Turk J Vet Anim Sci
28 (2004) 779-784

Research Article

Replacement of Fetal Calf Serum with Synthetic Serum Substitute in the In Vitro Maturation Medium: Effects on Maturation, Fertilization and Subsequent Development of Cattle Oocytes In Vitro

Hakan SAĞIRKAYA*, Mehmet YAĞMUR, Zekariya NUR, Mustafa Kemal SOYLU
Department of Reproduction and Artificial Insemination, Faculty of Veterinary Medicine, Uludağ University, 16059 Görükle, Bursa - TURKEY

Received: 23.06.2003

Abstract: The aim of this study was to investigate the possibility of using synthetic serum substitute (SSS) instead of fetal calf serum (FCS) in maturation medium to stimulate in vitro maturation (IVM), fertilization (IVF) and subsequent development of bovine oocytes. Ovaries were obtained from a local slaughterhouse. Selected oocytes were matured in tissue culture medium 199 (M-199) supplemented with 2 mM glutamine + 0.25 mM Na-pyruvate + 0.5 µg/ml of FSH + 5 µg/ml of LH + 25 µg/ml of gentamycin and 10% FCS or 10% SSS (FCS and SSS groups, respectively) for 22 h. Matured oocytes were fertilized in vitro using frozen bull sperm. Fertilization day was taken as day 0 in the present study. Forty-eight h after IVF, the numbers of 2-4-cell stage embryos were recorded and they were transferred into CR1aa culture medium for in vitro culture until day 8. In addition, blastocyst numbers were recorded on day 8. A total of 255 and 250 oocytes were used for the FCS and SSS groups, respectively. The cleavage rate of the FCS group (73.7%; 188/255) was significantly higher ($P < 0.001$) than that of the SSS group (30.0%; 75/250). While the blastocyst formation rate of the FCS group was 16.1% (41/255), no blastocyst development was observed in the SSS group. The difference between the groups in terms of blastocyst formation was also significant ($P < 0.001$). These results show that serum supplementation of the IVM medium is necessary to obtain a higher cleavage rate and development rate to the blastocyst stage of immature bovine oocytes recovered from ovaries obtained from slaughterhouses. In conclusion, further studies are required to replace FCS with SSS in the maturation medium, and supplementation with growth factors might improve cleavage and development rates in maturation medium supplemented with SSS.

Key Words: In vitro maturation, cattle oocytes, fetal calf serum, synthetic serum substitute, embryo

İn Vitro Olgunlaştırma Medyumunda Fötal Buzağı Serumu Yerine Sentetik Serumun Kullanılması: Sığır Oositlerinin İn Vitro Maturasyon, Fertilizasyon ve Sonraki Gelişimleri Üzerindeki Etkileri

Özet: Bu çalışmanın amacı sığır oositlerinin in vitro olgunlaştırılmasını, fertilizasyonunu ve sonraki gelişimini stimüle etmek için olgunlaştırma medyumunda fötal buzağı serumu (FCS) yerine sentetik serumun (SSS) kullanılabilirliğini incelemektir. Ovaryumlar lokal mezbahadan elde edilmiştir. Seçilen oositler 2 mM glutamin + 0,25 mM Na-piruvat + 0,5 µg/ml FSH + 5 µg/ml LH + 25 µg gentamisin ve % 10 FCS ya da % 10 SSS (sırasıyla FCS ve SSS grupları) içeren hücre kültür medyumu (M-199) içerisinde 22 saat süreyle olgunlaşmaya bırakılmıştır. Olgunlaştırılmış oositler dondurulmuş sperm kullanılarak in vitro fertilize edilmiştir. Bu çalışmada fertilizasyon günü 0. gün olarak değerlendirilmiştir. IVF'dan 48 saat sonra, 2-4-hücre aşamasındaki embriyoların sayıları kaydedilmiş ve 8. güne kadar in vitro kültür için CR1aa kültür medyumuna transfer edilmiştir. Ayrıca, sekizinci günde gelişen blastosistlerin sayıları da kaydedilmiştir. FCS ve SSS grupları için sırasıyla 255 ve 250 adet oosit kullanılmıştır. FCS grubunun bölünme oranı (% 73,7; 188/255) SSS grubunun bölünme oranından (% 30,0; 75/250) önemli derecede yüksek bulunmuştur ($P < 0,001$). FCS grubunun blastosist gelişim oranı % 16,1 (41/255) iken; SSS grubunda blastosist gelişimi gözlenmemiştir. Blastosist gelişimi bakımından gruplar arasındaki fark önemli bulunmuştur ($P < 0,001$). Bu sonuçlar mezbahalardan elde edilen ovaryumlardan kazanılan olgun olmayan sığır oositlerinden daha yüksek bölünme ve blastosiste gelişim oranı elde etmek için IVM medyumuna serum katkısının gerekli olduğunu göstermektedir. Sonuç olarak, olgunlaştırma medyumunda FCS'un yerine SSS'un kullanılması için daha geniş çaplı çalışmalara gereksinim vardır ve bazı büyüme faktörlerinin SSS ilave edilmiş olgunlaştırma medyumuna ilave edilmesi bölünme ve gelişme oranlarını iyileştirebilir.

Anahtar Sözcükler: İn vitro olgunlaştırma, sığır oositleri, fötal buzağı serumu, sentetik serum, embriyo

* Correspondence to: hakans@uludag.edu.tr

Introduction

In vitro embryo production (IVP) using slaughterhouses as a source of oocytes is of great importance for the mass production of cattle embryos for research purposes and the improvement of cattle populations. A constant and high rate of blastocyst development in the IVP system is very important for laboratories and requires strict precautions and precise standards in every aspect of the IVP system, such as the collection of oocytes, chemicals, hormones and water used for the preparation of media, the sperm used for fertilization, culture conditions. Even though the same technique and media are used by various laboratories, the rate of development to the blastocyst stage obtained through the IVP system differs from one laboratory to another (1,2).

In vitro maturation (IVM) of oocytes obtained from follicles 2-8 mm in diameter is one of the most important steps in the cattle IVP system. The most common medium used for the IVM of cattle oocytes is tissue culture medium 199 (M-199). The maturation of mammalian oocytes, which starts during fetal development progresses until the first meiotic arrest at the dictyate stage of the prophase, just before or immediately after birth. Resumption of meiosis is activated either after hormonal stimulation in situ or spontaneously after the release of a cumulus-oocyte complex from the follicle. Developmental competence of bovine oocytes seems to be low under common IVM conditions. Improvement of the developmental competence of cattle oocytes by supplementing IVM medium with different additives has been the subject of many studies. The most common additives used for IVM of oocytes in M-199 are FSH, LH, fetal calf serum (FCS), and recently some growth factors, such as epidermal growth factor (EGF) and the insulin-like growth factor-I (IGF-I). Supplementation of IVM media with gonadotropins has been shown to be essential for the acquisition of developmental capacity of oocytes in cattle (3-5). Supplementation of IVM media with FCS or bovine serum albumin (BSA) has also been found to be beneficial for achieving high in vitro maturation, fertilization and subsequent development rates for cattle oocytes (6,7).

It has been reported that the effectiveness of FCS in IVM and IVF can vary considerably from one batch to another (8). Ingredients of FCS, such as amino acids, hormones, growth factors, cytokines, vitamins and many other substances, exhibit wide variations (9). These variations cause different results in IVM, IVF and subsequent development. Factors improving embryo production in media supplemented with FCS are not clearly understood. However, they include some positive embryotrophic factors and play roles in the inactivation of embryotoxic agents, such as free radicals, heavy metals. Moreover, bovine-derived sera or proteins are especially avoided in the human IVF system because of the recent appearance of bovine spongioform encephalopathy and viral or prion contamination risk. Therefore, defined culture conditions supporting a high developmental rate are important to obtain constant results. For this purpose, there has recently been a trend to use more defined proteins, such as BSA, human serum albumin (HSA) and synthetic serum preparations instead of poorly known natural serum preparations like FCS and oestrus cow serum (OCS) (10).

The aim of the present study was to explore the possibility of using synthetic serum substitute (SSS) in place of FCS to stimulate maturation, fertilization and subsequent development of cattle oocytes in vitro.

Materials and Methods

Oocyte recovery

Bovine ovaries were obtained from a local slaughterhouse in Bursa and transported to the laboratory in a thermos filled with physiological saline (0.9% w/v NaCl) within 3 to 5 h of recovery. The thermos temperature on arrival was approximately 34 ± 2 °C. Ovaries were rinsed several times with warm tap water (approximately 35 °C) before aspiration in the laboratory. Cumulus oocyte complexes (COCs) were aspirated from 2 to 8 mm antral follicles by means of an 18-gauge needle attached to a 10 ml syringe. Aspirated follicular contents were collected in a 50 ml centrifuge tube and then the sediment was placed in 100 mm plates and examined for oocytes with a homogeneously granulated cytoplasm and at least 3 layers of compact cumulus cells under a stereomicroscope in a warm culture room. The COCs were washed 3 times with TL-HEPES medium (11) containing 3 mg/ml of polyvinyl pyrrolidone (PVP 40). All the chemicals used in the present study were purchased from Sigma Chemicals Co., St. Louis, MO USA unless otherwise indicated.

In vitro maturation

The principle medium used for IVM was tissue culture medium 199 with Earle's salts (M-199) supplemented with 2 mM glutamine + 0.25 mM Na-pyruvate + 0.5 µg/ml of FSH + 5 µg/ml of LH + 25 µg/ml of gentamycin. In addition, the medium was also supplemented with 10% FCS or 10% SSS (Irvine Scientific, Santa Ana, CA, USA), which contains 6% total protein composed of approximately 84% HSA and 16% alpha and beta globulins with no more than 11% gamma globulins in normal saline. About half of the COCs were cultured in medium supplemented with FCS and the other half were cultured in medium supplemented with SSS. Eight to twelve COCs were cultured in 50 µl of medium under 9 to 10 ml of mineral oil in 60 mm petri dishes at 39 °C in 5% CO_2 in air with saturated humidity (12,13). After 22 h of IVM, maturation of COCs was evaluated by checking cumulus cell expansion.

In vitro fertilization

The motile fraction of frozen-thawed semen from a single bull was separated using the density gradient system (14): 0.5 ml of 90% Percoll was pipetted to the bottom of a 1.5 ml Eppendorf tube, and 0.5 ml of 45% Percoll was carefully placed on top. Frozen sperm (presented by the Department of Reproduction and Artificial Insemination, Faculty of Veterinary Medicine, İstanbul University) was first thawed at 35 °C for 1 min, and then layered onto the Percoll gradient. The tube containing the sample was then centrifuged at 700 x g for 15 min at room temperature. The pellet containing the live sperm was recovered, and the sperm concentration was determined using a hemocytometer. Sperm was then diluted to 50 x 10^6 spermatozoa/ml in TL-HEPES (15). After the maturation period, oocytes were washed twice in TL-HEPES and then transferred to the fertilization drops in groups of 8 to 12. The final concentration was 2 x 10^6 spermatozoa/ml in 50 µl drops of fertilization medium, glucose-free TALP, supplemented with 0.2 mM pyruvate, 6 mg/ml of fatty acid free BSA (BSA-FAF), 25 µg/ml of gentamycin, 20 µM penicillamine, 10 µM hypotaurine, 1 µM epinephrine (12,16-19) and 2 µl of 2 µg/ml heparin (20,21). Oocytes and sperm were co-cultured for 48 h.

In vitro culture

At the 48th h after insemination, the cumulus cells were removed by placing the embryos in a 1.5 ml Eppendorf tube and vortexing them at high speed for 3 min. The number of embryos cleaved at least once during 48 h culture in fertilization medium after fertilization was recorded and cleaved embryos (2- to 4-cell stage) were transferred into 50 µl of CR1aa culture medium (19,22,23) after washing them 3 times in TL-HEPES. Table 1 shows the preparation of the CR1aa culture medium. Fertilization day in the present study was taken as day 0. A second evaluation of embryos was carried out on days 7-8 and the number of embryos reaching the blastocyst stage was also recorded.

Table 1. Preparation of CR1aa culture medium.

Chemicals	Sigma catalog number	10 ml
NaCl	S-5886	67 mg
KCl	P-5405	2.3 mg
$NaHCO_3$	S-5761	22 mg
BSA (FAF)	A-6003	30 mg
L-glutamine	G-3126	1.5 mg
Na-pyruvate	P-5280	0.4 mg
L-lactate	L-4388	5.5 mg
Gentamycin	G-3632	5 µl
MEM amino acids*	M-7145	100 µl
BME amino acids*	B-6766	200 µl

* Add these ingredients using sterile technique after filtration

Statistical Analysis

Statistical analysis of the data was carried out using the Graphpad Software Program (Version 2.02, LSU Medical Center, USA). Differences between the groups were compared with the chi-square and Fisher's exact tests. Differences of $P < 0.001$ were considered statistically significant.

Results

Cleavage and blastocyst formation rates of in vitro matured oocytes in TCM-199 supplemented with FCS or SSS were evaluated. Experiments were replicated 3 times. In the FCS and SSS groups, a total of 255 and 250 oocytes were fertilized and 188 of 255 (73.7%) and 75 of 250 (30.0%) oocytes cleaved within 48 h of in vitro culture, respectively. The difference between cleavage rates was significant ($P < 0.001$). After culturing 2- to 4-cell stage embryos in CR1aa culture medium,

development rates to the blastocyst stage were 16.1% (41/255) and 0.0% (0/250) for the FCS and SSS groups, respectively. The difference between the rates of development to the blastocyst stage was also significant ($P < 0.001$, Table 2).

Discussion

The maturation of immature bovine oocytes in vitro is probably the most important step affecting the success of IVF and the subsequent development rate (10). It was shown that during IVM of COCs, the constituents of the media could have an extreme effect on the developmental capability of in vitro produced bovine embryos (24). There have been many studies investigating the effects of protein supplementation of media used for IVP (25-27). Sera (i.e. FCS and OCS) and BSA are the most common protein supplements for IVM media. The scientific reason for the beneficial effect of adding serum is not clearly understood but it is commonly accepted that one major biological role of serum is to compensate for whatever essential elements are missing from the medium by serving as a reservoir for many of the beneficial components, such as different energy substrates, steroids, amino acids, fatty acids, vitamins and growth factors. Serum also serves as a protective compound against scavenging ions and small molecules secreted from the developing embryo (28). However, safety requirements restrict the use of these products, especially in the human IVF system. Therefore, synthetic serum substitutes are produced commercially, and using these substitutes in culture media has allowed scientists to develop well-defined culture media for in vitro embryo production.

It has been shown that the supplementation of IVM media with gonadotropins is essential for the developmental capacity of bovine oocytes (3-5). In addition, there is recent evidence that some kinds of growth factors and cytokines play a role as intraovarian regulators in vivo and at the follicle level the action of gonadotropins is regulated by locally generated paracrine and autocrine acting growth factors (29-31). It has also been reported that granulosa and cumulus cells have receptors for EGF and IGF-I (32,33).

The results of the present study support previous studies reporting that serum supplementation is beneficial for the IVM, IVF and subsequent development of bovine oocytes (6,7,34,35). Rates of cleavage and development to the blastocyst stage in the FCS group were significantly higher than those in the SSS group ($P < 0.001$, Table 2). SSS, a protein additive specifically developed for human IVF applications, was tested as an alternative to FCS since the biological activity of commercially available FCS can vary enormously from one batch to another.

Russell et al. (36) used SSS for the IVM of immature human oocytes. In addition, Chanson et al. (10) reported a study comparing SSS with FCS for the IVM of immature bovine oocytes. In their study the cleavage rate of the control group supplemented with FCS and hormones was reported as 31.5% (47/149) and the development rate to the blastocyst stage was 7.4% (11/149). In addition, when FCS was replaced with SSS, the cleavage rate and development rate to the blastocyst stage were reported as 33.1% (60/181) and 11.0% (20/181), respectively. In this study, the cleavage rate and development rate to the blastocyst stage in the FCS group (73.7% and 16.1%, respectively) were higher than the results for the control group of Chanson et al. (10). For the SSS groups in both studies, the cleavage rates were similar but no development to the blastocyst stage was observed in the present study.

The logical explanation for the differences between the cleavage rates and blastocyst formation rates of the FCS and SSS groups is the differences between the constituents of FCS and SSS. As mentioned above, FCS contains some growth factors, such as EGF and IGF-I. Replacement of natural serum with synthetic products

Table 2. Cleavage and blastocyst formation rates of bovine oocytes matured in M-199 supplemented with FCS and SSS.

Serum used for IVM	Fertilized oocyte number (%)	2- to 4-cell embryo number (%)	Blastocyst number (%)
FCS	255	188 (73.7)[a]	41 (16.1)[a]
SSS	250	75 (30.0)[b]	0 (0.0)[b]

Different superscripts in the same column indicate significant differences ($P < 0.001$).

would be very useful to obtain constant results and some synthetic serum substitutes have already been made available for use. However, our results showed that serum supplementation of the IVM medium is necessary to obtain a higher cleavage rate and development rate to the blastocyst stage of immature bovine COCs recovered from ovaries obtained from slaughterhouses. In conclusion, further experiments with synthetic serum substitutes should be carried out by also supplementing IVM medium with growth factors to optimize the fertilization rate and development of bovine IVM oocytes.

References

1. Leibfried-Rutledge, M.L., Dominko, T., Critser, E.S., Critser, J.K.: Tissue maturation in vivo and in vitro: Gamete and early embryo ontogeny. In: Karow, A. Ed. Reproductive Tissue Banking. Academic Press, New York, 1997; 23-138.
2. Gardner, D.K.: Changes in requirements and utilization of nutrients during mammalian preimplantation embryo development and their significance in embryo culture. Theriogenology, 1998; 49: 83-102.
3. Fukushima, M., Fukui, Y.: Effects of gonadotropins and steroids on the subsequent fertilizability of extrafollicular bovine oocytes cultured in vitro. Anim. Reprod. Sci., 1985; 9: 323-332.
4. Brackett, B.G., Younis, A.I., Fayrer-Hosken, R.A.: Enhanced viability after in vitro fertilization of bovine oocytes matured in vitro with high concentrations of luteinizing hormone. Fertil. Steril., 1989; 52: 319-324.
5. Choi, Y.H., Carnevale, E.M., Seidel Jr, G.E., Squires, E.L.: Effects of gonadotropins on bovine oocytes maturated in TCM-199. Theriogenology, 2001; 56: 661-670.
6. Leibfried-Rutledge, M.L., Critser, E.S., First, N.L.: Effects of fetal calf serum and bovine serum albumin on in vitro maturation and fertilization of bovine and hamster cumulus-oocyte complexes. Biol. Reprod., 1986; 35: 850-857.
7. Holm, P., Booth, P.J., Schmidt, M.H., Greve, T., Callesen, H.: High bovine blastocyst development in a static in vitro production system using SOFaa medium supplemented with sodium citrate and myo-inosotol with or without serum-proteins. Theriogenology, 1999; 52: 683-700.
8. Kane, M.T.: Culture media and culture of early embryos. Theriogenology, 1987; 27: 49-57.
9. Gordon, I.: Laboratory Production of Cattle Embryos. CAB International, Wallingford, 1994: 30-142.
10. Chanson, A., Nocera, D., Senn, A., De Grandi, P., Germond, M.: Development of well-defined medium for the in vitro maturation of immature bovine cumulus-oocytes complexes. J. Assist. Reprod. Gen., 2001; 18: 97-105.
11. Bavister, B.D., Leibfried-Rutledge, M.L., Lieberman, G.: Development of preimplantation embryos of golden hamster in a defined culture medium. Biol. Reprod., 1983; 28: 235-247.
12. Sağırkaya, H.: Effects of CR1aa and TCM-199 media on in vitro embryonic development until blastocyst stage in cattle. Uludağ Üniv. Vet. Fak. Derg., 1998; 17: 55-63.
13. Birler, S., Pabuççuoğlu, S., İleri, İ.K., Alkan, S., Evecen, M.: Sığır oositlerinin in vitro olgunlaştırılmasında farklı sürelerin etkisi. Turk. J. Vet. Anim. Sci., 1998; 22: 551-557.
14. Parrish, J.J., Krogenaes, A., Susko-Parrish, J.L.: Effect of bovine sperm separation by swim up or percoll on success of in vitro fertilization and embryo development. Theriogenology, 1995; 44: 859-870.
15. Parrish, J.J., Susko-Parrish, J.L., Leibfried-Rutludge, M.L., Critser, E.S., Eyestone, W.H., First, N.L.: Bovine in-vitro fertilization with frozen-thawed semen. Theriogenology, 1986; 25: 591-600.
16. Leibfried, M.L., Bavister, B.D.: Effects of epinephrine and hypotaurine on in vitro fertilization in the golden hamster. J. Reprod. Fertil., 1982; 66: 87-93.
17. Ball, G.D., Leibfried, M.L., Lenz, R.W., Ax, T.L., Bavister, B.D., First, N.L.: Factors affecting successful in vitro fertilization of bovine follicular oocytes. Biol. Reprod., 1983; 28: 717-725.
18. Susko-Parrish, J.L., Wheeler, M.B., Ax, R.L., First, N.L., Parrish, J.J.: The effect of penicillamine, hypotaurine, epinephrine and sodium metabisulfite on bovine in vitro fertilization. Theriogenology, 1990; 33: 333.
19. Dogan, I., Sagirkaya, H., Soylu, M.K., Nur, Z., Yerlikaya, H.: Development of in vitro produced bovine embryos in four different culture media. Indian J. Anim. Sci., 2002; 72: 227-229.
20. Parrish, J.J., Susko-Parrish, J.L., First, N.L.: Effect of heparin and chondroitin sulfate on the acrosome reaction and fertility of bovine sperm in vitro. Theriogenology, 1985; 24: 537-549.
21. Tekin, N., Daşkın, A., Akçay, E.: Boğa spermatozoonlarında in vitro kapasitasyon ve fertilizasyon. Turk. J. Vet. Anim. Sci., 2001; 25: 349-358.
22. Rosenkrans Jr, C.F., Zeng, G.Q., McNamara, G.T., Schoff, P.K., First, N.L.: Development of bovine embryos in-vitro is affected by energy substrates. Biol. Reprod., 1993; 49: 459-462.
23. Rosenkrans Jr, C.F., First, N.L.: Effects of free amino acid and vitamins on cleavage and developmental rate of bovine zygotes in-vitro. J. Anim. Sci., 1994; 72: 434-437.
24. Rose, T.A., Bavister, B.D.: Effect of oocyte maturation medium on in vitro development of in vitro fertilized bovine embryos. Mol. Reprod. Dev., 1992; 31: 72-77.
25. Chauhan, M.S., Palta, P., Das, S.K., Katiyar, P.K., Madan, M.L.: Replacement of serum and hormone additives with follicular fluid in the IVM medium: effects on maturation, fertilization and subsequent development of buffalo oocytes in vitro. Theriogenology, 1997; 48: 461-469.

26. Dugan, K.J., Shalika, S., Smith, R.D., Padilla, S.L.: Comparison of synthetic serum substitute and fetal cord serum as media supplements for in vitro fertilization: a prospective, randomized study. Fertil. Steril., 1997; 67: 166-168.

27. Smitz, J., Nogueira, D., Albano, C., Cortvrindt, R., Devroey, P.: Improving in vitro maturation of oocytes in human taking lessons from experiences in animal species. Reprod. Dom. Anim., 2001; 36: 11-17.

28. Ben-Yosef, B., Yovel, I., Schwartz, T., Azem, F., Lessing, J.B., Amit, A.: Increasing synthetic serum substitute (SSS) concentrations in P1 glucose/phosphate-free medium improves implantation rate: a comparative study. J. Assist. Reprod. Gen., 2001; 18: 588-592.

29. Bevers, M.M., Dieleman, S.J., van den Hurk, R., Izadyar, F.: Regulation and modulation of oocyte maturation in the bovine. Theriogenology, 1997; 47: 13-22.

30. Sakaguchi, M., Dominko, T., Leibfried-Rutledge, M.L., Nagai, T., First, N.L.: A combination of EGF and IGF-I accelerates the progression of meiosis in bovine follicular oocytes in vitro and fetal calf serum neutralizes the acceleration effect. Theriogenology, 2000; 54: 1327-1342.

31. Mtango, N.R., Varisanga, M.D., Dong, Y.J., Rajamahendran, R., Suzuki, T.: Growth factors and growth hormone enhance in vitro embryo production and post-thaw survival of vitrified bovine blastocyst. Theriogenology, 2003; 59: 1393-1402.

32. Singh, B., Rutledge, J.M., Armstrong, D.T.: Epidermal growth factor and its receptor gene expression and peptide localization in porcine ovarian follicles. Mol. Reprod. Dev., 1995; 40: 391-399.

33. Spicer, L.J., Stewart, R.E.: Interaction among basic fibroblast growth factor, insulin and insulin-like growth factor-I (IGF-I) on cell numbers and steroidogenesis of bovine thecal cells: Role of IGF-I receptors. Biol. Reprod., 1996; 54: 255-263.

34. Lonergan, P., Carolan, C., Mermillod, P.: Development of bovine embryos in vitro following oocyte maturation under defined conditions. Reprod. Nutr. Dev., 1994; 34: 329-339.

35. Lorenzo, P.L., Illera, M.J., Illera, J.C., Illera, M.: Role of EGF, IGF-I, sera and cumulus cells on maturation vitro of bovine oocytes. Theriogenology, 1995; 44: 109-118.

36. Russell, J.B., Knezevich, K.M., Fabian, K.F., Dickson, J.A.: Unstimulated immature oocyte retrieval: early versus midfollicular endometrial priming. Fertil. Steril., 1997; 67: 616-620.