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Determination of optimal dose of EGF for bovine oocyte maturation and subsequent in vitro fertilization and culture in two media

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Abstract: The aim of this study was to determine the optimal dose of EGF on in vitro maturation of bovine oocytes and to investigate the effect of different IVF and IVC media on development of bovine embryos matured with EGF. Oocytes collected from a slaughterhouse were randomly divided into 4 groups. Each group of oocytes was incubated with different doses of EGF (Control: 0 ng/mL, Group 1: 1 ng/mL, Group 2: 10 ng/mL, and Group 3: 100 ng/mL) for 24 h to determine the optimal dose. Matured oocytes were fertilized with frozen-thawed bull sperm by using the swim-up method. The oocytes in each group were divided into 2 subgroups and cultured in CR1aa or G1.3/G2.3 media. Embryos were evaluated for cleavage (48th h), and 5-8 cell embryo (day 3) and blastocyst development (day 8) after fertilization. The rates of cumulus expansion and nuclear maturation were higher ($P < 0.01$) in GI, GII, and GIII compared to the control group. However, no significant difference was observed on oocyte maturation rates between different doses of EGF. Cleavage rates were higher ($P < 0.01$) in EGF groups than in the control group. No significant differences were obtained in blastocyst development between CR1aa and G1.3/G2.3 media ($P > 0.1$).

Key words: Bovine, oocyte, EGF, maturation, in vitro culture

Sığır oositlerinin maturasyonunda epidermal büyüme faktörünün optimal dozunun belirlenmesi ve sonrasında iki farklı vasatta in vitro fertilizasyonu ve kültürü

Özet: Bu çalışmanın amacı, sığır oositlerinin in vitro maturasyonunda EGF'nin en uygun dozunun saptanması ve EGF ile mature edilen oositlerin farklı IVF ve IVC vasatlarında inkübe edildikten sonra kültür vasatlarının embryo gelişimi üzerine etkilerinin araştırılmasıdır. Bu çalışmada mezbahadan elde edilen sığır oositleri rastgele seçilerek 4 gruba ayrıldı. Her bir gruptaki oositler EGF'nin optimum dozunu belirlemek amacıyla EGF'nin farklı dozlarında 24 saat süreyle inkübe edildi (Kontrol: 0 ng/mL, Grup 1: 1 ng/mL, Grup 2: 10 ng/mL ve Grup 3: 100 ng/mL). İnkübasyon sonrası mature olan oositler dondurulmuş boğa sperması ile swim up metodu kullanılarak fertilize edildi. Dört gruptaki fertil oositler ikiye ayrılarak yarısı CR1aa ve diğer yarısı G1.3/G2.3 vasatlarında kültüre edildi. Kültür sonrası embriyolar bölünme (48. saat), 3. günde 5-8 hücreli embriyo ve fertilizasyon sonrası 8. günde blastosist aşamaları açısından incelendi. Maturasyon kriterleri olan kumulus ekspansiyonu ve nükleer maturasyon oranları Grup 1, Grup 2 ve Grup 3'de kontrol grubuna

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oranla yüksek bulundu (P < 0,01). Ancak farklı EGF dozlarında oosit maturasyonu açısından fark olmadığı tespit edildi. Bölünme oranları, kontrol grubuna göre EGF eklenen gruplarda önemli ölçüde yüksek bulundu (P < 0,01). Blastosist gelişimi açısından, CR1aa ve G1.3/G2.3 kültür vasatlarında inkübe edilen embryolar arasında önemli farklılık saptanmadı (P > 0.1).

Anahtar sözcükler: Sığır, oosit, EGF, maturasyon, in vitro kültür

Introduction

Mammalian oocytes collected from antral follicles can complete meiotic maturation in media in vitro. However, subsequent developments of cattle oocytes matured and embryos cultured in vitro are quite lower than those matured and developed in vivo. This is mainly due to media that can not provide optimal conditions for the oocytes. In vivo conditions cannot be mimicked totally under in vitro situations, but developmental capabilities of oocytes can be improved by supplementation of various hormones, growth factors, serum, cells, follicular fluid, and other substances added to the maturation media and various culture media (1-3).

Sera contain many components including hormones, trace elements, and growth factors. Among the latter, epidermal growth factor (EGF) has been implicated in development. Lonergan et al. (4) showed that when the IVM medium was supplemented with EGF, there was a significant improvement in the maturation rate of cattle oocytes, an altered pattern of protein biosynthesis during maturation, and improved embryo development.

Oocyte maturation and fertilization conditions and embryo culture environments can have major effects on embryo development rates, yield, and quality (5). Techniques of in vitro maturation (IVM), in vitro fertilization (IVF), and in vitro culture (IVC) of bovine oocytes have been continuously studied in recent years to obtain information on developmental mechanisms of the embryo for mass production for research and transfer (2,6). Bovine embryos are commonly produced using many different in vitro systems (5). The most common media used in culture systems are SOF (synthetic oviduct fluid), KSOM, and CR1aa; nevertheless, other media, such as G1/G2, CR2aa, and TCM 199, can also be used (7). One such serum-free culture system is the sequential media G1.2 and G2.2 system. These media were formulated specifically to prevent intracellular stress to the embryo, thereby maintaining embryo viability. Additionally, these media take into account the changing carbohydrate and amino acid requirements of the embryo. As a result these media are able to support high rates of blastocyst development in culture of embryos from many species (8).

The objective of the present study was to determine the optimal dose of EGF in TCM 199 medium for in vitro maturation and to compare different IVF and IVC conditions.

Materials and methods

All the chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise indicated.

Oocyte recovery

Bovine oocytes were obtained by aspiration of 2 to 8 mm follicles from ovaries collected from a slaughterhouse. Only oocytes having a dense cumulus cell mass and homogeneous cytoplasm were selected for the study. Oocytes were washed 3 times with Dulbecco's phosphate-buffered saline. For all culture experiments, maturation of oocytes took place in TCM 199 + 10% heat-inactivated FCS + 50 IU/mL penicillin and 50 µg/mL streptomycin.

In vitro maturation (IVM)

Oocytes were randomly assigned to individual treatment drops (200 mL, 10-15 COCs/drop) and incubated for 24 h for: Control, 0 ng/mL (n = 74), 1 ng/mL (n = 73), 10 ng/mL (n = 82), and 100 ng/mL EGF (n = 78) IVM media in culture plates. All incubations were performed at 39 °C in media under paraffin oil in a humidified atmosphere of 5% CO_2 in air for 24 h. After maturation, cumulus expansion (CE) from the 4 groups was visually assessed under a stereomicroscope (100× and 400×). To assess nuclear maturational status, oocytes were denuded by repeated pipetting, fixed in acetic acid: ethanol (1:3),

and stained with aceto-orcein (1%). Each oocyte was examined (100× and 400×) for metaphase II (M II) chromosome configurations. No assessment was made for the oocytes in those groups used for fertilization.

In vitro fertilization (IVF) and in vitro culture (IVC)

Culture I

Motile spermatozoa were selected by a swim-up technique (9). Matured oocytes were washed twice in HEPES TALP medium for cumulus cell detachment. Oocytes were transferred into IVF-TALP supplemented with 6 mg/mL fatty acid-free BSA media. Sperm were capacitated using 10 mg/mL heparin that was added to the fertilization media. For each mL of the culture media, 1×10^6 spermatozoon and 25 mL penicillamine-hypotaurine-epinephrine (PHE) mixture (comprising 20 mM D-penicillamine, 10 mM hypotaurine, and 1 mM epinephrine) were added into wells (9). For fertilization, media covered by mineral oil were incubated at 39 °C under an atmosphere of 5% CO_2 in air for 20 h. Presumptive zygotes were denuded from cumulus cells and washed 4 times in TL-HEPES and transferred, into 1 mL CR1aa medium supplemented with 3.00 mg EFAF BSA, 0.5 µL gentamicin (50 µg/mL), 100×-50 µL non-essential amino acids, and 50×-100 µl essential amino acids and covered with mineral oil (9). Fertilized oocytes were cultured in CR1aa medium under standard conditions. Embryos were examined under a stereomicroscope (100× and 400×) for cleavage (48 h), 5-8 cell embryos on day 3, and blastocyst stage on day 8 after insemination.

The number of oocytes utilized in Culture I was 112, 104, 123, and 108 for 0 (Control), 1, 10, and 100 ng/mL EGF, respectively.

Culture II

Oocytes were matured and sperm were procured in the same conditions as in Culture I. Next, the oocytes transferred into G-FERT covered by mineral oil were incubated at 39 °C under 5% CO_2 in air for 20 h (in wells). At 20 h after insemination, presumptive zygotes were washed in GMOPS for detaching cumulus cells. After fertilization, presumptive fertilized oocytes were transferred for 72 h of culture in G1 version 3 medium, which contained 2 mg/mL BSA, MEM nonessential amino acids, 1 mM glutamine, 0.1 mM EDTA, and 0.1 mM taurine, and then, for the remaining 120 h of culture in G2 version 3 medium, which contained 2 mg/mL BSA, MEM nonessential and essential amino acids, and 1 mM glutamine. Both G1 version 3 and G2 version 3 media were adjusted to pH 7.6 with 0.1 M NaOH after addition of amino acids. Adjustments to pH were made to culture medium to stabilize bicarbonate during storage. The numbers of oocytes utilized in the Culture II were 107, 125, 117, and 95 for 0 (Control), 1, 10, and 100 ng/mL EGF, respectively. After co-culture, the presumptive embryos were examined under a stereomicroscope (100× and 400×) for cleavage and development at 72 h and 8 d after insemination, respectively.

Statistical analysis

Duplicates of oocytes were assigned to 1 of 4 doses of EGF in 2 cultures in a complete randomized design. Data were subjected to PROC FREQ procedure of SAS and the culture effect was attained using the Chi-square test. Also, the polynomial contrast option was employed to determine the dose-response relationship for EGF.

Results

Twenty four hours of oocyte culture in 10% FCS with TCM 199 containing various concentrations of EGF [1 ng/mL (93.2%; 86.3%), 10 ng/mL (95.1%; 91.5%), and 100 ng/mL EGF (93.6%; 88.5%)] resulted in higher cumulus expansion ($P < 0.01$) and M II ($P < 0.01$) rate than the control (87.8%; 82.4%, respectively) (Figure 1). Regardless of concentration, EGF addition to media positively affected percentages of cumulus expansion and M II. Regression equation revealed that change in cumulus was $y = 90.1 + 0.59x - 0.006x^2$, with R^2 of 0.99 and $P < 0.02$ for intercept and $P < 0.44$ for slope and change in M II was $y = 83.8 + 0.86x - 0.008x^2$, with R^2 of 0.90 and $P < 0.01$ for intercept and $P < 0.22$ for slope in response to EGF concentration, respectively, where y = change in cumulus expansion in the former and y = change in M II phase in the latter, whereas x = EGF dose. Moreover, there was a positive correlation between cumulus and M II ($r = 0.94$, $P < 0.06$).

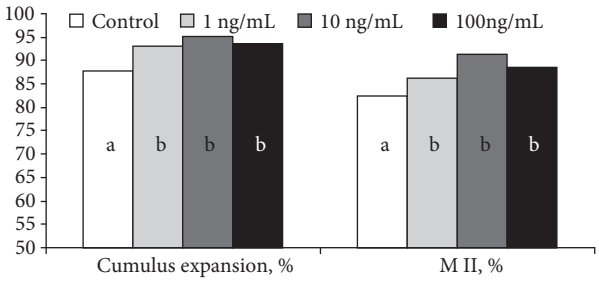

Figure 1. Cumulus expansion and M II percentages in response to EGF concentration in IVM medium.

a-b: Means without common superscripts differ (P < 0.05).

For G1.3/G2.3, the cleavage rate of embryos was significantly different among the control (43.0%), 1 (52.0%), 10 (61.5%) and 100 ng/mL EGF (59.0%) (Figure 2A).

When IVF TALP bovine embryos were cultured with CR1aa containing 0 (control), 1, 10, and 100 ng/mL EGF, the rate of cleavage was significantly different among the groups (Control: 47.3%; 1 ng/mL 60.6%; 10 ng/mL 65.8% and 100 ng/mL 60.2%). Increasing EGF concentration did not affect the cleavage percentage. However, inclusion of EGF to IVM media resulted in increased cleavage (P < 0.01). There was no IVF and IVC media effect on the cleavage ratio (Figure 2A). As shown in Figure 2B there were no differences in the number of 5-8 cell embryos between G1.3/G2.3 and CR1aa embryo cultures supplemented EGF on day 3 after fertilization. However, there were differences (P < 0.01) between EGF and control groups. Although IVM media with EGF yielded more 5-8 cell embryos than the control, the numbers of 5-8 cell embryos among EGF doses were not different. Overall response was quadratic (P < 0.01). Different IVF and IVC procedures did not influence 5-8 cell embryo numbers (Figure 2B).

There were no differences in blastocyst rates between G1.3/G2.3 and CR1aa embryo cultures supplemented EGF on day 8. However, there were differences (P < 0.01) in EGF and control groups. Although IVM media with EGF yielded more blastocysts than control, blastocysts among EGF doses were not different. The overall response was quadratic (P < 0.05). Different IVF and IVC procedures did not influence blastocysts percentage on day 8 (Figure 2C).

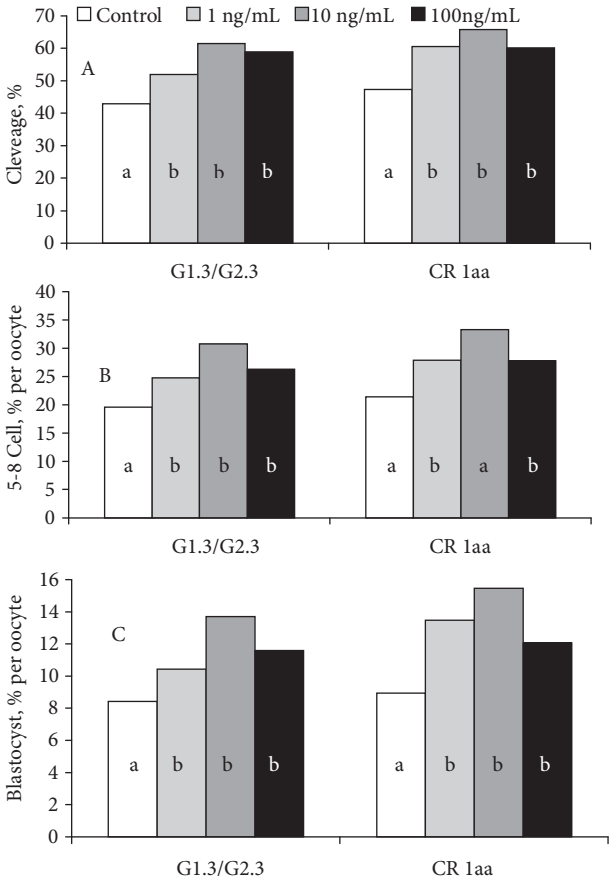

Figure 2. Response of cleavage (A), 5-8 cell embryo (B), and blastocyst production (C) to different media (G1.3/G2.3, CR1aa) on bovine embryos added with EGF concentration.

a-b: Means within media without common superscripts differ (P < 0.05).

Discussion

In this report, EGF at different concentrations was employed in 2 media: CR1aa and G1/G2 version 3, to analyze the effects exerted by EGF on maturation, fertilization and developmental competence in vitro of bovine oocytes using a 5% CO_2 in air environment.

Nagar and Purohit (10) found that cumulus expansion of oocytes increased significantly with EGF supplementation in a dose-dependent manner up to 50 ng mL^{-1}. However, there were no differences in serum-free or serum-supplemented media. The proportion of oocytes showing +++ cumulus expansion was higher for all the supplements

compared to control media. However, the dose dependent effect of EGF supplementation in increasing the proportion of oocytes reaching metaphase II was evident only up to 20 ng mL^{-1}. In accordance with the above mentioned studies supplementation of EGF resulted in a higher ($P < 0.01$) proportion of oocytes reaching M II compared to the control. In the study of Lonergan et al. (4), addition of EGF, irrespective of concentration, or 10% FCS to M199 stimulated cumulus expansion as well as significantly increasing the proportion of oocytes attaining metaphase II. Im and Park (11) reported that in IVM, the EGF + FCS combination significantly increased normal fertilization rates. Unknown factors in FCS may give rise to a synergistic effect of EGF on oocyte maturation (12). The results of the present study indicate that cumulus expansion and nuclear maturation rate of bovine oocytes are significantly higher when they are cultured in a medium (10% FCS with TCM 199) supplemented with various doses (1, 10, 100 ng/mL EGF) than without EGF. However, there were no differences between different doses of EGF supplementation.

In terms of blastocysts produced, the most successful bovine IVM systems have employed bovine serum to optimize oocyte developmental capacity. As pointed out by Harper and Brackett (13), EGF in serum is possibly one of the undetermined components contributing to enhanced oocyte maturation. Lonergan et al. (4) reported that blastocyst rates on days 7 and 9 were significantly improved for oocytes matured in the presence of EGF. In the present study, the rate of embryos developing to cleavage was significantly different among the control, 1, 10 and 100 ng/mL EGF and the rate of embryos developing to cleavage was similarly significantly different from the control. Increasing EGF concentration did not affect cleavage percentage. However, inclusion of EGF to IVM media resulted in increased cleavage ($P < 0.001$).

Some nutrients in culture media have been used in an attempt to meet the nutritional requirements of embryos, but unfortunately media for embryo development are still not optimized and may cause molecular and phenotypic alterations in embryo, fetuses, and neonates (14,15). Xu et al. (16) compared proliferation and apoptosis of mouse embryo in different culture media, including CZB, KSOM, MTF, G1.2/G2.2 sequential culture media, and in human oviductal cell coculture. The authors reported that sequential culture using G1.2/G2.2 was superior to KSOM, MTF, and CZB/CZB-C with respect to the formation of 3 - 4 cell embryos, morulae and blastocysts. G1.2/G2.2 cultured blastocyst had significantly more proliferating blastomeres and higher total cell numbers per blastocyst than those cultured in KSOM or CZB/CZB-C. In our study, there were no great differences among the embryos cultured in G1/G2 and CR1aa media on day 3 after fertilization. However, there were differences ($P < 0.001$) with control groups and culture groups with EGF.

In conclusion, supplementation of EGF in maturation media positively affected cumulus expansion and maturation to MII. However, there were no differences on oocyte maturation rates due to different doses of EGF. Epidermal growth factor supplementation increased embryo development rates. In addition, both the G1.3/G2.3 sequential culture system and CR1aa showed similar support for bovine embryo development in a 5% CO_2 in air environments.

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