

Activation of Bovine Oocytes Following ICSI and Effect of Activation on Embryo According to Developmental Stages^[1]

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Summary

In the present study the aim was investigate to Ca ionophore activation in fertilized oocytes by using Intracytoplasmic sperm injection (ICSI) method and the effects of activation considering the stages of embryo development. Cumulus-oocyte complexes (COCs) were collected by aspiration method from the ovaries of cattle and heifers slaughtered at an abattoir. *In vitro* maturation of A quality COCs (n=500) was performed in TCM 199 medium at 39°C in an atmosphere of 5% CO₂ in air with maximum humidity for 22 h. Subsequently, ICSI was carried out to oocytes and randomly divided into two groups. In Group I (n=250; study group) consisted of oocytes activated with 5 µM Ca ionophore at 38.5°C for 5 min, and in Group II (n=250; control group) contained oocytes without any activation. In Group I cleavage, morula and blastocyst rates were determined as 44.8%, 17.2% and 11.6%, respectively. In Group II the rates were established as 26.8%, 9.2% and 3.2%, respectively. Statistically the difference between groups considering the cleavage rates were obtained significantly (P<0.001); however, difference between groups considering morula and blastocyst rates was not determined significantly (P>0.05). In conclusion, the activation of bovine oocytes after ICSI is obligated to apply, and Ca ionophore was a practical and rapid activator to incite cleavage, but inadequate for embryos to reach the morula and blastocyst stages.

Keywords: Activation, Bovine, Ca ionophore, Intracytoplasmic sperm injection, Oocyte

Sığır Oositlerinin ICSI Sonrası Aktivasyonu ve Embriyonun Gelişim Aşamalarına Göre Aktivasyonun Etkileri

Özet

Bu çalışmada amaç ICSI uygulanan oositlerin Ca iyonofor ile aktivasyonu ve bu aktivasyon yönteminin embriyonik gelişim aşamalarına göre etkilerinin araştırılmasıdır. Çalışma materyalini mezbahada kesilen inek ve düve ovaryumlarından aspirasyon metodu ile toplanan kumulus oosit kompleksleri oluşturdu. A kalite kumulus oosit komplekslerinin (n=500) *in vitro* maturasyonu TCM-199 vasatında, 39°C'de %5 CO₂ içeren maksimum neme sahip ortamda 22 saat süreyle inkübe edilerek sağlandı. Matur oositler seçilerek rastgele iki gruba ayrıldı ve ICSI işlemi gerçekleştirildi. Grup I'de (n=250) bulunan oositler 5 µM Ca iyonofor ile 38.5°C'de, 5 dak. süreyle aktive edildi. Grup II'de (n=250) bulunan oositler ise kontrol grubu olarak alındı ve herhangi bir aktivasyon işlemi uygulanmadan ICSI sonrası kültür vasatına kondu. Grup I'de bölünme, morula ve blastosist oranları sırasıyla %44.8, %17.2 ve %11.6 olarak bulunurken, Grup II'de bu oranlar sırasıyla %26.8, %8.2 ve %3.2 olarak belirlendi. Bölünme oranları açısından gruplar arasındaki fark önemli bulunurken (P<0.001), morula ve blastosist oranlarında gruplar arasında istatistiksel olarak anlamlı bir fark bulunamadı (P>0.05). Sonuç olarak, sığır oositlerinde ICSI sonrası aktivasyonun zorunlu olduğu, bölünmenin başlatılmasında Ca iyonofor ile aktivasyonun pratik ve hızlı bir yöntem olduğu fakat embriyoların morula ve blastosist aşamalarına ulaşmasında yetersiz kaldığı belirlendi.

Anahtar sözcükler: Aktivasyon, Sığır, Kalsiyum iyonofor, İntrasitoplazmik sperm enjeksiyonu, Oosit



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INTRODUCTION

Intracytoplasmic sperm injection (ICSI) is a technique used to place mechanically a single spermatozoon directly into oocyte cytoplasm. Cumulus cell - hyaluronic acid matrix barrier, zona pellucida and ooplasmic membrane are skipped with the use of this technique ¹. The procedure of ICSI has expanded the possibilities of assisted reproduction technologies in both animals and humans and has provided a facility for investigation of basic aspects of fertilization such as mechanisms of gamete interaction, sperm-induced oocyte activation and first cell cycle control ².

In several species such as humans ³, rabbits ⁴, mice ⁵ and horse ⁶ do not need any artificial activation methods after ICSI. In mice 70% of injected oocytes develop to the blastocyst stage, while in bovine the rate of blastocyst development following ICSI is not higher than 12-20% ^{5,7}. Bovine ICSI is characterized by difficulties in pronuclear formation. Therefore, increasing *in vitro* development of produced ICSI embryos is considered to suppose use of an artificial activation treatment after injection of sperm ^{8,9}. With the aim of improving activation of bovine oocytes ICSI has been combined with physical (electric stimulation) or chemical activation (Calcium ionophore ¹⁰, ionomycin ¹¹, ethanol ¹², 6-dimethylaminopurin-DMAP ¹³, cycloheximide-CHX ¹⁴ and puromycin ¹⁵). Oocyte activation is a calcium dependent development and Ca ionophore provides a direct calcium influx. Ca ionophore was reported as a very useful chemical to investigate calcium dependent processes in the oocyte activation ¹⁶.

The present study aims to investigate Ca ionophore activation of oocytes fertilized using intracytoplasmic sperm injection (ICSI) method, and the effects of activation considering the stages of embryo development.

MATERIAL and METHODS

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

Collection and In Vitro Maturation of Oocytes

Bovine ovaries were obtained from the local abattoir and transported to the laboratory in 0.9% saline supplemented with 100 IU/ml penicillin, 100 µg/ml streptomycin and 25 µg/ml amphotericin-B at 30-35°C within 3-4 h of collection. The ovaries were washed three times with 0.9% saline, and the contents of the visible follicles (2-8 mm in diameter) were aspirated with a disposable syringe using an 18 G needle. Immature COCs were washed with IVM medium (TCM-199 supplemented with 25 mM HEPES, 5% newborn calf serum-NCS, 0.2 mM sodium pyruvate, 100 IU/ml penicillin, 100 µg/ml streptomycin and 25 µg/ml amphotericin-B) in three different dishes. Selection of COCs were performed under a stereomicroscope (10.X20 - 10.X30) as described by Brackett and Zuelke ¹⁷; afterwards, uniformly dark cytoplasm and surrounding by four or more cumulus cell layers were accepted to be of A quality. A group of 10-15 COCs were put in a drop (100 µl) of the maturation medium and cultured for 22 h at 39°C in 5% CO₂ humidified air. The oocytes with expanded cumulus cells were freed from the cumulus by treatment first with 0.2% hyaluronidase for 1-2 min with gentle pipettings. Only the oocytes with a visible first polar body were selected and transferred into the drops of HEPES TALP medium under mineral oil.

Preparation of Spermatozoa

Three straws of frozen spermatozoa from bulls of proven fertility were thawed in a water bath (37°C) for 30 s. Swim-up method described by Hansen ¹⁸ was used in the selection of motile spermatozoa.

Intracytoplasmic Sperm Injection

Leica DMIL inverted microscope and Narishige MMN1 micromanipulator systems were used for sperm injection. Intracytoplasmic sperm injection was performed in 60 mm plastic dishes. There were 12 rows of medium droplets in the dish. Ten of them consisted of 5 µl droplets of IVF TALP, each containing one oocyte. One row consisted of 40 µl sperm suspension and in the last row was 10 µl



Fig 1. The stages of bovine ICSI. First polar body (arrow) is visible at 6 o'clock (A), Drilling of zona pellucida by using injection pipette (B), Sperm injection into the ooplasm (C)

Şekil 1. Sığırdaki ICSI aşamaları. Birinci kutup hücrenin saat 6 yönünde görünümü (A), Enjeksiyon pipeti ile zona pellusidanın delinmesi (B), Ooplazma içine sperm enjeksiyonu (C)

10% PVP for pipette washing. The injection pipette had an outer diameter of 10-12 µm and the inner diameter of the holding pipette was 10-20 µm. Both pipettes were connected to syringe systems, filled with oil. An individual spermatozoon was immobilized by breaking its tail, with the tip of the injection needle, aspirated tail first and injected into the ooplasm through the zona pellucida. The polar body was either in the 6 or 12 o'clock position and the injection pipette were in the 3 o'clock position (Fig. 1).

Activation of Bovine Oocytes

The injected oocytes were activated according to the method described by Ware et al.¹¹. In Group I (n=250), the injected oocytes were exposed to 5 µM Ca ionophore in Ca-, Mg-free PBS for 5 min, and then washed three times in TCM 199 supplemented with 5% NCS. Injected oocytes in Group II (n=250) were taken to the culture media without any activation as control group.

Embryo Culture

Sperm injected oocytes cultured in groups of 10-15 in 100 µl drops of CR1aa medium for 3 days at 39°C in an atmosphere of 5% CO₂, followed by culture to blastocysts for further 5 days in the CR1aa medium supplemented with 5% NCS. Cleavage, morula and blastocyst rates were assessed 48 h, 120 h and 168 h after ICSI, respectively. Embryonic development was observed by using Leica DFC 280 and M205C stereomicroscopes.

Statistical Analysis

For statistical comparison of data for cleavage, development to morula and blastocyst were analyzed by the chi-square test, Statistic Packet of Social Science (SPSS 14) software. Differences were considered significant at P<0.05.

RESULTS

Following maturation, cumulus expansion was determined in 473 (94.6%) and first polar body expulsion was observed in 438 (87.6%) of 500 COCs (Table 1).

In Group I, the cleavage rate of oocytes activated by Ca ionophore was 44.8%, morula rate was 17.2% and the blastocyst rate was 11.6%. In Group II, the cleavage rate of non-activated oocytes was 26.8%, morula rate was 9.2% and the blastocyst rate was 3.2%.

The rates of cleavage, morula and blastocyst were higher

Table 1. In vitro maturation rates

Tablo 1. In vitro maturasyon oranları

Maturation Criteria	Number	Rate %
Cumulus expansion	473	94.6
First polar body	438	87.6

in Group I than Group II. Cleavage rate was significantly affected by the oocyte activation procedure (P<0.001). Statistically, any significant differences were detected between the groups considering the rates of embryos at morula and blastocyst stages (P>0.05; Table 2).

Table 2. Cleavage, morula and blastocyst rates between groups

Tablo 2. Gruplar arasında bölünme, morula ve blastosist oranları

Stages of Embryo Development	Group I		Group II	
	Number	Rate (%)	Number	Rate (%)
Cleavage	112	44.8 ^a	67	26.8 ^b
Morula	43	17.2	23	9.2
Blastocyst	29	11.6	8	3.2

a, b: Lines labelled with different letters are significantly different (P<0.001)

DISCUSSION

During the fertilization period, a series of morphological and biochemical transformations, known as oocyte activation, take place between the sperm cells and secondary oocytes. In many animal species, calcium signals are key mechanisms in this process¹⁹. Activation is especially required in the ICSI procedure performed to bovine oocytes. For this purpose, many studies have been performed using different activation methods and chemicals. However, currently in which stage of embryo culture Ca ionophore (A23187) is more effective have not been determined. Rho et al.²⁰ carried out a study using ICSI procedure without applying any activation method to spermatozoon and oocyte; subsequently, no pronucleus formation was observed in 28 (35%) out of 79 oocytes. In the other hand, only one pronucleus was obtained following the ICSI procedure without any activation, and the rate was reported as 2.8%¹. Furthermore Keefer et al.⁸, were detected in 1 (4%) of 27 oocytes cleaved, while 23 oocytes were observed to be degenerated. Oikawa et al.²¹ reported a cleavage rate of 3% in oocytes following the ICSI procedure without performing any activation; however, none of embryos can reach the blastocyst stage. Li et al.²² performed a study that investigation the effects of heparin on oocyte activation in embryo medium, and determined the division rate as 74% and blastocyst rate as 28% following the ICSI procedure in the culture medium not contain heparin.

In the present study; cleavage, morula and blastocyst rates were determined as 26.8%, 9.2% and 3.2%, respectively, in the control group oocytes without activation after ICSI. The cleavage rate obtained in the study was higher than the rates reported by Rho et al.²⁰, Chung et al.¹, Keefer et al.⁸ and Oikawa et al.²¹; however, it was lower than the rate determined by Li et al.²².

For activating the oocytes, most frequent preferred

method is Ca ionophore proceeding. It was considered that embryo development rates could be increased by changing activation times with the doses of Ca ionophore. Keefer et al.⁸ reported that activation with Ca ionophore was necessary to obtain formation of male pronucleus and cleavage after ICSI. Chung et al.¹ determined cleavage rate as 27.3% in the oocytes activated by 5 μ M Ca ionophore for 5 min after ICSI activation; however, in these studies oocytes at morula and blastocyst stages could not be obtained. Rho et al.²⁰ determined the cleavage, morula and blastocyst rates as 16.5%, 7.5% and 3.8%, respectively with 5 μ M ionophore application. Keskinetepe et al.¹³ investigated the effects of oocyte activation with different ionomycin doses on embryo development, and determined cleavage, morula and blastocyst stages as 45.7%, 19.3% and 12.1%, respectively, with 5 μ M ionomycin activation after ICSI; however, they determined these rates as 52.1%, 15.7% and 10.7% with 10 μ M ionomycin activation. Goto²³ obtained 15.1% of cleavage rate, 7.4% of morula and 7.3% of blastocyst rate in the oocyte activation with 50 μ M Ca ionophore; on the other hand, they determined 5.6% of cleavage rate in 100 μ M Ca ionophore activation, and no embryo was determined at morula or blastocyst stages.

In the present study, A quality oocytes were subjected to activation with 5 μ M Ca ionophore for 5 min after ICSI, and subsequently, embryonic development stages were observed. Cleavage, morula and blastocyst rates were determined as 44.8%, 17.2% and 11.6%, after activation. These results were found higher than the findings of Chung et al.¹, Rho et al.²⁰ and Goto²³, while they were similar to the findings of Keskinetepe et al.¹³. The difference between the present and previous studies, it was considered that the cleavage, morula and blastocyst rates determined after oocyte activation is caused by the amount of Ca ionophore, activation time and differences in IVC media.

In conclusion, it has been established that oocyte activation is necessary after ICSI in bovine oocytes; in addition, Ca ionophore activation has positive effects on oocyte cleavage; however, when it is insufficient for embryos to reach the morula and blastocyst stages, additional activation methods should be used.

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