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Research Article

# Somatic Cloning in Cats Using MI or MII Oocytes [1]

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## Abstract

Animal production via SCNT provides a unique tool for protection of valuable individuals, conservation of vulnerable and endangered species and production of transgenic animals. A total of 167 MI and 219 MII stage oocytes were used as the material of the study. The oocytes were enucleated at 44 h after in vitro maturation by aspiration of the polar body and the MI or MII plates. Cycling granulosa cells were used for nuclear transfer. Cell fusion was induced with DC pulses of 2.0 kV/cm 60µs, 0.1s apart (2x) delivered by a BTX Electrocell Manipulator 200 (BTX, San Diego, CA, USA). After fusion, the embryos were activated by 1.0 kV/cm 20µs DC pulses 0.1s apart (2x) followed by 2 mM 6-DMAP (6-dimethylaminopurine) incubation in culture medium for 4 h in a humidified atmosphere of 5% $CO_2$, 5% $O_2$, and 90% $N_2$ at 38°C. The somatic cell transferred embryos were cultured for 8 days in mSOF medium supplemented with 0.4% BSA in a humidified 5% $CO_2$, 5% $O_2$, and 90% $N_2$ atmosphere at 38°C. After in vitro culture period, all embryos transferred to HSOF containing Hoechst 33342 (5 µg/mL) and the cell numbers were counted under ultraviolet light using a fluorescent microscope. The fusion (66.66 vs 21.55%) and cleavage rates (15.75 vs 11.11%) were significantly higher in MII stage oocytes than MI stage oocytes ($P<0.02$). While SCNT embryos were developed to morula stage in MII group (14; 9.58%), all the cleaved embryos were arrested at the 2-4 cell stage in MI group. None of the embryos was developed to blastocyst stage in both groups.

***Keywords:*** *Cat, SCNT, In vitro, MI - MII oocytes*

# Kedilerde MI ve MII Oositleri Kullanilarak Somatik Klonlama

## Özet

Somatik klonlama yoluyla hayvan üretimi, üstün değerdeki bireylerin korunması, savunmasız ve tehlike altında bulunan türlerin korunması ile transgenik hayvanların çoğaltılmasına hizmet eder. Çalışmanın materyalini 167 adet MI ve 219 adet MII dönemdeki oosit oluşturdu. Polar cisimciklerin (MII) ve kromatin setlerin (MI ve MII) enükleasyonu, 44 saatlik in vitro olgunlaştırma periyodunun ardından gerçekleştirildi. Nükleer transfer amacıyla siklik dönemlerdeki granüloza hücreleri kullanıldı. Oosit-somatik hücre komplekslerinde füzyon işlemi, DC akımın sağlandığı 2.0 kV/cm 60 µs, 0.1s ara (2x), BTX Electrocell Manipulator 200 (BTX, San Diego, CA, USA) ile gerçekleştirildi. Aktivaston işlemi için ise, 1.0 kV/cm 20µs DC akım 0.1s ara (2x) kullanıldı ve ardından 2 mM 6-DMAP (6-dimethylaminopurine) içerisine alınarak, 38°C'lik sıcaklık ve %5 $CO_2$, %5 $O_2$ ve %90 $N_2$ gaz karışımının sağlandığı inkübatörde 4 saat süresince kültüre edildi. Somatik hücrelerin nakledildiği klon embriyolar daha sonra aynı inkübatör koşullarında %0.4 BSA katkılı mSOF medyumu içerisinde 8 gün boyunca in vitro kültüre bırakıldılar. Ardından klon embriyolar, embriyonik hücre sayılarının tespiti amacıyla Hoechst 33342 (5 µg/mL) içeren HSOF medyumu içerisine alındı ve ultraviyole küplü floresan mikroskobunda değerlendirildi. Sonuçta, füzyon (%66.66-21.55) ve yarıklanma oranlarının (%15.75-11.11) MII dönem oositleri lehine önemli derecede üstün olduğu belirlendi ($P<0.02$). MII grubunda 14 adet embriyonun (%9.58) morula döneme kadar geliştiği gözlenirken, MI grubunda ise, yarıklanan tüm embriyoların 2-4 hücreli dönemde kaldığı ve her iki gruptan da hiçbir embriyonun blastosist aşamasına ulaşamadığı gözlendi.

***Anahtar sözcükler:*** *Kedi, SCNT, In vitro, MI-MII oosit*

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## INTRODUCTION

Most of the wild felid species are described as endangered, rare or vulnerable because of human destruction of natural ecosystems and habitat loss. Domestic cats are commonly used as a model to develop assisted reproductive technologies for the protection of endangered felids and for biomedical research [1]. They may also allow a recipient female for embryo transfer (ET) and recipient cytoplasm for nuclear transfer (NT) from several species of small wild cats [2,3]. Animal production by somatic cell nuclear transfer (SCNT) and interspecies somatic cell nuclear transfer (iSCNT) serves a sole tool for protection of precious individuals, genetically modified animals, conservation of endangered species and a number of opportunities for simple and practised research in human medicine [3-5]. Cats are often used to represent normal physiology and human diseases which are especially located in nervous system and kidneys in studies [4-6]. After the first mammalian Dolly the sheep was produced from a somatic cell from an adult animal [7], studies evolved rapidly and many other cloned animals have been produced by the use of MII oocytes as cytoplasts (sheep [8], ferret [9], dog [10], horse [11], mule [12], cat [13], pig [14], cattle [15], goat [16] and mouse [17]) so far.

In meiotic maturation period, many cytoplasmic and nuclear changes occur that arrange the oocytes for fertilization. During this period, the changes start by activation of mitogen-activated protein (MAP) kinase and maturation-promoting factor (MPF) [18]. In the recipient oocytes, the MPF activity is critical for the reprogramming of nuclei of reconstructed embryos [19]. Also the MPF is thought to be a critic factor in maintaining meiotic resumption [20,21]. The nuclear membrane of donor cells' with low activity of MPF stays stable at the pre-activated oocytes. The DNA synthesis occurs dependently to the stage of the original cell cycle at the time of nuclear transfer [19] and reprogramming of nucleus takes place during the expansion of donor nucleus [22]. After parthenogenetic activation, the nuclear membrane reorganised and DNA synthesis starts [23]. It is stated that MPF activity is greatest at both MI and MII throughout maturation period and because of the high activity of MPF the nuclear membrane of the donor cell is broken down and the chromosomes are condensed [19]. In the amphibian it is found that the most advanced tadpoles generated from MI compared to MII stage oocytes [24]. Moreover in a recent study [25], it is showed that somatic cell transferred porcine MI oocytes are developed to blastocyst stage.

To our knowledge there is no study about the ability of reprograming the somatic cell nuclei of MI stage cat oocytes so far. This study was performed to evaluate the development of domestic cat embryos reconstructed by transferring somatic cells into enucleated MI or MII oocytes *in vitro*.

## MATERIAL and METHODS

The experiment was performed in accordance with guidelines for animal research from Istanbul University Ethics Committee on Animal Research (2011/84).

### *Chemicals*

Chemicals were purchased from Sigma Chemical Co. (St. Louis, MO), unless otherwise indicated.

### *Collection of Ovaries and Oocyte Recovery*

The oocytes were obtained from ovaries of 29 ovariohysterectomised cats at local veterinary clinics. These operations were performed under general anaesthesia and the ovaries were transported to the laboratory in 2-3 h with Phosphate Buffered Saline (PBS) at 35°C. The cumulus oocyte complexes (COCs) were collected by slicing and washing the ovarian surface with oocyte washing medium. The COCs were selected according to their structure of cumulus cells and ooplasm integrity [26].

### *In Vitro Maturation (IVM)*

The maturation medium was Nutrient Mixture F-10 Ham (Ham's F-10) (N-6635). The medium was supplemented with 10 µg/mL FSH (F-2293), 10 µg/mL LH (L-5269), 20 ng/mL EFG (Invitrogen; 13247-051), 0.4% BSA and antibiotics. The selected COCs were maturated in four-well petri dishes (NUNCR, Denmark) in 500 µL maturation medium at 38°C in a humidified 5% $CO_2$, 5% $O_2$, and 90% $N_2$ atmosphere for 44 h. For each experimental group, 30-40 COCs were placed for each perti dish well.

### *Source of Somatic Cells*

Cumulus cells were provided from immature cat oocytes. They were disaggregated mechanically by gentle pipetting in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% FCS (v:v) and 75 mcg/mL penicillin G, 50 µg/mL streptomycin, washed by centrifugation and the pellet was suspended in handling DMEM medium supplemented with 15% FCS (v:v). The cumulus cells were cultured *in vitro* until confluent, then were passaged 2 to 3 times before being used for nuclear transfer (NT).

### *Preparation of Recipient Cytoplasm and Somatic Cell Nuclear Transfer (SCNT)*

The *in vitro* maturated oocytes were denuded by vortexing in HSOF medium plus 11.5 mg/mL hyaluronidase for 1 min. Oocytes with an extruded first polar body were considered mature (MII stage; *Fig. 1*) and the oocytes without polar body were separated for an examination under a fluorescent microscope after Hoechst (33342) staining. Oocytes having a MI spindle were selected as MI stage and oocytes with germinal vesicle (GV), germinal vesicle breakdown (GVBD), undetermined nuclear material (UDNM) or degeneration

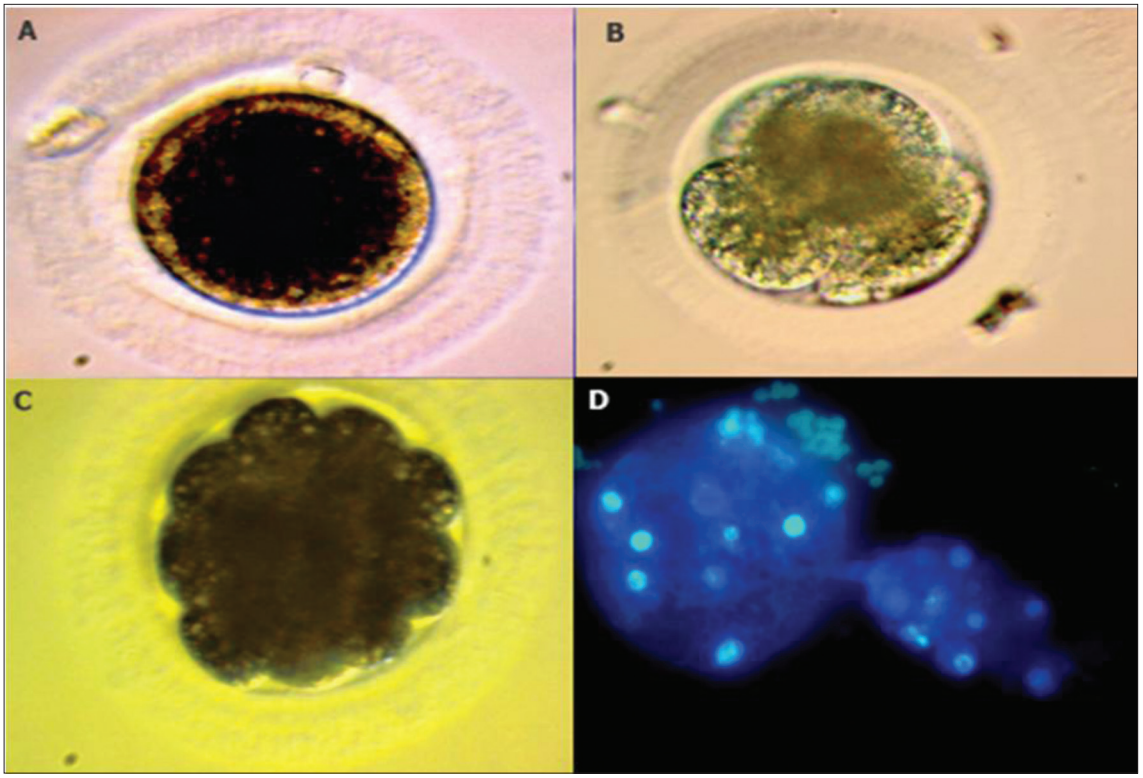


**Fig 1.** *In vitro* matured cat oocyte and embryos developed after SCNT. **A-** M II stage oocyte with extruded first polar body (x200), **B-** 4-cell stage embryo (x200), **C-** Morula (x200), **D-** Morula stained with Hoechst

**Şekil 1.** *İn vitro* olgunlaştırılmış kedi oositi ve SCNT sonrası embriyoların gelişimi. **A-** Birinci polar cisimciği ile M II dönemde bir oosit (x200), **B-** 4hücreli dönemde bir embriyo (x200), **C-** Morula (x200), **D-** Hoechst ile boyanmış bir morula (x200)

were discarded. All the selected oocytes (MI or MII) were placed in HSOF media supplemented with 7.5 µg/mL cytochalacin B and were enucleated by aspirating their first polar body and the MI and MII plates with a small volume of surrounding cytoplasm. Successful enucleation was confirmed under ultraviolet light using a fluorescent microscope. After enucleation procedure, the cytoplasts were washed in Calcium free SOF medium and were held in this medium nearly 1 h until somatic cell injection [27]. Micromanipulation was performed by inserting a small size (14-16 µm) individual cumulus cell into the perivitelline space of each enucleated oocyte.

### *Electrofusion and Activation*

The electrofusion and activation protocols were performed according to Yin et al.[27]. Briefly, somatic cell-cytoplast complexes were transferred to a fusion chamber with two electrodes 500 µm apart, filled with fusion medium (0.3 M mannitol, 0.1 mM $CaCl_2$, and 0.1 mM $MgSO_4$ and 0.05% fatty acid–free BSA) at room temperature. Cell fusion was induced with two DC pulses of 2.0 kV/cm 60µs, 0.1s apart delivered by a BTX Electrocell Manipulator 200 (BTX, San Diego, CA, USA). The fused NT embryos were incubated in HSOF plus 1% essential and 1% non-essential amino acids for 1 h. Then, the cytoplasts were activated by exposure of two 1.0 kV/cm 20 µs DC pulses 0.1 s apart by followed 2 mM 6-DMAP (6-dimethylaminopurine) in culture medium for 4 h in a humidified atmosphere of 5% $CO_2$, 5% $O_2$, and 90% $N_2$ at 38°C.

### *In Vitro Culture*

The NT embryos were cultured in mSOF medium supplemented with 0.4% BSA in a humidified 5% $CO_2$, 5% $O_2$, and 90% $N_2$ atmosphere at 38°C with high humidity. At day 3 of culture, all cleaved embryos were transferred to a fresh mSOF medium supplemented with 1.5 mM glucose (G 6152) and cultured for an additional 3 d. After IVC period, all the embryos transferred to HSOF containing Hoechst 33342 (5 µg/mL) for 30 min and the cell numbers were counted under ultraviolet light using a fluorescent microscope *(Fig.1)*.

### *Statistical Analysis*

The experiment was replicated 10 times. Statistical analysis was performed using by a "Mann Whitney U" test by SPSS for Windows version 13.0.

## RESULTS

A total of 386 oocytes (167 at MI and 219 at MII stage) were used as the material of the study. The number of somatic cell-cytoplast complexes, the number of cleaved NT embryos after fusion and the developmental stage of

***Table 1.*** *Developmental rate of cat embryos reconstructed by transferring cumulus cells into enucleated M I and MII oocytes*
***Tablo1.*** *Enüklee edilmiş olan MI ve MII dönem oositleri içerisine kumulus hücreleri enjekte edilerek klonlanan kedi embriyolarının gelişim oranları*

| Oocyte Stage | No. of Complexes | | No. of Cleaved (%) | No. of Embryos Developed to (%) | | | |
|---|---|---|---|---|---|---|---|
| | n | Fused (%) | | 2-4 Cell | 4-8 Cell | Morula | Blastocyst |
| M I stage | 167 | (21.55)[a] 36 | (11.11)[a] 4 | (11.11) 4 | (0.00) 0 | (0.00)[a] 0 | (0.00) 0 |
| M II stage | 219 | (66.66)[b] 146 | (15.75)[b] 23 | (6.16) 9 | (0.05) 3 | (9.58)[b] 14 | (0.00) 0 |

*[a,b] Different letters indicate statistical differences among columns (P<0.002)*

SCNT embryos' are summarized in *Table 1*. The fusion and the cleavage rates of MII stage oocytes were significantly higher than MI oocytes (P<0.02). 14 (9.58%) SCNT embryos were developed to morula stage in MII group. All the cleaved embryos of MI group are arrested at the 2-4 cell stage.

## DISCUSSION

Although offspring production from SCNT has been noticed in different mammalian species, the overall success rates are under the expected levels (1-11%). There are too many known and unknown factors and suboptimal situations affecting the production of live healthy animal *in vitro* [28,29]. In cats, it is demonstrated that the fusion (45-66%) and blastocyst (3-8%) rates of cycling fetal fibroblast, adult fibroblast, muscle and cumulus cells were similar [28]. It is stated that the mammalian cell cycle stage of the donor cell nucleus has multiple effects on embryo re-construction and is a main factor in the achievement of NT [19]. There are many different methods for synchronizing cells cycle such as; serum starvation, contact inhibition, chemical cell cycle inhibitors [1,29]. Some researchers achieved pregnancy in sheep [30] and produced calves [31] by transferring both cycling and non-cycling somatic cells and they suggested that cell synchronization by serum starvation is not obligatory. In this study, somatic cells were used without having serum starvation. In cats, it is stated that although the source of the donor nucleus affected the rate of blastocyst development, the cell cycle synchronization method did not [3]. There are some live cloned cat offspring have been acquired after the transfer of embryos reconstituted with cells synchronized by serum-starvation [2,27] and cycling cells [27]. The somatic cells (fibroblasts and cumulus cells) of the cat have a naturally long G0/G1 phase and it is claimed that serum starvation induces the apoptosis process [32].

The fusion and cleavage rates of couplets vary according to the meiotic stage, the quality and the way of maturation of the oocyte, the somatic cell type, the cell synchronization method, the fusion technique and *in vitro* culture conditions [25,28,29]. Both *in vivo* and *in vitro*-matured cat oocytes have been used as recipient cytoplasts for production of cloned embryos and the fusion rates of *in vivo* matured oocytes found higher than the *in vitro* matured oocytes [28]. In this study, *in vitro* matured cat oocytes were used as cytoplast and the fusion rates in MII stage couplets were significantly higher than the MI couplets (21.55 vs 66.66%). The fusion rate of the MII stage couplets were similar to the researchers (45-66%) that they used cycling cumulus cat cells [28], but the MI stage rates (21.55%) were lower than the results in pig results (29-60%) using MI stage oocytes [25]. In the present study, the cleavage rates of couplets were found higher in MII than the MI stage oocytes (15.75 vs 11.11%) and 14/146 of MII stage couplets were developed to morula stage (9.58%). However, all the cleaved MI stage couplets (11.11%) were arrested at early stages (2-4 cell) and others were degenerated. Although Miyoshi et al.[25] showed that porcine MI oocytes have a potential to develop into blastocysts (1.5%) after nuclear transfer of somatic cells, we observed that MI stage cat oocytes have the potential to cleave to early stages (2-4 cell); however, they have no potential to subsequent development. The low developmental rate of MI oocytes in this study can be concluded as a result of difference among animal species.

It is reported that 1 to 11% of cat cloned embryos reached to the blastocyst stage *in vitro*, regardless of synchronization method or the cell type that used [28]. It is known that the highest proportion of mature oocytes is reached between 42 and 45 h of *in vitro* culture [28,33]. Therefore, the oocytes were cultured for 44 h for *in vitro* maturation in this study. However, researchers [34] stated that a prolonged maturation period of 43 h influenced *in vitro* development of reconstructed cat embryos. The extending period leads to lower fusion rates, lower development of embryos to the morula and blastocyst stages. Although MII stage oocytes were cleaved and some of them (9.58%) reached to morula cell stage, they couldn't reach the morula and blastocyst stages in this study. This situation could be related to the length of *in vitro* maturation, and the possible variations of *in vitro* conditions. It is demonstrated that nutrition is important factors on the maturation, fertilization and further development of oocyte and embryos *in vitro* [35]. However, the poor nutritional conditions of the spayed street cats which have been oocyte donors could be another affecting factor.

Although nuclear transfer technique is developed

rapidly, it is not clear how much progress can be attained by optimizing the available procedures. The mechanism of reprogramming somatic cells after nuclear transfer is complex and still unclear. In the present study, it is concluded that the reprogramming events in MI and MII oocytes could have important differences and although using MI oocytes as cytoplasts may provide the opportunity of increasing donor cell numbers. However, it is not supposed to be likely.

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