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A comparative study of parthenogenetic activation and in vitro fertilization of in vitro matured bovine oocytes

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Abstract: The objective of this study was to determine the effects of 3 chemical agents used sequentially and electrical stimuli on parthenogenetic activation of in vitro matured bovine oocytes and comparison with a standard IVF protocol for embryo developmental rates. For this purpose, oocytes were matured in tissue culture medium-199 (TCM-199) at 39 °C and 5% of CO_2 in humidified air. For IVF, matured oocytes were fertilized in Modified Tyrode-Lactate Medium. In the parthenogenetic activation process, direct current (DC) was pulsed (133 V/500 µm) for 25 µs and oocytes were sequentially activated with calcium ionophore (CaI) for 10 min, cycloheximide (CHX) + cytochalasin D for 1 h, and CHX for 5 h. After that, all embryos (both IVF and parthenogenetic) were cultured in G1.3/G2.3 media containing 6% CO_2, 5% O_2, and 89% N_2 in humidified air at 39 °C.

Cleavage rate was not significantly different following parthenogenetic activation compared to IVF ($P > 0.05$). Morula, blastocyst development, and blastocyst cell numbers were not also significantly different between parthenogenetic activation and IVF ($P > 0.05$). These results showed that bovine oocyte activation with electrical stimulation and chemical agents gave the desirable results, and the culture medium (G1.3/G2.3) supported both parthenogenetic and IVF embryo development.

Key words: Maturation, in vitro fertilization, parthenogenetic oocyte activation, embryo culture

İn vitro mature edilmiş sığır oositlerinin partenogenetik aktivasyonu ve in vitro fertilizasyonunun karşılaştırmalı çalışması

Özet: Bu çalışmanın amacı, in vitro mature edilmiş sığır oositlerinin elektriksel uyarım ve kimyasal ajanlar ile aktive edilmiş partenogenetik aktivasyonu ile standart IVF protokolünün, embriyo gelişim oranları yönünden karşılaştırılmasıdır. Bu amaçla, oositler 39 °C ve % 5 CO_2'li ortamda Tissue Culture Medium–199 (TCM–199)'da mature edildi. IVF işlemi, mature oositlerin modifiye edilmiş Tyrode-Lactate Medium'unda fertilize edilmesi ile yapıldı. Partenogenetik aktivasyonda, oositler 25 mikrosaniye süreyle elektriksel uyarıma (133 V/500 µm) tabi tutuldu ve daha sonra sırayla 10 dakika calcium ionophore (CaI), 1 saat cycloheximide (CHX) + cytochalasin D karışımı ve son olarak da 5 saat süreyle CHX ajanlarında tutulmak suretiyle aktivasyonları tamamlandı. Tüm embriyolar (IVF ve partenogenetik) G1.3/G2.3 mediumunda ve 39 °C'de % 6 CO_2, % 5 O_2 ve % 89 N_2 gaz karışımı ortamında kültüre edilmişlerdir.

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Partenogenetik aktivasyon ve IVF sonrası bölünme oranı yönünden yapılan karşılaştırmada önemli bir farklılık saptanmamıştır (P > 0,05). Morula, blastosist oranları ve blastosist hücre sayıları, IVF ve partenogenetik aktivasyon uygulamaları için karşılaştırıldığında aynı şekilde fark önemsiz bulundu (P > 0,05). Bu sonuçlar göstermiştir ki, sığır oositlerinin aktivasyonunda kimyasal ajanlar ile kombine edilmiş elektrik stimülasyonu uygulaması arzu edilen sonuçlar vermektedir ve kullanılan kültür mediumu (G1.3/G2.3) hem partenogenetik ve hem de IVF embriyolarının gelişimini desteklemiştir.

Anahtar sözcükler: Maturasyon, in vitro fertilizasyon, partenogenetik oosit aktivasyonu, embriyo kültürü

Introduction

Animal cloning by somatic cell nuclear transfer (SCNT) has the potential to improve the productivity of livestock and may eventually prove to be a more economical venture than current breeding methods (1-3).

Ovulated or in vitro matured bovine oocytes are activated in metaphase II (MII) by spermatozoa or by artificial stimulus (4,5). When a spermatozoon activates an oocyte, it promotes multiple and periodic oscillations of intracellular free calcium. These pulses provoke a cortical reaction, resumption of meiosis, maternal mRNA recruitment, pronuclear development, and mitotic cleavage. For an artificial treatment to be efficient, all steps of activation should be achieved (6-8).

Furthermore, parthenogenetic activation is also relevant to cloning research because artificial activation of oocytes is an essential step of nuclear transfer protocols (9-11). This is easily achieved by multiple electric pulses or protein synthesis and phosphorylation inhibitors or by combinations of different treatments. Among several activating artificial agents, some promote intracellular calcium increase, e.g. strontium, ionomycin, electric pulse, and ethanol, and others inhibit protein synthesis (e.g. cycloheximide) or protein phosphorylation (5,7,8,12). Cytochalasin was reported to inhibit extrusion of the second polar body (1). Electrical stimulation is an alternative to chemical activation to induce Ca^{2+} influx through the formation of pores in the plasma membrane. The success of this procedure depends on not only the size of the pores formed but also the ionic content of the medium and the cell type (4,13).

In the present study, we examined the ability of 4 parthenogenetic agents (electrical stimuli (ES) + calcium ionophore (CaI), cycloheximide (CHX) + cytochalasin D, and CHX to activate in vitro matured bovine oocytes and compared them with the standard IVF protocol for embryo developmental rates in the same culture conditions.

Materials and methods

Chemicals

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

Recovery of bovine oocytes

Bovine ovaries were obtained from a local slaughterhouse and transported in 3-4 h to the laboratory in a thermos filled with physiological saline solution (0.9% w/v NaCl) at 34 ± 2.0 °C. Ovarian follicles measuring 2-8 mm in diameter were aspirated with the aid of a vacuum pump armed with an 18-gauge needle (100 mmHg; 28 mL/min). Only oocytes surrounded by compact, dense cumulus cell layers were selected for the maturation by stereomicroscope.

In vitro maturation

Selected cumulus-oocyte complexes (COCs) were washed 3 times in TL Hepes medium and placed into 500 µL of maturation medium in 4-well dishes (Nunc, Roskilde, Denmark), 25-35 oocytes per well. Each well of maturation medium was covered with 400 µL of mineral oil. The medium used for maturation of COCs was TCM-199 containing Earle salts, 2.2 g/L sodium bicarbonate, 10% FBS (fetal bovine serum), 5.5 µg/mL sodium pyruvate, 1% (v:v) penicillin-streptomycin (10,000 U/mL penicillin G, 10,000 µg/mL streptomycin), 5.0 µg/mL bLH (bovine luteinizing hormone), 0.5 µg/mL bFSH (bovine follicle stimulating hormone), and 10 ng/mL EGF (epidermal growth factor) under 400 µL of mineral oil

(14). The pH was adjusted to 7.4 and the osmolality was adjusted to 280 mOsm/kg. Oocytes were incubated at 39 °C in air containing 5% CO_2 with saturated humidity for parthenogenetic activation and IVF process for 19 and 24 h, respectively. Assessment of maturation was done by the degree of expansion of cumulus cell mass and extrusion of the first polar body.

Parthenogenetic activation

Oocytes were stripped of their cumulus 19 h after the start of maturation by vortexing with TL Hepes solution, and those with homogeneous cytoplasm and an extruded first polar body were selected. Oocytes were exposed to a direct current (DC) pulse of 133 V/500 µm for 25 µs (Fusion Machine BLS, CF-150/B, Hungary) and then were activated by CaI (5 µM for 10 min), cytochalasin D (2.5 µg/mL) + CHX (10 µg/mL) for 1 h, and CHX alone for 5 h (9). Electrical stimulation was performed in an electrofusion chamber with 2 stainless-steel electrodes 0.5 mm apart filled with Zimmerman fusion buffer. Following activation, oocytes were washed 3 times with TL Hepes medium, and were transferred to the wells with the culture medium (20 oocytes per well) and incubated for 9 days at 39 °C in humidified atmosphere containing 6% CO_2, 5% O_2, and 89% N_2. The experiments were replicated 4 times.

In vitro fertilization

After 22-24 h of in vitro maturation, COCs were washed twice in TL Hepes and then transferred into 44 µL drops (10-12 oocytes/drop) of fertilization medium (Modified Tyrode-Lactate Medium, 280-300 mOsm/kg) supplemented with 6 mg/mL fatty acid-free BSA, 25 µg/mL gentamicin solution, and 0.25 mM pyruvate (15).

Subsequently, a straw of frozen bull spermatozoa (which was previously tested for IVF) was thawed at 36 °C for 1 min. The thawed semen was layered over 2 mL of both 45% and 90% Percoll discontinuous density gradient in a 15 mL centrifuge tube. The tube was centrifuged at 1200 × g for 15 min to obtain the motile fraction of spermatozoa. The pellet containing the motile sperm fraction was carefully removed from the bottom of the tube with a 1 mL pipette. Sperm was then diluted to 50×10^6/mL sperm in TL Hepes (15). The sperm concentration was counted by hemocytometer using a Thoma counting chamber at 400× magnification and checked for acceptable motility (i.e. at least 80% progressively motile). The percentage of sperm motility was visually evaluated using a phase-contrast microscope at a magnification of 400×. The oocytes were inseminated with 2.0×10^6 spermatozoon/mL (2µL), 2 µg/mL heparin (2 µL), and PHE (2 µL) (penicillamine, 20 mM; hypotaurine, 10 mM; epinephrine 1 mM) (16-18) under 5% CO_2, saturated humidity in air at 39 °C. The experiments were replicated 4 times.

In vitro culture

The culture of parthenogenetic and IVF embryos were performed with 50 µL culture droplets (10-12 embryo/drop) under mineral oil. The culture environment consisted of 6% CO_2, 5% O_2, and 89% N_2 in humidified air at 39 °C. The embryos were cultured for the first 72 h in G1.3, and then embryos were transferred to the G2.3 until the end of the culture period (G1.3/G2.3, Version 3, Vitrolife, Englewood, Colorado, USA) (plus 8 mg/mL fatty acid free-BSA). Cleavage, morula, and blastocyst development rates were evaluated from the presumptive zygotes on days 3, 7, and 9 using a stereomicroscope. The number of nuclei of the blastocysts was determined according to Arat et al. (19). The blastocysts in all experiments were stained with Hoechst 33342 and checked under ultraviolet light to examine the number of nuclei of the embryos.

Statistical analysis

Cleavage rate, morula, blastocyst stages, and cell number of blastocysts in parthenogenetic activation and IVF groups were analyzed using by generalized linear models linked with log likelihood and distribution Poisson by GENMOD procedure in SAS/Proc GENMOD (20).

Results

Recovery of oocytes and in vitro maturation

During the entire study, 1502 ovaries were collected and 1382 (92.01%) used for aspiration of oocytes; the remaining 120 (7.99%) ovaries that were either devoid of follicles or cystic were discarded. Mean recovery of total and maturable oocytes from each ovary was 2.41 ± 0.75 and 1.77 ± 0.65,

respectively, and 73.30% of oocytes matured following IVM.

In vitro fertilization and chemical activation of in vitro matured oocytes

In vitro matured oocytes subjected to IVF, which were considered as controls for chemical activation subsequent cleavage, were assessed. A total of 943 in vitro matured oocytes were subjected to both IVF and chemical activation during the entire study.

Data on cleavage, morula, blastocyst, blastocyst/cleavage rate, and blastocyst cell numbers of parthenogenetically activated and IVF embryos are presented in the Table. A comparison of cleavage rates following parthenogenetic activation 65.0 ± 9.0 and IVF 32.0 ± 25.0 revealed no significant difference ($P > 0.05$). Further development of embryos to morula (34.0 ± 15.0 versus 10.0 ± 9.0) and blastocyst stages (16.0 ± 7.0 versus 17.0 ± 13.0), and blastocyst cell number (95.3 ± 22.3 versus 98.1 ± 19.0) was also not significantly different between parthenogenetic activation and IVF groups ($P > 0.05$). Furthermore, difference in the blastocyst/cleavage rate in the IVF (30.0 ± 4.0) and parthenogenetic activation (20.0 ± 9.0) was also not statistically different ($P > 0.05$) (Table).

Discussion

In the present study ES combined with chemical agents was used to induce oocyte activation in order to evaluate development to blastocysts and development rate of parthenogenetic and IVF embryos was compared to investigate the efficiency of activation protocol. The results of the study showed that oocytes activation by using electrical stimulation combined with chemical agents and IVF produced the same blastocyst rate.

At fertilization in mammals, the sperm cell induces a large release of intracellular calcium in the oocyte. This intracellular calcium release triggers oocyte activation and early development in embryos. In addition to these factors, ages of recipient oocytes and activation agents affect the success of animal cloning (4,12,13,21). Combined treatments with different chemicals for parthenogenetic activation have been widely used for reconstructed oocytes in sheep, cattle, and goats to increase intracellular Ca^{2+} concentration (such as electrical pulse, ethanol, calcium ionophore, or ionomycin) and inhibit protein synthesis or MPF (metaphase promoting factor) activity (8).

Tosti et al. (6) demonstrated that calcium stores decrease during maturation and they state that this may be related to the oocyte quality. The higher electrical events induced by spermatozoa and sperm factor in matured oocytes indicate activation competence at maturation (4,6).

Oocyte maturation and quality are important factors for further development. When we compared the maturation rate with other studies (12,13) the rate was high (73.30%) in our study. This result may be due to supplementation of LH, EGF, and FSH in the maturation medium. Similarly, Liu et al. (22) report that 83%-85% maturation was obtained by addition of LH and EGF to the maturation media in buffalo oocytes.

Table. In vitro embryo development rates following in vitro fertilization and parthenogenetic activation of bovine oocytes matured in vitro.

Experiments	No. of embryos cultured (n)	Embryos cleaved (%)	Morula (%)	Blastocyst (%)	Blastocyst/cleaved embryos (%)	Blastocyst cell number ± SEM
Part. Act.	753	65.0 ± 9.0	34.0 ± 15.0	16.0 ± 7.0	20.0 ± 9.0	95.3 ± 22.3
IVF	190	32.0 ± 25.0	10.0 ± 9.0	17.0 ± 13.0	30.0 ± 4.0	98.1 ± 19.0

Data represent arithmetic means ± SEM. Values within rows are not significantly different ($P > 0.05$).

Part. Act: Parthenogenetic activation, IVF: In vitro fertilization, SEM: Standard Error of Means.

The improved efficiency obtained with combined activation (electric stimuli and chemical agents) versus IVF suggests that bovine oocytes become competent after IVM and that the low cleavage rate recorded in this species is likely to be related to deficiencies in the IVF system (10,21,22). Although we have shown that bovine MII oocyte activation induced by ES, CaI, CHX + cytochalasin D, and CHX did not significantly improve cleavage rates and blastocyst production when compared with the standard IVF protocol, this artificial activation had a similar effect on oocytes with natural ones induced by IVF.

In cattle, depending on the activation method, percentages of oocyte activation vary from 29% to 52% (9). Similarly, in pigs, oocyte activation measured by the presence of pronuclei varies from 22% to 74%, while in rabbits it is lower (from 10% to 38%) (3).

In our study, cleavage rates following parthenogenetic activation (65.0 ± 9.0) and IVF (32.0 ± 25.0) revealed no significant difference ($P > 0.05$) (Table). The cleavage rate was lower than those reported by Arat et al. (9), higher than those reported by Wang et al.(11) and similar to those reported by Liu et al. (22), Dinnyés et al. (23), Booth et al. (5), and Bhak et al. (2). The cleavage rate following different activation treatments (ET + CHX, ET + DMAP; ET + CHX + DMAP) was significantly higher (44.4%-52.5%) than the respective controls (IVF; 23.4%-36.5%), whereas the cleavage rates within the various chemical activation and control groups were not significantly different from each other (22). It is difficult to explain the differences in development rates among ours and other studies since different activation protocols, oocyte sources, and maturation and culture media were used in each study.

The cleavage rate of activated oocytes and their potential for further embryonic development depends on several factors, like species, source and quality of oocytes, IVM conditions, type and composition of culture media, and activating agent. The culture system may also contribute to the low embryo development. The efficiency of the culture system may be expressed in terms of the percentage of embryos that cleaved and reached more than the 8-cell stage (16).

When the interval between fusion and activation of the reconstructed oocytes was prolonged to 3 or 5 h, the blastocyst rates of the reconstructed bovine oocytes activated with A23187 + CHX were significantly decreased (8). Short, high voltage DC electric field pulses cause the formation of temporary pores in the plasma membrane, thus allowing the exchange of extracellular and intracellular ions and molecules (10).

According to Saikhun et al. (21), low percentages of cleavage and blastocyst development from IVF of swamp buffalo oocytes may be due to the low efficiency of the in vitro production (IVP) system. Parthenotes activated by a combined treatment of ES and chemical agents had a lower total blastocyst cell number than that of IVF embryos. However, activation by a combination of ethanol and cytochalasin D resulted in blastocyst development with the total cell number comparable to the IVF embryos.

In this study, the blastocyst development rates were lower than those reported by Liu et al. (22), Dinnyés et al. (23), Booth et al. (5), and Arat et al. (9), higher than those reported by Wang et al. (11), and similar to those reported by Bhak et al. (2). However, the blastocyst cell numbers were higher than those reported by Arat et al. (9) and similar to those reported by Wang et al. (11). The cell number of blastocysts is a good indicator of the quality of embryos. In accordance with the present results, previous studies confirmed that parthenogenetic bovine blastocysts have a significantly lower total cell number than IVF blastocysts. Liu et al. (22) reported that sequential combined activation procedures of CaI + 6-dimethylaminopurine, and CaI + CHX + Cyto-D were effective for the activation and development of young bovine oocytes.

Considering the fact that cleavage and subsequent embryo development following chemical activation [ES + CaI + (CHX + Cyto-D) + CHX] was significantly higher than following IVF, our study indicates that bovine oocytes had better developmental competence and that the poor cleavage and embryo development following IVF may be partly due to the poor quality of frozen/thawed sperm, improper sperm capacitation and/or fertilization.

In conclusion, ES + CaI + CHX + Cyto-D + CHX treatment and IVF produced the same development rates in terms of cleavage, morula and blastocyst production rates, and blastocyst cell numbers. Oocyte activation is a sequence of events triggered by the fertilizing sperm that are required to initiate embryonic development. Parthenogenetic activation induced by ES and chemically is an artificial oocyte activation mimicking fertilization. Therefore, the results indicated that the protocol used in our study was effective on bovine oocyte activation. In addition, G1.3/G2.3 culture medium used for both parthenogenetic and IVF embryos supported embryo development at the same rate. This result showed that G1.3/G2.3, which was developed for human embryo culture, could be used for bovine embryo culture and gave good results. Since parthenogenetic activation is a critical step for embryo development after nuclear transfer and intracytoplasmic sperm injection (ICSI), our activation protocol can be recommended for animal cloning and ICSI for further studies.

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