

The effect of macromolecule and growth factor combinations on in vitro development of bovine embryos

Alper KOÇYİĞİT¹, Mesut ÇEVİK^{2*}, Uğur ŞEN³, Mehmet KURAN⁴

¹Department of Reproduction and Artificial Insemination, Faculty of Veterinary Medicine, Cumhuriyet University, Sivas, Turkey

²Department of Reproduction and Artificial Insemination, Faculty of Veterinary Medicine, Ondokuz Mayıs University, Samsun, Turkey

³Department of Agricultural Biotechnology, Faculty of Agriculture, Ahi Evran University, Kırşehir, Turkey

⁴Department of Agricultural Biotechnology, Faculty of Agriculture, Ondokuz Mayıs University, Samsun, Turkey

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Abstract: This study was conducted to determine the effects of different macromolecule sources added to synthetic oviduct fluid (SOF) culture medium supplemented with growth factors on the development of bovine embryos and blastocyst morphology. Zygotes were distributed into 5 treatment groups. Cleavage, morula, and blastocyst rates were evaluated under a stereomicroscope. Trophectoderm (TE) and inner cell mass (ICM) cells were determined by differential staining method. It was found that bovine serum albumin (BSA), either alone or in combination with growth factors, as compared to the control or polyvinyl-alcohol (PVA) resulted in higher embryo yield and faster development during early bovine embryo culture. The quality of bovine embryos, based on the number of blastocyst cells and the ratio of ICM to total blastomeres, was affected by the sources of macromolecules and their combinations with growth factors. Growth factors supplemented to SOFaa media with BSA and PVA significantly increased the number of ICM cells and the ratio of ICM cells to total number of cells. In conclusion, replacing BSA with PVA depressed the blastocyst rate and cell numbers, and the number of blastomeres and ICM and TE cell numbers were affected by both the type of macromolecule and the growth factor supplements.

Key words: Bovine, embryo culture, blastocyst quality, macromolecules, growth factors

1. Introduction

In vitro fertilization (IVF) and development of bovine embryos up to the blastocyst stage has been successfully achieved with a variety of culture systems. Numerous studies have been performed to improve developmental competence of mammalian embryos by supplementation of culture media with gonadotropins, steroid hormones, serum, and growth factors. Therefore, current culture media usually are supplemented with various proteins (1,2). The protein supplements with the addition of embryo culture are thought to play several important roles in the culture media: 1) reducing embryo toxicity, 2) as a source for basic nutritive requirements of early embryos, and 3) as a source of growth factors promoting embryonic development directly or indirectly via cumulus-cell proliferation (3-5).

It is well defined that serum and bovine serum albumin (BSA) are complex and undefined mixtures of different proteins containing various energy substrates, small peptides or growth factors that play an important role in embryonic development (6). In several studies it has been

shown that serum or BSA has been replaced with synthetic macromolecules such as polyvinyl-alcohol (PVA) or polyvinylpyrrolidone (PVP) to prevent the stickiness of the embryos that occurs when protein-free medium is used (7,8). These synthetic polymers are used widely as a serum/BSA substitute in chemically defined embryo culture media, especially for bovine embryos. Among these polymers, PVA has been preferred as an additive, particularly having surfactant activity similar to that of albumin. PVA is more frequently used in culture media (1,9) than PVP (10) because its surface-active properties are greater, while the use of PVP in embryo freezing media (6,11) is attributed with colloidal rather than surface-active properties (12). More recently, this list has been extended to include glycosaminoglycans, in particular hyaluronic acid, already known to stimulate cattle embryo development in vitro (13,14).

Growth factors like epidermal growth factor (EGF) and insulin like growth factor-I (IGF-I) are known to have effects on preimplantation development by stimulating the metabolism and growth of embryos (5,15). In bovine

* Correspondence: cevikm@omu.edu.tr

embryos, poor morphology is generally associated with hatched blastocysts with low inner cell mass (ICM) cell numbers. The number of blastomeres and the ratio of ICM cells to total cells are considered to be potential indicators of embryo quality (16). The culture conditions influence the cell allocation in different species (7,17). The objective of the present study was to replace serum with BSA as a less complex protein source or with a synthetic macromolecule (PVA) and growth factors and to observe the effects of additional culture supplements on morphologic quality and cell allocation of bovine embryos.

2. Materials and methods

All chemicals and media used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA), except where otherwise indicated.

2.1. Collection and in vitro maturation of oocytes

Bovine ovaries were collected from a local slaughterhouse and transported to the laboratory at approximately 36 ± 2.0 °C in physiological saline solution supplemented with gentamycin sulfate (0.1 µL/mL). Cumulus oocyte complexes (COCs) were recovered from follicles 2–8 mm in diameter by aspiration. The COCs were collected in 3–4 mL of HEPES-buffered Medium-199 containing Earle's salts and supplemented with 1% v/v antibiotic-antimycotic solution. Before in vitro maturation, the COCs were assessed morphologically and only oocytes with compact, nonatretic cumulus investment and evenly granulated cytoplasm were selected for maturation. Maturation medium was sodium bicarbonate-buffered Medium-199 with sodium pyruvate (5.5 µg/mL), antibiotic-antimycotic solution (1% v/v), and heat-inactivated fetal calf serum (FCS, 10% v/v). The COCs were matured for 22 h in a humidified atmosphere of 5% CO₂ in air at 38.5 °C.

2.2. Spermatozoa preparation and in vitro fertilization

After a 22-h maturation period, oocytes were transferred into 44-µL fertilization drops. The fertilization medium was glucose-free modified TALP supplemented with bicarbonate (25 mM), Na-lactate (22 mM), Na-pyruvate (1 mM), fatty acid-free BSA (6 mg/mL), heparin-sodium salt (184 U/mg heparin, 10 mg/mL), and antibiotic-antimycotic solution (0.5 µL/mL) (pH 7.4 and 280–300 mOsm/kg). Frozen-thawed semen was used for the fertilization of oocytes. A Percoll density gradient system was used for the separation of the motile fraction of the frozen-thawed semen (18). Sperm were then diluted to 50×10^6 spermatozoa/mL in TL-HEPES, including 2×10^6 spermatozoa/mL as the final concentration. The fertilization procedure was completed by adding 2 µL of diluted sperm, 2 µL of heparin (5 µg/mL), and 2 µL of PHE solution (20 µM penicillamine, 10 µM hypotaurine, and 1 µM epinephrine in final concentration) into the fertilization drops containing oocytes. The oocytes were

fertilized with 2 µL of diluted semen per fertilization drop for 22 h in a humidified atmosphere of 5% CO₂ in air at 38.5 °C.

2.3. In vitro culture

Cumulus cells surrounding the oocytes were removed from presumptive zygotes at approximately 22 h after insemination by vortexing for 3 min. The zygotes were transferred in groups of 20–30 for the culture droplets. The mSOF medium was supplemented with pyruvate (0.4 mM), BSA-FAF (4 mg/mL), 100X MEM (20 µL/mL), 50X BME (10 µL/mL), penicillin (100 U/mL), and streptomycin (100 µg/mL) on the day of use. Macromolecules and growth factors added to the culture medium were as follows: 1) essentially fatty-acid free BSA (4 mg/mL), 2) BSA + IGF-I (100 ng/mL) + EGF (10 ng/mL), 3) PVA (1 mg/mL), 4) PVA + IGF-I + EGF, and 5) FCS (10% , v/v), for 9 days under low oxygen tension (5% CO₂, 5% O₂, 90% N₂ atmosphere) at 38.5 °C. Cleavage, morula, and blastocyst development rates were evaluated from the zygotes on days 3, 5, and 8.

2.4. Determining of inner cell mass and trophectoderm

Expanding blastocysts characterized by zona pellucidae showing signs of thinning and with slightly increased embryo diameters by day 7 or 8 were stained as described by Van Soom et al. (16). Embryos were examined under a fluorescence microscope (Nikon Invert Microscope Eclipse Ti-FL). ICM nuclei labeled with bisbenzimidide appeared blue and trophectoderm (TE) nuclei labeled with both bisbenzimidide and propidium iodide appeared pink to red. The numbers of ICM and TE nuclei were counted directly under the fluorescence microscope.

2.5. Statistical analysis

Embryos were randomly allocated to each treatment group and all experiments were replicated six or seven times. Statistical data analysis was performed using the GLM procedure of SAS (19). One-way analysis of variance (ANOVA) followed by Duncan's multiple comparison test was used for statistical comparison of the groups. All values are reported as least-squares means \pm SEM, and statistical differences were considered as significant when P-values were less than 0.05.

3. Results

3.1. Embryonic development

The effects of different macromolecule sources alone or combined with growth factors on in vitro bovine embryo development potential were evaluated. As shown in Table 1, there were significant differences among culture groups in terms of cleavage and morula rates and development to the blastocyst stage ($P < 0.001$). BSA alone or combined with growth factors and PVA supplemented with EGF and IGF-I resulted in the highest cleavage rate in contrast to the FCS- and PVA-alone treatments. Furthermore, the

Table 1. Effect of growth factors and macromolecules on in vitro developmental competence of bovine embryos.

Culture groups	Presumptive zygotes (n)	Percentage of embryos developed to		
		Cleavage (% mean \pm SEM)	Morula (% mean \pm SEM)	Blastocyst (% mean \pm SEM)
SOF+BSA	281	79.71 \pm 2.40 ^a	37.72 \pm 2.89 ^a	23.13 \pm 2.51 ^{ab}
SOF+BSA+IGF+EGF	308	69.48 \pm 2.62 ^b	37.01 \pm 2.75 ^a	28.89 \pm 2.58 ^a
SOF+PVA	245	54.69 \pm 3.18 ^c	19.59 \pm 2.54 ^b	11.42 \pm 2.03 ^c
SOF+PVA+IGF+EGF	283	71.73 \pm 2.68 ^b	24.38 \pm 2.55 ^b	15.19 \pm 2.13 ^c
SOF+FCS	230	54.78 \pm 3.28 ^c	24.78 \pm 2.85 ^b	16.52 \pm 2.45 ^{bc}

^{abc}: Rates and values with different letters in the same column are statistically significant in bovine IVF embryos ($P < 0.001$).

results of our study demonstrated that bovine embryos in the culture medium containing BSA developed faster and had increased blastocyst cell counts when compared to bovine embryos cultured in 1 mg/mL PVA-supplemented media (105.15 and 88.57, $P < 0.05$). However, BSA alone or supplemented with IGF-I and EGF yielded similar results (23.1% and 28.9 %, respectively) in terms of development to the blastocyst stage. Additionally, FCS, PVA alone, or PVA supplemented with IGF-I and EGF combination resulted in similar blastocyst rates (16.5%, 11.4%, and 15.1%, respectively). FCS had the lowest percentage of embryos that cleaved, and it resulted in 24.8% morula development, suggesting that FCS delays embryonic cleavage compared to the BSA and growth factor-supplemented culture media. Similarly, there was a difference in the overall blastocyst yield at days 7–9 (range: 11%–28%) among the groups.

3.2. Effects of culture supplements on cell allocation

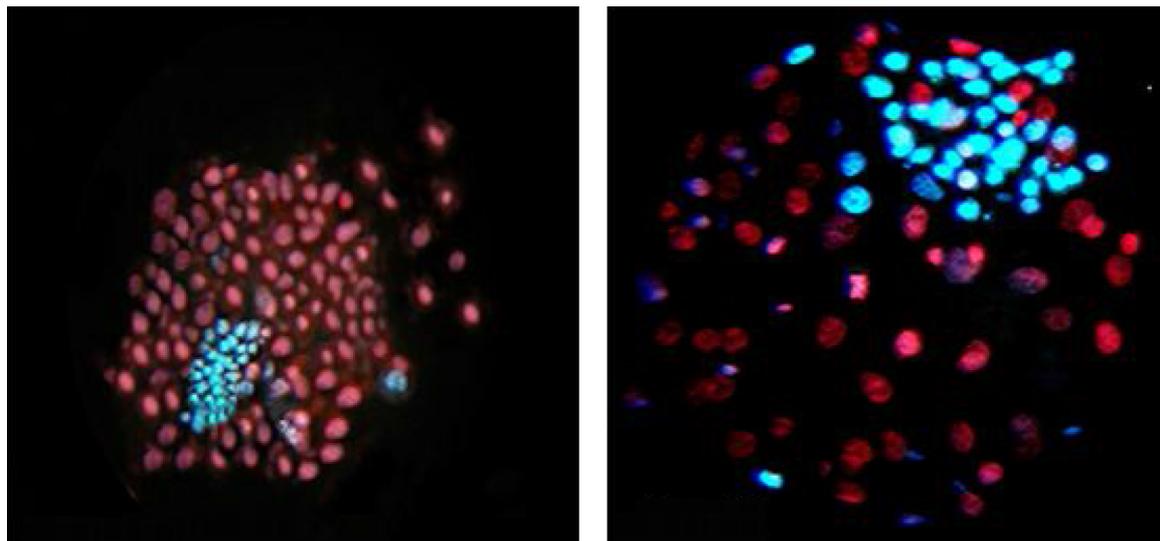
The data on cell allocation of ICM and TE cells are presented in Table 2. There were significant differences between treatment groups in terms of the number of

blastomeres and ICM cells ($P < 0.05$), and in the ratio of ICM cells to total cells ($P < 0.001$) harvested on days 7, 8, and 9 of culture after fertilization. Numbers of ICM and TE cells were influenced by both the type of macromolecule and the growth factor supplements. As shown in Table 2, successive use of BSA alone or in combination with IGF-I and EGF, and also FCS in the SOF medium, significantly increased the number of blastomeres in blastocysts (103.4, 105.1, and 102.5 cells/blastocyst, respectively) (Figure 1). In general, embryos produced in PVA alone or supplemented with growth factors had a lower number of cells in blastocysts ($P < 0.05$) (Figures 2). However, significant differences were found in the numbers of blastomeres and ICM cells ($P < 0.05$) and in TE cells and the ratio of ICM cells to total cells among the treatment groups ($P < 0.001$). A higher ratio of ICM cells to total cells was observed when embryos were cultured in the media supplemented with FCS, PVA alone, or growth factors compared to embryos cultured in the BSA alone and the BSA plus growth factors combination (45.3 and 49.3 and 53.9 versus 33.4 and 36.9) ($P < 0.001$).

Table 2. Differential cell counts of blastocysts cultured in medium containing different macromolecules and growth factors.

Culture groups	Stained blastocyst (n)	Number of cells			
		Total cells (mean \pm SEM)	Trophectoderm (mean \pm SEM)	Inner cell mass (mean \pm SEM)	ICM/total cells (% mean \pm SEM)
SOF+BSA	19	105.15 \pm 5.18 ^a	70.31 \pm 4.01 ^a	34.84 \pm 1.79 ^b	33.46 \pm 1.17 ^c
SOF+BSA+IGF+EGF	18	103.44 \pm 4.56 ^{ab}	65.00 \pm 3.77 ^{ab}	38.33 \pm 3.58 ^{ab}	36.97 \pm 2.75 ^{bc}
SOF+PVA	19	88.57 \pm 3.30 ^c	44.00 \pm 3.80 ^c	44.05 \pm 4.14 ^{ab}	49.33 \pm 3.74 ^a
SOF+PVA+IGF+EGF	20	91.10 \pm 3.51 ^{bc}	42.15 \pm 3.31 ^c	48.95 \pm 3.18 ^a	53.98 \pm 2.91 ^a
SOF+FCS	20	102.50 \pm 4.31 ^{ab}	56.30 \pm 4.86 ^b	46.20 \pm 4.29 ^a	45.39 \pm 3.62 ^{ab}

^{abc}: Rates and values with different letters in the same column are statistically significant in bovine IVF embryos.



Figures 1 and 2. Differentially stained (Hoechst 33342 and propidium iodide) bovine blastocysts with blue nuclei representing the inner cell mass (ICM) and pink to red nuclei representing outer cells (TE). 1- Cell allocation of blastocyst in BSA group, 2- cell allocation of blastocyst in PVA group.

4. Discussion

The purpose of the present study was to replace serum with BSA as a less complex protein source or with PVA as a synthetic macromolecule and to observe the effects on early embryonic development and morphologic quality in preimplantation bovine embryos. The present study showed that different macromolecule sources (especially BSA) alone or combined with growth factors were significantly effective on *in vitro* bovine embryo development, but no effect of PVA alone or in combination with growth factors on cleavage and blastocyst rates and blastocyst cell number was observed compared other culture groups.

Recent studies focused on amino acid or growth factor supplementation to *in vitro* culture media of mammalian embryos. Therefore, a wide variety of epigenetic factors, including ions, energy substrates, amino acids, vitamins, growth factors, cytokines, and hormones, play an important role in early embryonic development (7,11). Defined media with PVP and PVA, semidefined media with BSA, and undefined media with FCS have all been added to mammalian embryo media as energy sources. In a modified SOF culture system, the successive use of BSA and FBS during early and late preimplantation development was the most effective regime. BSA may also provide as of yet undefined embryotrophic compounds, function as a heavy metal ion chelator/free radical scavengers, protect cellular constituents against the effect of toxins, and regulate redox potential, pH, and osmolarity. The serum contains not only a variety of energy sources (3,6) but also growth factors (8,13) needed for *in vitro* embryonic development. The beneficial effect of more complex protein supplements is evident after the

activation of the embryonic genome and probably due to the presence of growth factors (10). One of the most commonly used supplements, BSA is an embryotrophic macromolecule used in embryo culture media, which is commonly replaced with synthetic compounds, such as PVA. However, the polyvinyl polymers PVP and PVA have been commonly used as substitutes for BSA in media for the culture of mammalian preimplantation embryos. The more frequent use of PVA in culture media (1,9,20) than PVP (10) could be related to its greater surface-active properties or may be related to colloidal rather than surface-active properties (3,12).

The present study showed that exposure of bovine embryos to BSA alone or in combination with growth factors accelerated progression of blastocyst in bovine embryos. These data are in agreement with several researchers, such as Lonergan et al. (4) and Rieger et al. (21). Some of the beneficial effects of BSA may be due to its action as a chelating agent, regulator of oxidation reduction potential, cell surface protector, or enzyme protector. Furthermore, our results demonstrated that there was no effect of PVA alone or combined with growth factors on cleavage and blastocyst rates and blastocyst cell number in comparison with other groups. These results are in agreement with the findings of Kuran et al. (20) and Duque et al. (7), but in contrast to those of Wrenzycki et al. (8), Krisher et al. (11), and Mingoti et al. (13). Eckert and Niemann (2) demonstrated that embryos cultured in SOFaaBSA consumed more oxygen than their SOFaaPVA counterparts, despite having significantly lower pyruvate uptake. This suggests that embryos grown in PVA may have reduced viability. In this study, the reduction in

blastocyst quality in medium supplemented with PVA was associated with reduced blastocyst cell numbers.

EGF and IGF-I have been reported to be mitogens inducing a positive effect on preimplantation development and stimulating metabolism and development of embryos (5,22,23). Moreover, these factors are involved in the compaction and formation of the blastocyst, activation of transport systems responsible for the uptake of glucose, enhancement of endocytosis, and probably protein turnover (24,25).

Neira et al. (26) reported that IGF-I accelerated development of bovine blastocysts to hatched blastocysts and a combination with growth factors and cytokines added to SOF medium produced greater percentages of blastocysts and hatched blastocysts than the others. According to several researchers (5,21,24), IGF-I and EGF had mitogenic effects and stimulated the growth and metabolism of embryos. These findings are consistent with our results. In our study, the data observed suggest that when culture media are supplemented with growth factors, the culture system produces more blastocysts and embryos produced have a higher number of cells. Furthermore, the addition of serum to embryo culture medium was similar in its effect in terms of embryonic development with only BSA or BSA supplemented with growth factors, while it was similar to PVA alone or PVA supplemented with growth factors in terms of blastocyst cell allocation.

In assessing the viability of the embryos, morphological observations are most widely used as a gross indicator of embryo viability, but the cell number of the blastocysts and the ICM/total cells ratio are valid indicators of the viability of preimplantation embryos, while morphological criteria alone are poor indicators (27,28). Van Soom et al. (16) reported that blastocyst formation is the first differentiation process during early embryonic development in mammals, yielding the ICM and TE cells. The ICM cells will differentiate into all tissues of the developing fetus. It is well known that the ICM cells contribute to all embryonic tissues and to a part of the extraembryonic membranes, whereas the TE cells mainly form the outer layer of the placenta. Both cell lineages are vital for embryonic and fetal survival (17). In bovine embryos, poor morphology is associated with blastocysts with low numbers of ICM cells (28,29).

According to Koo et al. (17), some factors in the culture media, such as growth factors present in the serum, can modify the distribution of the embryonic cells in favor of the TE cells, which can lead to pregnancy abnormalities encountered after transfer of in vitro cultured bovine and ovine embryos. However, IVF-derived embryos also showed a moderate increase in the ICM/TE cell ratio as compared with in vivo derived embryos. Diaz-Cueto and Gerton (22) reported that IGF-I and IGF-II receptors are only localized in the ICM. Stimulation of cell proliferation is specific to the ICM and the TE cells are not affected. The present study indicates that the addition of BSA and/or PVA supplemented with EGF and IGF-I into the embryo culture medium led to increased ICM cell differentiation. The ICM/total cell ratios of the bovine blastocysts obtained in the present study were similar to those of the previous findings of Choi et al. (3) and Orsi and Leese (30), but lower than in the studies of Duque et al. (7) and higher than those of Krisher et al. (11) and Shirazi et al. (14). Differences between the present study and others may be related to differences in culture and laboratory conditions. Van Soom et al. (16) reported that especially ICM cell numbers were sensitive to environmental influences; precisely for this reason, significantly lower ICM cell numbers were detected in in vitro produced bovine embryos. A difference in allocation of inner cells was noted for in vitro produced bovine embryos, depending on the medium combinations.

In conclusion, the present study demonstrated that defined medium supplemented with PVA can be used in in vitro culture of bovine embryos, but both blastocyst rates and the numbers of cells were significantly lower than those of undefined medium. Bovine embryos cultured in SOF medium supplemented with BSA alone or together with growth factors cleaved faster and reached blastocyst stage earlier than culture medium supplemented with PVA alone or with growth factors. The addition of BSA alone or with growth factors to culture medium also resulted in better blastocyst quality than in the other groups.

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