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RESEARCH ARTICLE

Effect of Different Activation Techniques on Immature and *In Vitro* Matured Cat Oocytes [1]

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Summary

This study was conducted to determine the most successful techniques on inmature and *in vitro*-matured cat oocytes that were parhtenogenically activated using 6-dimethylaminopurine (6-DMAP) and cycloheximide (CHX), in combination with electrical stimulation and calcium ionophore. After 44 h of *in vitro* maturation, the oocytes with a polar body were separated as mature (M II) and those without a polar body were considered as immature. Four different activation treatments and two control groups were used for parthenogenetic activation with both mature and immature cat oocytes. After 48 h of activation, the oocytes were examined and the non-cleaved oocytes removed. The cleaved oocytes/embryos were cultured *in vitro* in mSOF medium for an additional four days. After six days of *in vitro* culture (IVC), embryo quality was evaluated. The results in the present study suggested that (I) both *in vitro* matured and immature cat oocytes have a potential to develop to morula and blastocyst stages after parthenogenetic activation, (II) electrical stimulation + 6-DMAP is a more useful technique for both matured and immature cat oocytes and (III) to our knowledge, this is the first report that describes morula and blastocyst formation from parthenogenetically activated immature cat oocytes.

Keywords: *Parthenogenetic, Activation, Cat, Oocyte, Immature oocyte*

Farklı Aktivasyon Tekniklerinin Olgun Olmayan ve *In Vitro* Olgunlaştırılmış Kedi Oositleri Üzerine Etkisi

Özet

Bu çalışma olgun olmayan ve *in vitro* olgunlaştırılmış kedi oositlerinin partenogenetik aktivasyonu üzerine 6-Dimetilaminopurin (6-DMAP) ve Sikloheksimidin (CHX), elektik uyarımı ve kalsiyum ionoforla birlikte farklı kullanım kombinasyonlarının denendiği bir çalışma olarak tasarlandı. *İn vitro* olgunlaştırma (IVM) aşamasının 44. saatinde polar cisimciği attığı gözlenen oositler olgun (MII), atmayanlar ise olgun olmayan (MI) olarak kabul edildi. Aktivasyon sonrası kültüre aktarılan oositler 48 saat sonra değerlendirildi ve yarıklanmayanlar kültürden çıkarıldı. Yarıklanan oositler/embriyolar Modifiye Sentetik Ovidukt Medyumu (mSOF) içerisinde dört gün daha kültüre devam ettirildi. Kültürün altıncı gününde de embriyolar kaliteleri yönünden değerlendirilerek kaydedildi. Bu çalışma sonuçları göstermiştir ki, (I) gerek olgun, gererekse de olgun olmayan kedi oositleri parthenogenetik aktivasyon sonrasında morula ve blastosist aşamasına ulaşabilmektedir, (II) elektrik ve 6-DMAP'ın birlikte kullanıldığı aktivasyon tekniği, olgun ve olgun olmayan oosit aktivasyonu gruplarının her ikisinde de en başarılı sonuçları vermiştir, (III) çalışmada olgun olmayan oositlerin aktivasyonundan elde edilen morula ve blastosist aşamasındaki embriyolar bu alanda ilktir.

Anahtar sözcükler: *Partenogenetik, Aktivasyon, Kedi, Oosit, Olgun olmayan oosit*

INTRODUCTION

Animal production by somatic cell nuclear transfer (SCNT) provides a number of opportunities for basic and applied research and genetic protection in human medicine [1]. Domestic cats are a useful research model to develop

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assisted reproductive technologies for the conservation of endangered felids and for use in biomedical research [2]. They are the preferred species for studies of normal physiology and human diseases, particularly in neuroscience and kidney diseases [3-6]. In the process of fertilization, interaction between a sperm cell and an oocyte triggers off a series of morphological and biochemical transformations, known as oocyte activation [7].

Oocyte activation is induced under natural conditions by the sperm penetrating to the egg and plays a key role in meiosis. Meiosis in the matured mammalian egg is blocked at the metaphase II (MII) stage, when the first polar body is extruded from the egg. Further progress of meiosis depends on the activating stimulus. This stimulus is brought into the egg by the sperm during fertilization and it involves mechanisms inducing the oscillation of intracellular levels of free calcium ions [8].

Artificial oocyte activation is a very important step determining the success of SCNT studies. An artificial stimulus causes pulsatile increases in intracellular calcium concentrations, induce activation and inhibit MPF activity and mitogen activation promoter factor (MAPK) of the oocytes without penetration of the sperm. These causes depolarization of the oocyte membrane, a release of cortical granule contents and a hardening of the zona pellucida [8,9]. It is suggested that activating stimulus alone is not adequate to completely activate mammalian oocytes [10,11] and it was effectively demonstrated that electroporation alone efficiently triggered the inactivation of M-phase promoting factor (MPF) but not that of MAPK. For the attainment of low MAPK activity, electrical activation should be followed by protein synthesis inhibitors [11].

Several different activating stimulants such as ionomycin, ethanol or electrical pulses, protein synthesis inhibitors such as cycloheximide (CHX) and 6-(Dimethylamino) purine (6-DMAP) are widely used to induce the artificial activation of mammalian eggs [12]. CHX and 6-DMAP are the protein synthesis inhibitors responsible for decreasing MPF and MAPK activity of the oocyte and restarting meiosis [13]. Alternatively, the effect of electrical stimulation influences movement of calcium ions in the oocyte and ionomycin establishes a complex that transports calcium ions through the membrane and into the oocytes [7,8,14].

In recent years, several different animal species have been cloned by using MII oocytes as the recipient cytoplasm in sheep [15,16], cattle [17], mouse [18], goat [19], pig [20], dog [21] and cat [22,23].

It is known that MPF activity is important for the reprogramming of the nucleus in reconstructed embryos [24,25]. It was identified that MPF activity during oocyte maturation is maximal at MII [26]. Nevertheless, it was demonstrated that MI oocytes may also reprogram somatic cell nuclei [27]. In the amphibian, although adults could not be generated from such reconstructed embryos, it was observed that the greatest output of tadpoles came from differentiated somatic cell nuclei injected into immature MI oocytes [24,28].

Although, several publications on artificial activation of cat oocytes exist [10,14,29], most of them are related to activation of MII stage oocytes during nuclear transfer studies. Only few studies had compared different methods of activation and their effectiveness, as measured by the number of activated oocytes and parthenogenetic embryos [7,13]. So far, a common activation method for cats has not been developed as the process is highly species specific. Nevertheless, there is no data in the literature about immature cat oocytes being used for parthenogenetic activation or SCNT purposes.

Because parthenogenetic activation protocols are still not well described in cats, the aim of the present study was to evaluate parthenogenetic activation in *in vitro*-matured and non-matured cat oocytes, using 6-DMAP and cycloheximide, combined with electrical stimulation and calcium ionophore.

MATERIAL and METHODS

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

Collection of Ovaries and Recovery of Oocytes

Ovaries were collected from 26 domestic cats (crossbred of different breeds; ages 1-3 years) by routine ovariohysterectomy following anesthesia at different local veterinary clinics and maintained in Phosphate Buffered Saline (PBS) at 35°C for 3 h. The ovaries were sliced with a scalpel blade and rinsed in washing medium [30] at room temperature in order to obtain cumulus oocytes complexes (COCs). COCs were washed three times with Ham's F-10 medium [31]. The oocytes with dark homogenously pigmented ooplasm and completely surrounded by at least four layer of cumulus cells were selected for *in vitro* maturation (IVM) *(Fig. 1)*.

In vitro Maturation (IVM)

Nutrient Mixture F-10 Ham (Ham' F-10) (Sigma; N-6635) was used as a maturation medium, supplemented with 10 µg/ml FSH (Sigma; F-2293), 10 µg/ml LH (Sigma; L-5269), 20 ng/ml EFG (Invitrogen;13247-051), 0.4% BSA and antibiotics. Selected COCs were maturated at 38°C for 44 h in four-well petri dishes (NUNCR, Denmark), which included 500 µl maturation medium under mineral oil in each well [22,32]. Incubations for IVM were carried out in a humidified 5% CO_2, 5% O_2, and 90% N_2 atmosphere with 100% humidity. For each experimental group, 30-40 COCs were separately placed in each well according to the number of obtained oocytes per replication. After

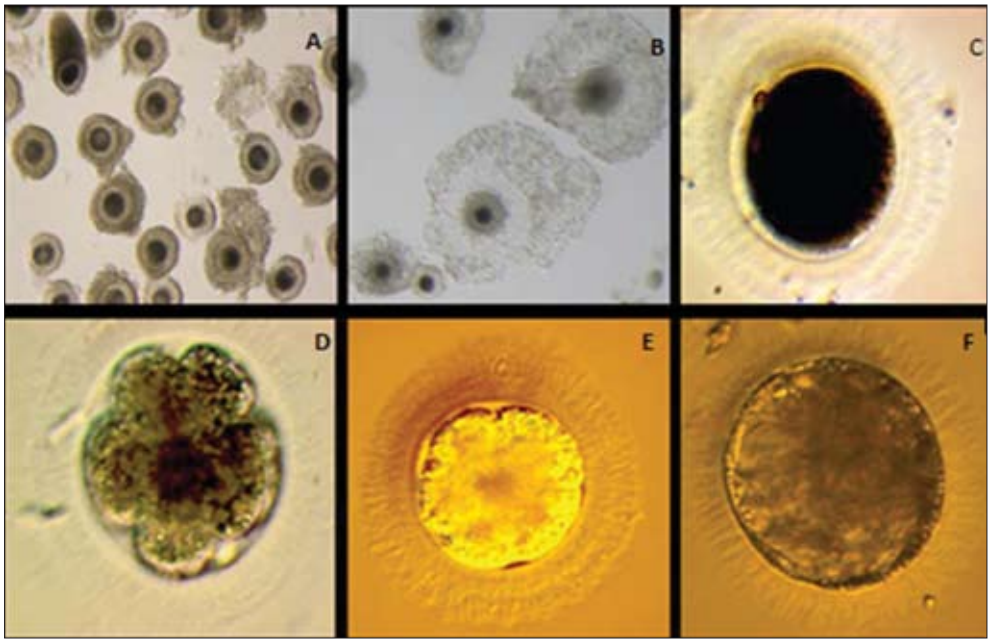

Fig 1. A- immature cat oocytes (x8), **B**- oocytes with expanded cumulus oophorous after *in vitro* maturation (x8), **C**- M II stage oocyte with extruded first polar body (x200), **D**- 8-cell stage embryo (x200), **E**- compact morula (x200), **F**- expanded blasocyst (x200)

Şekil 1. A- olgun olmayan kedi oositleri (x8), **B**- *in vitro* olgunlaşma sonrası kumulus ooforus genişlemesi gösteren oositler (x8), **C**- birinci polar cisimciği atmış M II aşamasında bulunan oosit (x200), **D**- 8-hücreli aşamada bulunan embriyo (x200), **E**- kompakt morula (x200), **F**- genişlemiş blastosist (x200)

IVM, oocytes denuded by vortexing in synthetic oviduct fluid (SOF) medium with Hepes (hSOF) plus 11.5 mg/ml hyaluronidase for 1 min allowed for the selection of intact immature and M II oocytes.

Experimental Design

After *in vitro* maturation, the oocytes were separated into two groups according to maturity. The oocytes with a first polar body were separated as mature MII and those without a polar body were considered as immatured. Five replicates were performed for each treatment.

Two control and four activation groups' experiments were performed. In the control groups, oocytes were cultured without activation treatment to confirm that parthenogenetic activation was promoted by the proposed protocols and not by the culture conditions. Both the mature and immature oocytes were subjected to the following same treatments.

Control Group 1: Oocytes were incubated in culture medium for 5 min and then transferred into the fresh culture medium (mSOF) and cultured for a further 6 days.

Control Group 2: Oocytes were incubated in fusion medium for 5 min and then transferred into the culture medium (mSOF) and cultured for a further 6 days.

In both control groups 1 and 2, oocytes were cultured in the absence of any activation agent.

The treatment groups included electrical stimulus and chemical agents as described below:

Treatment Group 1 (Electrical Stimulation + 6-DMAP): 1.0 kV/cm 20 µs DC pulses 0.1 s apart (2x) + 2 mM 6-DMAP (sigma D 2629).

Treatment Group 2 (Electrical Stimulation + CHX): 1.0 kV/cm 20 µs DC pulses 0.1 s apart (2x) + 10 µg/ml CHX (sigma C4859).

Treatment Group 3 (CaI + 6-DMAP): 5 µg/ml CaI (sigma C 7522) + 2 mM 6-DMAP.

Treatment Group 4 (CHX + 6-DMAP): 5 µg/ml CaI + 10 µg/ml CHX.

In treatment groups 1 and 2, the electricial stimulation to induce oocyte activation was performed by the cell fusion apparatus BTX 830 electro cell manipulator (BTX, San Diego, CA, USA). The chamber used contained two parallel platinum wire electrodes spaced 0.5 mm apart and overlaid with cell fusion medium including 0.3 M mannitol, 0.1 mM $CaCl_2$, 0.1 mM $MgSO_4$, and 0.05% fatty acid–free BSA at room temperature [33]. One hour after electrical stimulation, the oocytes were incubated in a synthetic oviductal fluid plus 1% essential and 1% non-essential amino acids (mSOF) supplemented with 2 mM 6-DMAP for group 1; and 10 µg/ml CHX for group 2. The incubation lasted 6 h at 38.5°C in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2. For the treatment groups 3 and 4, the oocyes were activated by using 5 µg/ml CaI for 5 min and additionally exposed to 10 µg/ml CHX and 2 mM 6-DMAP for 6 h. After 48 h of activation, the oocytes were examined and the non-cleaved oocytes accepted as non-activated and removed. The cleaved oocytes/embryos were *in vitro* cultured for an additional 4 days.

In vitro culture of activated oocytes

Activated oocytes were cultured in mSOF medium supplemented with 0.4% BSA [34]. At day 3 of culture, all cleaved embryos were transferred to a fresh SOF medium supplemented with 1.5 mM Glucose (G 6152; Sigma) and

cultured for an additional 3 days to evaluate their ability to develop to the morula and blastocyst stages.

Statistics

Each experiment was replicated 5 times. Kruskal-Wallis test was applied to compare the activation and developmental rates of oocytes *in vitro*.

RESULTS

A total of 571 immature and 422 *in vitro* matured oocytes were activated and 322 oocytes allocated to the control groups. We evaluated the cleaved embryos as the total number of parthenogenetic activated oocytes. *Table 1* and *Table 2* show the number of oocytes and the rates of cleavage of the mature and immature oocytes activated by each treatment group.

In this study, the positive effects of both activator agents (Electrical stimulus and CaI), plus two protein synthesis inhibitors (6-DMAP and Cycloheximide) on the parthenogenetic activation of *in vitro* matured (with polar body) cat oocytes was demonstrated. Although, all four treatments effectively induced parthenogenetic activation of *in vitro* matured domestic cat oocytes, the first treatment group (Electric + 6-DMAP) was the most successful group, giving higher rates for the formation of morula-blastocyst stages *(Table 1)*.

For the immature oocyte (without polar body) groups, although there were no significant differences for their activation rates among the first three groups, the 6-DMAP + Electrical stimulus and CaI treatment groups were the most successful for the formation of morula-blastocyst rates. For the Electric stimulus + CHX group no oocyte reached to morula-blastocyts stage ($P<0.05$) *(Table 2)*. Moreover, the highest degenerated oocyte rates were found in Electric stimulus + CHX groups in both mature and immature oocyte groups *(Table 1* and *Table 2)*.

DISCUSSION

In this study, no statisticall differences on parthenogenetically activated rates between all MII oocyte treatment groups were observed. Parallel to our findings, others [10,35] reported improved results when calcium ionophore was used in combination with protein synthesis inhibitors in cat and cattle.

Although there were no differences on parthenogenetic activation rate among all treatment groups in *in vitro* matured oocytes exposed to the protein synthesis inhibitor 6-DMAP after being subjected to electrical stimulus, the attainment of morula-blactocyst rate was increased (12.38%). Although researchers [13], reported satisfactory morula attainment results (22-25%) in CHX exposed cat oocytes, in our study the lowest morula-blastocyst rates were obtained in electrical stimulation and calcium ionophore

Table 1. *The results of parthenogenetic activation and in vitro embryonic development of mature (M II) stage cat oocytes*
Tablo 1. *Olgun (M II) kedi oositlerinin pathenogenetik aktivasyon ve embriyonik gelişim sonuçları*

Treatment Groups	Technique	n	Activated Oocytes (%)	Morula-Blastocyst (%)	Fragmentated Oocytes (%)	Degenerated Oocytes (%)
1	Electric + 6-DMAP	105	39 (37.14)^{a}	13 (12.38)^{a}	16 (15.24)^{bc}	6 (5.71)^{ab}
2	Electric + CHX	111	36 (32.43)^{a}	3 (2.70)^{b}	19 (17.11)^{bc}	14 (12.61)^{a}
3	Cal + 6-DMAP	95	25 (25.51)^{ab}	9 (9.47)^{ab}	16 (16.32)^{bc}	2 (2.04)^{b}
4	Cal + CHX	108	30 (27.77)^{a}	4 (3.70)^{b}	4 (3.70)^{c}	5 (4.62)^{ab}
5	Fusion Medium Control	77	8 (10.39)^{bc}	1 (1.29)^{b}	17 (22.07)^{ab}	0 (0.00)^{b}
6	Negative Control	92	6 (6.52)^{c}	1 (1.08)^{b}	34 (36.95)^{a}	2 (2.17)^{b}

Values with different superscripts in the same column are significantly different (a,b,c) (P<0.05)

Table 2. *The results of parthenogenetic activation and in vitro embryonic development of immature (MI) cat oocytes*
Tablo 2. *Olgun olmayan (M II) kedi oositlerinin pathenogenetik aktivasyon ve embriyonik gelişim sonuçları*

Groups	Technique	n	Activated Oocytes (%)	Morula - Blastosist (%)	Fragmentated Oocytes (%)	Degenerated Oocytes (%)
1	Electric + 6-DMAP	108	31 (28.70)^{a}	8 (7.40)^{a}	6 (5.55)^{bc}	6 (5.55)^{a}
2	Electric + CHX	118	23 (19.49)^{ab}	0 (0.00)^{b}	1 (0.84)^{bc}	13 (11.01)^{a}
3	Cal + 6-DMAP	220	67 (30.45)^{a}	15 (6.81)^{ab}	13 (5.90)^{bc}	13 (5.90)^{a}
4	Cal + CHX	125	16 (12.80)^{ab}	1 (0.80)^{ab}	1 (0.80)^{c}	11 (8.80)^{a}
5	Fusion Medium Control	93	11 (11.82)^{b}	0 (0.00)^{b}	18 (19.35)^{a}	6 (6.45)^{a}
6	Negative Control	70	12 (17.14)^{ab}	2 (2.85)^{ab}	7 (10.00)^{b}	5 (7.14)^{a}

Values with different superscripts in the same column are significantly different (a,b,c) (P<0.05)

groups exposed to CHX in both immature and matured oocyte groups (2.7-3.7%). These differences may depend on the use of different activator agents such as roscovitine, and strontium in the Rascadoa et al.[13] study. In contrast, others demonstrated that aging oocytes undergo artificial activation more rapidly than oocytes matured for shorter (24 h) intervals [36]. It was previously reported that cat oocytes matured *in vitro* for 24 h had concentrations of MAPK and MPF significantly higher than those matured for longer periods [37]. However, it was demonstrated that maturation of cat oocytes for longer periods (42-45 h), reduced post-fertilization embryonic development rates [22,29]. In the present study, domestic cat oocytes were matured *in vitro* for 44 h and subjected to four different activation protocols. The low morula-blastocyts rates in our study may be releated to the long maturation period.

It was previously suggested that embryo viability strongly supports the morphological observations signifying that no chromosomal damage is induced by 6-DMAP treatment after activation [38]. Our findings are supportive of these results. Although there were no statistical differences among immature oocyte treatment groups, using 6-DMAP provides higher benefit than CHX on the viability (non degeneration) of oocytes. The observed differenceses were significant among MII oocyte treatment groups.

It is reported that the process of nuclear reprogramming may be different between mature and immature oocytes, since developmental abilities of reconstructed embryos vary between the maturation stages of recipient oocytes [27]. Parallel to these statements, in the present study the morula-blastocyst rates in MII stage oocytes were higher than the same clusters of immature oocytes for all activation groups. Some researchers [12] found positive effects of the protein synthesis inhibitor CHX on the activation and subsequent parthenogenetic development of *in vitro* matured pig oocyte, activated by calcium ionophore. In our study, the protein synthesis inhibitors 6-DMAP and CHX although shown to have similar positive effects on activation rates and in terms of morula-blastocyst formation rates, 6-DMAP was more effective than CHX in both MII and imature stage oocytes.

Although the developmental abilities of reconstructed embryos vary between the maturation stages of recipient oocytes, it shown that porcine MI oocytes have a potential to develop into blastocysts after SCNT and this situation may be associated with the differencies in the process of nuclear reprogramming between MI and MII oocytes [27]. Our findings are parallel to these results where immature cat oocytes have a potential to develop to morula and blastocyst stages after parthenogenetic activation. This observation demonstrates that this was not a species-specific event. To our knowledge, this is the first report that describes morula and blastocyst formation from parhenogenetically activated immature cat oocytes.

In conclusion the current study demonstrates that (I) both matured (MII) and immature cat oocytes have a potential to develop to morula and blastocyst stages after parthenogenetic activation; (II) there was no statistical difference in the development to the morula and blastocyst stage for the CaI + 6-DMAP and Electrical stimulation + 6-DMAP group for matured (MII) and immatured cat oocytes; (III) the current study indicated that immature oocytes can develop to morula and blastocyst stage and therefore should be tested as nuclear recipients for SCNT.

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