendonckt, A., I. Donnay, N. Schuurbiers, P. Auquier, C. Carolan, A. Massip and F. Dessy, 1997. Effects of supplementation with fetal calf serum on development of bovine embryos in synthetic oviduct fluid medium. J. Reprod. Fertil., 109: 87-93. PMID: 9068418.

Van Soom, A., A.R. Mahmoudzadeh, A. Christophe, M.T. Ysebaert and A. De Kruif, 2001. Silicone oil used in microdrop culture can affect bovine embryonic development and freezability. Reprod. Domest. Anim., 36: 169-176. DOI: 10.1046/j.1439-0531.2001.00281.x. PubMed: 11555364. http://www3.interscience.wiley.com/journal/119022522/abstract?CRETRY=1&SRETRY=0.

Yoshioka, K., A.M. Othman, T. Taniguchi, H. Yamanaka and K. Sekikawa, 1997. Differential patterns of blastulation in bovine morulae cultured in synthetic oviduct fluid medium containing FCS or BSA. Theriogenol., 48: 997-1006. DOI: 10.1016/S0093-691X(97)00326-9. PMID: 16728189. http://www.sciencedirect.com/science?_ob=ArticleURL&_udi=B6TCM-3RTS1PV-9&_user=10&_rdoc=1&_fmt=&_orig=search&_sort=d&view=c&_version=1&_urlVersion=0&_userid=10&md5=66351eccdd2245711251293679ea58a7.