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Effects of serum starvation and ionomycin activation on the development of somatic cell nuclear transfer embryos in sheep

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Summary: Synchronization of donor cells and activation of the reconstructed oocytes are important factors affecting the success rate in somatic cell cloning. In this study, it was aimed to investigate the effects of serum starvation in donor cell synchronization and ionomycin treatment in the activation of reconstructed oocytes after somatic cell nuclear transfer in Kıvırcık sheep. Cumulus cells were obtained from a slaughtered sheep ovaries and used as donor cells after serum starvation for 4 days (0.5% FCS; SS) or without serum starvation (10% FCS; S). After reconstruction, oocytes were activated by ionomycin for 5 min plus 6-dimethylaminopurine for 3 h (I+) or only with 6-dimethylaminopurine for 3 h (I-). All cleaved embryos (n= 44) at the second day of in vitro culture were transferred into synchronized recipient ewes (n= 10). Cleavage rates of the embryos were 37.3, 44.1, 34.6 and 44.7% in SS/I+, S/I+, SS/I- and S/I- groups, respectively. Recipient ewes had serum progesterone levels >1 ng/ml at 18th day were 33.3, 50.0, 50.0 and 100.0%, respectively. Only one pregnancy in the S/I- group continued after 40 days however the cloned lamb (7.1%, regarding to embryos transferred) died 10 days before term due to a maternal problem (uterine torsion). The results of this study reveal that somatic cell synchronization by serum starvation and ionomycin treatment for the activation of oocytes can be omitted for the success of somatic cell nuclear transfer in sheep.

Keywords: Ionomycin, serum starvation, sheep, somatic cell nuclear transfer.

Serum açlığı ve ionomisin aktivasyonunun koyunlarda somatik hücre nükleer transfer embriyolarının gelişimi üzerine etkileri

Özet: Donör hücrelerin senkronizasyonu ve yeniden yapılandırılmış oositlerin aktivasyonu, somatik hücre klonlamasındaki başarı oranını etkileyen önemli faktörlerdir. Bu çalışmada, Kıvırcık koyunlarında somatik hücre nükleer transferinde serum açlığının donör hücre senkronizasyonuna ve ionomisinin yeniden yapılandırılmış oositlerin aktivasyonuna olan etkileri araştırılmıştır. Donör hücre olarak kullanılan kumulus hücreleri mezbahadan alınan koyun ovaryumundan elde edildi. Bu hücreler çalışmada 4 gün serum açlığına bırakılarak (%0.5 FCS; SS) veya serum açlığına bırakılmadan (%10 FCS; S) kullanıldı. Yeniden yapılandırma sonrası oositlerin aktive olması için ionomisinle 5 dk ve 6-dietilaminopurin ile 3 saat (I+) veya sadece 6-dietilaminopurin ile 3 saat (I-) inkübe edildi. İn vitro kültürün ikinci gününde, bölünmüş embriyolar (n=44) senkronize edilmiş alıcı koyunlara (n=10) transfer edildi. Embriyoların yarıklanma oranları SS/I+, S/I+, SS/I- ve S/I- gruplarında sırasıyla % 37.3; % 44.1; % 34.6 ve % 44.7 bulundu. 18. günde yapılan progesteron analizine göre kan progesteron seviyesi >1 ng/ml olan taşıyıcı koyun oranları sırasıyla % 33.3; %50.0; %50.0 ve %100.0 olarak tespit edildi. Gruplardan da sadece S/I- grubundan tek bir gebelik 40. günden sonra gelişimine devam edebildi. Ancak bu klon kuzu (transfer edilen embriyo sayısına göre, %7.1) doğuma 10 gün kala maternal bir problem (torsiyo uteri) sebebiyle öldü. Bu çalışmanın sonucunda koyunlarda somatik hücre nükleer transferinde serum açlığı ile somatik hücre senkronizasyon ve ionomisin ile oosit aktivasyon yöntemlerine gerek olmadan klon yavru elde edilebileceği ortaya konulmuştur.

Anahtar sözcükler: İonomisin, koyun, serum açlığı, somatik hücre nükleer transfer.

Introduction

The birth of the first mammal (Dolly, the sheep) produced by somatic cell nuclear transfer (SCNT) method showed that adult somatic cells can be reprogrammed to become totipotent (35) . After Dolly, cattle (12), goat (7), pig (28), cat (31), horse (16), mouse (32) and dog (22) were cloned by using SCNT technology. Although live clone births were obtained in many animal species, survival rates of cloned livestock animals remained below 4% except cattle (30, 34). The reasons of the developmental impairment experienced in somatic cell cloning are the failure of recipient cell failure to be synchronizationed with the donor cell, problems with the activation of the oocyte-somatic cell complex, and in vitro

culture conditions of somatic cells, oocytes and cloned embryos (1, 9). To overcome the synchronization problems, donor cells in G0 or G0/G1 phases are transferred to MII oocytes which contained the intense amount of maturation-promoting factor (MPF). The inhibition of chromosomal reduplication in donor cells by means of MPF may prevent the formation of embryonic developmental disorders (20). In order to synchronize donor cells, serum starvation (35), cyclin dependent kinase 2 (CDK2) inhibitor administration (17) and cell contact inhibition (4) methods are generally used in somatic cell cloning.

After oocyte-donor cell fusion, activation protocols are used to mimic the events occurring in oocyte cytoplasm during fertilization such as free calcium ion fluctuations. Chemical activators used for the oocyte activation are ethanol, calcium ionophore A23187, ionomycin, strontium chloride, or thimerosal (Thi)/dihithiothreiotal (DTT). In order to keep low MPF levels, calcium activators are used together with cycloheximide (CHX) or 6-dimethyl aminopurine (6-DMAP) (2, 9). In sheep, ionomycin and 6-DMAP (3, 8, 18, 21, 25), electrical activation and cytochalasin B (29, 35), calcium ionophore A23187, cycloheximide, and cytochalasin B (11), ionomycin, cycloheximide, and cytochalasin B (23), ionomycin and cycloheximide (3, 8) were used as different activation protocols. There was not superiority in terms of creating live clone animals between these activation protocols. During activation, free calcium ion enhancer chemicals are commonly used to calcium increase in the cell (2). This increase occurs with the influence of ionomycin as well and causes irreversible damage in the activation of cat oocytes (14, 33).

Therefore, the aims of this study were to compare the effects of (1) using serum starvation for the synchronization of donor cells, and (2) using ionomycin for the activation of reconstructed embryos in a 2x2 design on developmental competence of sheep SCNT embryos.

Materials and Methods

Chemicals: Unless otherwise indicated, all the products and chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA).

Preparation of Donor Cells: Cumulus-oocyte complexes (COCs) were obtained from ovaries of Kıvırcık ewes slaughtered. Cumulus cells were recovered from COCs by pipetting method and cultured in Dulbecco's modified Eagle's medium (DMEM), 15% (v/v) fetal bovine serum (FBS), 2 mM L-Glutamine, 1 mM sodium pyruvate, 1% (v/v) MEM nonessential amino acids, 75 mg/ml penicillin G, 50 mg/ml streptomycin in a 35 mm diameter petri dish at 38.5 °C in a humidified atmosphere with 5% CO_2. After 3 passages in cell culture, cumulus cells were frozen by using DMEM medium supplemented with 10% DMSO and 20% FBS and stored in liquid nitrogen. The cells were led up to fifth passage before using and (1) serum-starved at least for 4 days (0.5% FCS, SS) or (2) used without serum starvation (S). Donor cells in both groups were removed from the cell culture petri dishes by using 0.05% Trypsin-EDTA and were suspended. The suspended donor cells were placed into culture medium at 38.5 °C in a humidified atmosphere with 5% CO_2 an h before injection time.

Preparation of Oocytes: Sheep ovaries obtained from the slaughterhouse were transported to the laboratory and washed with 30°C PBS. COCs were obtained from the ovaries by the slicing method and those surrounded by three or more cumulus layers were selected for in vitro maturation. In vitro maturation was carried out in bicarbonate buffered TCM 199 10% FBS, 10 mg/ml of FSH, 10 mg/ml of LH and 0.1 mg/ml sodium pyruvate for 18-20 h according to Birler et al. (8). After maturation, cumulus cells were removed by vortexing the COCs for 2-3 min in Hepes-Synthetic Oviduct Fluid (HSOF) medium supplemented with 600 IU/ml of hyaluronidase. Oocytes with a first polar body and homogeneous cytoplasm were selected for enucleation.

Nuclear Transfer and Fusion: After maturation, oocytes were kept in HSOF medium containing 7.5 mg/ml Hoechst 33342 for 15 min in dark. First polar bodies and chromosome sets of oocytes were aspirated (Figure 1) under a micromanipulation system (TransferMan NK2, Eppendorf AG, Hamburg, Germany) by a micropipette with an internal diameter of 16 µm in a drop of HSOF medium containing 7.5 mg/ml cytochalasin B. Healthy enucleated oocytes were randomly divided into two groups and donor cumulus cells exposed to serum starvation (SS) or not exposed to serum starvation (S) were transferred into the perivitelline space of the oocytes (Figure 2). Somatic cell-oocyte complexes were put between two wires of the fusion petri dish with an interval of 0.5 mm inside (BTX fusion chamber catalog # 45-0103) filled with fusion medium consisted of 0.3 M mannitol, 0.1 mM $MgSO_4$, 0.05 mM $CaCl_2$, 0.5 mM HEPES and 1 mg/ml BSA. For the fusion of donor cell and oocyte complexes, 1.4 kV/cm DC current for a period of 40 µsec 2 pulses were applied (BTX 830, USA). After fusion, reconstructed embryos were incubated in synthetic oviduct fluid (SOF) medium at 38.5 °C, 5% CO_2 for 30 min. At the end of this period, non-fused somatic cell-oocyte complexes were applied with the same electric current dose once again.

*Activation and culture of reconstructed embryos***:** Somatic cell - oocyte complexes in SS and S groups were again divided into two activation groups, and (1) were incubated in HSOF medium containing 5 µM ionomycin for 5 min, then in SOF medium containing 2 mM 6-DMAP for 3 hours (SS/I+ and S/I+), or (2) were incubated in SOF

medium containing 2 mM 6-DMAP for 3 h (SS/I- and S/I-). All groups were then transferred to SOF culture medium drops at 38.5°C in a humidified atmosphere with 5% CO_2, 5% O_2. On the second day of culture, healthy cleaved embryos were selected for transfer into the oviducts of surrogate mothers.

Recipient synchronization and embryo transfer: This study was approved by the Istanbul University Veterinary Faculty Ethical Committee (2004-79).

Ten healthy surrogate Kıvırcık ewes were used for embryo transfer. For estrus synchronization, vaginal sponges containing 20 mg flugestone acetate (Chronogest Cr; MSD Intervet, Turkey) were used for 14 days, and on the day of sponge removal 500 IU PMSG (Chronogest; MSD Intervet, Turkey) was injected to each ewe.

Forty-eight h after sponge removal was considered as Day 0. The cloned embryos after 48 h of in vitro culture were transferred into the oviducts of surrogate mothers on the second day of the cycle (Table 2). Recipient ewes were sedated with xylazine and lumbosacral anesthesia, and embryo transfers were performed by midventral laparotomy. For each transfer, 3 to 5 embryos were placed to the top 2/3 side of oviduct ipsilateral to ovary with corpora lutea. On the 18th day of pregnancy, blood samples were taken from recipients and progesterone levels were evaluated by radioimmunoassay using a commercial kit (IM1188, Progesterone RIA Kit, Immunotech Inc., Prague, Czech Republic). Then, the results were read by a GAMMA counter (Genesys Genii Multi-Well, Laboratory Technologies Inc., Elburn, U.S.A). Recipient ewes having higher than 1 ng/ml progesterone level were considered as pregnant. On the 45th day of pregnancy, ultrasound examinations were performed by 7.5 MHz transrectal probe. Pregnant ewes were kept under control by transabdominal ultrasound examinations per month until the end of pregnancy.

Statistical Analysis: Experiments were repeated for 4 times. Comparison of significance between groups was performed using the Kruskal Wallis test. Statistical analysis was performed by SPSS for Windows version 13.0.

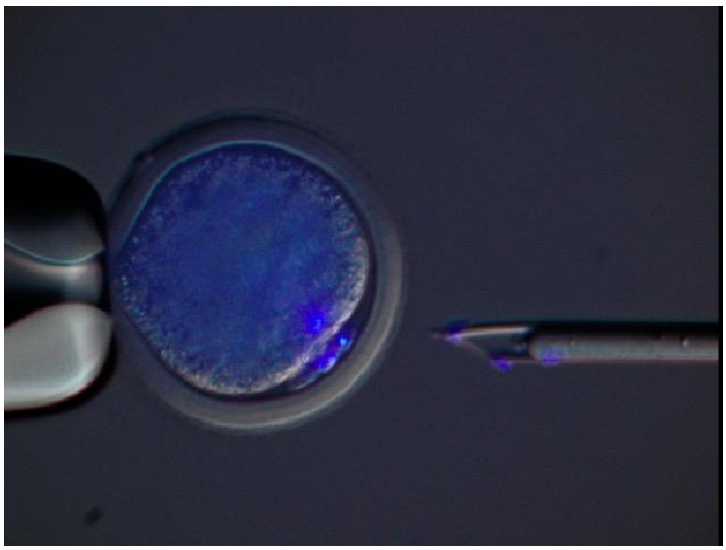

Figure 1. Hoechst 33342 stained metaphase plate and first polar body were removed by enucleation pipette.
Şekil 1. Hoechst 3342 ile boyanmış metafaz plağı ve birinci polar cisimciğin enükleasyon pipeti ile çıkarılması.

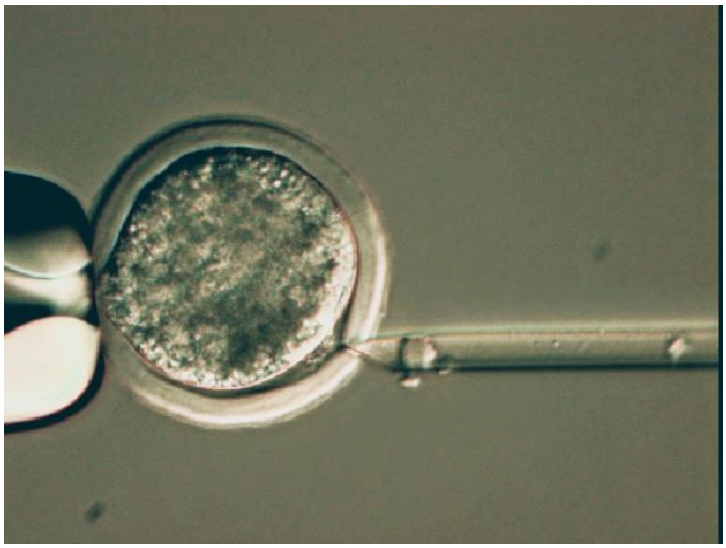

Fig 2. Each donor cell was introduced into a single enucleated oocyte by microinjection pipette.
Şekil 2. Donör hücrelerin mikroinjeksiyon pipeti ile eneküle edilmiş oositlerin içine yerleştirilmesi.

Table 1. Development rates of SCNT embryos in vitro
Tablo 1. SCNT embriyolarının in vitro gelişim oranları

Groups	Serum Starvation	Ionomycin Treatment	Fused Oocyte	Embryo Development Stages	
				Cleaved (%)	Fragmented (%)
SS/I+	Yes	Yes	51	19 (37.3)	2 (3.9)
S/I+	No	Yes	34	15 (44.1)	4 (11.8)
SS/I-	Yes	No	52	18 (34.6)	1 (1.9)
S/I-	No	No	47	21 (44.7)	1 (2.1)

SS/I+ : Serum starvation with ionomycin treatment; SS/I- : Serum starvation without ionomycin treatment; S/I+ : Ionomycin treatment without serum starvation; S/I- :Without serum starvation and ionomycin treatment.

Table 2. Pregnancy and offspring rates of SCNT embryos after transfer
Tablo 2. SCNT embriyolarının transfer sonrası gebelik ve yavru oranları

Groups	Serum Starvation	Ionomycin Treatment	Transferred Embryos	Recipient Ewes	Day 18 Ewes Progesterone levels >1,0 ng/ml (%)	Day 45 Pregnant Ewes (%)	Offspring Developed Near to Term (%)
SS/I+	Yes	Yes	11	3	1 (33.3)^{b}	-	-
S/I+	No	Yes	10	2	1 (50.0)^{b}	-	-
SS/I-	Yes	No	9	2	1 (50.0)^{b}	-	-
S/I-	No	No	14	3	3 (100)^{a}	1 (33.3)*	1 (7.1)‡

SS/I+ : Serum starvation with ionomycin treatment; SS/I- : Serum starvation without ionomycin treatment; S/I+ : Ionomycin treatment without serum starvation; S/I- :Without serum starvation and ionomycin treatment.
*: Lamb died at 140th day of pregnancy due to uterine torsion of recipient ewe.
‡: According to embryos transferred.
a,b: Data with different superscripts are statistically different ($P<0.05$).

Results

After somatic cell nuclear transfer, 51, 34, 52 and 47 fused embryos were cultured in vitro in SS/I+, S/I+, SS/I- and S/I- groups respectively. Developmental status of cloned embryos in in vitro culture are given in Table 1. There were not statistical differences in cell cleavage and fragmentation rates between the groups ($p>0.05$).

Cloned embryos were examined on the second day of the culture and a total of 44 healthy cleaved embryos were selected and transferred into the oviducts of 10 synchronized recipient ewes (Table 2). According to the blood progesterone levels, 6 recipients in 4 groups had more than 1 ng/ml blood progesterone levels (Table 2). Progesterone levels were higher in recipients in S/I- group than other three groups (3.80-23.99 ng/ml vs. 1.00-1.85; $p<0.05$). After ultrasound examinations at 45 days, it was observed that only one recipient in the S/I- group out of 6, was pregnant. That pregnancy was terminated 10 days prior to birth due to torsion of the uterus and the cloned lamb was lost. There were not any disorders regarding cloning in lamb and placenta during the pathological examination.

Discussion and Conclusion

Producing genetically identical animals in livestock species by somatic cell nuclear transfer technology can provide important improvements in basic and applied sciences. The main obstacles, however, against using cloning techniques widely are the high cost of SCNT studies, weak cloned offspring and lower success (1). Although due to the inherent potential of cloning studies the offspring rate could be raised up to 20-25% in cattle (26), live offspring from SCNT studies have still remained below 5% in sheep (8, 24, 35). Although blood progesterone levels of recipient ewes at Day 18 were found >1 ng/ml in 6 recipients in this study, only one recipient ewe in the S/I- group was pregnant (33.3%) at Day 45 based on ultrasound examinations, and the fetus (7.1%) reached nearly full term (Table 2).

In somatic cell nuclear transfer technology, the basic necessity for successful results is the synchronization between the donor cell and the oocyte. Somatic cells which are not subjected to serum starvation or contact inhibition are in G1 phase at a rate of up to 60% (19). Although G0 phase of the donor nucleus is thought to be

the best stage for nuclear reprogramming, G1 and M phases can be led to reconstructed embryos to full-term (19). There are also several reports that after serum starvation, apoptosis and DNA fragmentations can occur in donor cells and affect subsequent embryo development (13, 15, 27). In this study there are not significant differences in cleavage and pregnancy rates between groups ($p>0.05$). These results indicated that cumulus cells can be reprogrammed without using serum starvation and other techniques. Likewise, Cho et al. (10) showed similar cleavage and blastocyst rates by using cumulus cells as donor cells regardless of serum starvation in cattle while ear fibroblast cells having higher blastocyst rate after serum starvation.

Loi et al. (25) recommended using ionomycin treatment with 6-DMAP for activation of oocytes which is less toxic than ethanol even if blastocyst rates of parthenogenetic embryos are similar in both groups. There were not statistically significant differences in cleavage and fragmentation rates of cloned embryos activated using ionomycin with 6-DMAP or only 6-DMAP ($p>0.05$). However, the difference regarding progesterone levels >1 ng/ml at Day 18 was significant in S/I- group ($p<0.05$).

Prolonged high levels of calcium ions in the oocyte cytoplasm have toxic effects which can prevent by inducing calcium ion fluctuations into the cytoplasm similar to ones during the fertilization. However, in activation of sheep oocytes by using ethanol or ionomycin, a single wave of calcium comprises and increased levels continues for a long time at the same level (25). Wang et al. (33) indicated that different concentrations of ionomycin form a similar single calcium fluctuation and that increase continues for a long time by creating irreversible toxic effects in sheep. Asgari et al. (5) recommended using of ionomycin for oocyte activation which has been shown to be important to reach the blastocyst stage in sheep SCNT. Reaching to the blastocyst stage can also be affected by ionomycin concentration and duration, oocyte source (fresh or frozen) or amount of enucleated cytoplasm (MPF and Ca^{+2} ion loss) (5, 9). In sheep SCNT, ionomycin (5 µM concentration for 5 min) is generally used to obtain proper activation although causing some toxicity or developmental delay problems (3, 6, 8, 18, 23, 24, 25). Since ionomycin uses calcium stores of restructured oocyte's cytoplasm, it may not be suitable activator for oocytes with different calcium ion capacity. In this study, similar cleavage and pregnancy rates have been obtained without ionomycin after SCNT which showed no need for extra ionomycin in sheep. However, fragmentation rates in ionomycin used groups (I+) are higher than (I-) groups numerically, but these differences are not significant ($p>0.05$).

In conclusion, while there is a limited number of pregnancy results, the S/I- method can be a preferable option in somatic cell nuclear transfer studies in sheep. However, more studies are needed to obtain a more reliable conclusion.

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