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RESEARCH ARTICLE

Comparison of Two Different Media for *In vitro* Production of Dog Embryos [1]

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Summary

Embryo production via *in vitro* fertilization and nuclear transfer has been accomplished in the dog, and the transfer of the cloned embryos has recently resulted in the birth of puppies. However, the efficiency of these technologies is still very limited. Until now, only two morulas and single blactocyst have been achieved *in vitro*. Therefore, the aim of the present study was to examine the effects of two different media (mSOF and TCM 199) on *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and *in vitro* culture (IVC) of immature dog oocytes. The study was performed in two steps. At the first step, the effects of two different media, on IVM of dog oocytes were investigated. At the end of the IVM period, the nuclear maturation rates were evaluated by aceto-orcein staining method. At the second step, after the IVM period the oocytes inseminated with fresh spermatozoa for 24 h and left for IVC for 7 d. At the end of the IVC period, embryonic development was assessed by microscopic observation at 24 h intervals and then fixed and stained by the same method. Consequently, maturation rates of oocytes in mSOF medium were significantly higher than those of the TCM 199 (P<0.001). After 7 d of the IVC period, only one oocyte was cleaved in the TCM 199, while eight oocytes cleaved and one of them developed to morula stage in mSOF medium group (P=0.037).

Keywords: *Dog, Oocyte, In vitro, Maturation, Fertilization*

İn Vitro Köpek Embriyosu Üretimi Üzerine İki Farklı Medyumun Etkisi

Özet

Köpeklerde *in vitro* fertilizasyon yöntemi ile embriyo üretilebilmiş ve nükleer transfer yöntemiyle klonlanmış embriyolardan canlı bir doğum elde edilebilmiş olmasına karşın, bu alandaki teknolojilerin başarısı oldukça sınırlı düzeydedir. Bu güne kadar yapılan *in vitro* çalışmalarda, sadece iki adet morula ve bir adet blastosist elde edilebilmiştir. Sunulan çalışmanın amacı, iki farklı medyumun (mSOF ve TCM 199) köpek oositlerinin *in vitro* maturasyon (İVM), *in vitro* fertilizasyon (İVF) ve *in vitro* kültürü (İVK) üzerine etkilerini araştırmaktır. Çalışma iki aşamada gerçekleştirildi. Birinci aşamada, iki farklı medyumun köpek oositlerinin İVM'u üzerine etkisi araştırıldı. İVM sürecinin sonunda fikze edilen oositlerin nükleer olgunlaşma değerlendirmesi, aseto-orsein ile boyanarak yapıldı. Çalışmanın ikinci aşamasında ise, İVM sonrasında oositler taze sperma ile 24 saat İVF'a tabii tutuldular ve ardından yedi gün boyunca *in vitro* kültüre edildiler. Embriyoların gelişimsel kontrolleri her 24 saatte bir mikroskop bakısıyla kontrol edildikten sonra, yedinci günün sonunda aynı metotla fikze edilip boyanarak değerlendirildiler. Sonuç olarak, İVM oranları açısından mSOF medyum grubunda bulunan oositlerin TCM 199 grubundakilere nazaran daha başarılı olduğu belirlendi (P<0.001). İVK sürecinin sonunda TCM 199 medyum grubunda sadece bir adet oosit yarıklanma gösterirken, mSOF grubunda sekiz adet oositin yarıklandığı ve bunlardan birisinin de morulaya ulaştığı saptandı (P=0.037).

Anahtar sözcükler: *Köpek, Oosit, İn vitro, Olgunlaştırma, Fertilizasyon*

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INTRODUCTION

Biotechnological studies continue for the protection of genetic materials of both domestic and endangered exotic animals and these technologies are essential for genetic salvage program in endangered canid species [1]. The oocyte of the domestic dog is unique from that of other mammalian species. Ovulation occurs with the oocyte released at the germinal vesicle stage, and then completing nuclear and cytoplasmic maturation within the oviduct. *In vivo* meiotic maturation of the bitch oocyte is completed within 48-72 h after ovulation [2]. The first study on *in vitro* maturation (IVM) and fertilization (IVF) of dog oocytes was reported by Mahi and Yanagimachi [3]. In that study, 25% of oocytes obtained from bitches were able to resume meiosis and developed to metaphase I (MI) or to MII stages after being cultured for 48-72 h. *In vitro* insemination of cultured oocytes resulted in 70% of oocytes penetrated by spermatozoa and 20% containing decondensed sperm head(s). Since that report, little progress has been made on *in vitro* maturation and *in vitro* fertilization of dog oocytes *in vitro* [2]. Although the IVM and cleavage rates ranging between 20-40% and 8-37% respectively have been reported [4,5] the results of the studies on IVF and IVC are still very low. Until now, only two morulas [6,7] and a single blactocyst [8] have been achieved.

Although domestic dog puppies were recently produced from somatic cell nuclear transfer of *in vivo* matured oocytes [9] and in another study a single, non full-term pregnancy after transferring close to 100 presumptive zygotes to recipient bitches [10] was described, there has been no report on the production of live young from transferring IVM/IVF-derived embryos. The low rates of canine embryo development testify to the inefficient developmental competence of oocytes matured *in vitro* and justify the main focus of research in the dog to be IVM and IVF [4].

Due to these inherently novel traits, *in vitro* culture systems developed for maturing oocytes of other species have been found inadequate for the maturation of dog oocytes [2]. The rates of meiotic completion, IVF and IVC rates of canine oocytes have varied and still been very low, because the oocytes used for experiments have been collected from different sources and cultured with a variety of different culture systems and media [11]. Therefore, the aim of the current study was to examine the effects of two different media (modified synthetic oviductal fluid and tissue culture medium 199) on IVM, IVF and IVC of canine oocytes *in vitro*.

MATERIAL and METHODS

All chemicals and reagents were purchased from Sigma Chemical Company (St.Louis, MO, USA), unless otherwise indicated.

Ovaries were harvested from 55 mixed breed bitches (1-5 years of age) at the time of routine ovario-hysterectomy. Bitches were at various stages of the estrous cycle. The ovaries were placed in Dulbecco's phosphate buffered saline (PBS) at 4°C and transported to the laboratory within 2-3 h [12]. After keeping at room temperature for 10-15 min, the ovaries were minced and rinsed in a washing medium (heparin supplemented hepes modified TCM 199) at 37°C in order to obtain cumulus oocyte complexes (COCs) [12]. The oocytes with darkly pigmented ooplasm and complete surrounded by at least one layer of cumulus cells were classified as Grade I and were selected for IVM. A total of 775 Grade I COCs were used for IVM *(Table 1)*.

The *in vitro* maturation media employed in this study were, 1: Synthetic Oviduct Fluid (SOF) [13] supplemented with 10 µg/mL follicle stimulating hormone (FSH), 10 µg/mL luteinizing hormone (LH), 4% BSA (Fraction V), antibiotics, essential (MEM Amino Acids Solution, Biological Industries, 01-325-1) and non essential (MEM Non-Essential Amino Acids Solution, Biological Industries, 01-340-1) amino acids and 20 µg/ml Epidermal Growth Factor (EGF; Sigma, E 4127) (mSOF; 270 mOsm, pH:7.2), and 2: TCM 199 (Sigma, M 5017) supplemented with 4% BSA (Fraction V), 2.2 g/l $NaHCO_3$, 0.23 mM Na Pyruvate, FSH (10 µg/ml; Sigma, F-2293), LH (10 µg/ml; Sigma L-5269), antibiotics and 20 µg/ml Epidermal Growth Factor (EGF; Sigma, E 4127) (288 mOsm, pH:7.3). COCs were selected and transferred into four-well petri dishes (NUNC®, Denmark) containing 500 µL maturation medium under mineral oil in well for each maturation medium for maturation at 37°C for 72 h. Incubation atmosphere for IVM was humidified 5% CO_2, 5% O_2, and 90% N_2. Depending upon the number of obtained Grade I oocyte per replication, 40-60 COCs were left in each well for all experimental groups.

A healthy 3 years aged Turkish Shepherd Dog (Kangal) was used as a semen donor. Second fractions of the ejaculates were collected by manual stimulation. Semen were transferred into a 15 ml conical tube onto a two-layer discontinuous gradient formed by layering 1ml of the 45% Percoll solution on top of 1ml of 90%, and centrifuged at 500 g for 20 min at room temperature. The supernatant was removed and the sperm pellet

was washed with 5 ml of hepes supplemented SOF medium (hSOF) by centrifugation at 500 g for an additional 10 min. The pellet was then recovered after aspiration of the supernatant, and the spermatozoa were resuspended to give a final concentration of $1x10^6$ cells/ml in hSOF medium supplemented with 0.56 mg/ml heparin to induce capacitation [14]. For each 40-60 oocyte groups, that had been previously matured for 72 h and placed in 500 µL culture medium, were co-incubated with the sperm suspension in 37°C humidified 5% CO_2, 5% O_2, and 90% N_2 atmosphere for 24 h.

A total of 550 Grade I COCs were used for IVF (Table 2). At 24 h after IVF, presumptive zygotes were transferred into four-well petri dishes (NUNC®, Denmark) containing 500 µL culture medium under mineral oil in well (mSOF and TCM 199), for IVC at 37°C for 7 days. Incubation atmosphere for IVC was the same environmental conditions used for IVM and IVF. The culture media were changed every 48 h. *In vitro* embryonic development was assessed by microscopic observation at 24 h intervals for 7 days.

Following the IVM, oocytes were denuded completely by gentle pipetting in 0.2% (w/v) hyaluronidase and left in KCl solution (0.7% w/v) for chromatin dispersal for another 3-5 min at room temperature. Oocytes were positioned as described by Hewitt and England [15] and fixed for 2-4 days in acetic acid/ethanol fixative (1/3, v/v). Nuclear structures were visualized by staining with aceto-orcein (2% orcein in 45% acetic acid). Nuclear morphology was classified as germinal vesicle intact (GV), germinal vesicle breakdown (GVBD), metaphase I (MI), metaphase II (MII) and undetermined nuclear materials (UDNM) under a phase contrast microscope (400×original magnification). At seven days after insemination, oocytes/embryos was denuded using small glass pipettes, than fixed and stained according to the Hewitt and England's [15] method, and were evaluated by phase contrast microscope.

Chi-square test was applied in SPSS 13.0 program to compare the IVM rates of oocytes and IVF rates of embryos between mSOF and TCM 1999 groups.

RESULTS

In vitro meiotic maturation results are summarized in *Table 1*. A total of 381 and 394 oocytes were matured in mSOF and TCM 199 media respectively. Oocytes matured in mSOF medium had significantly higher maturation rates (GVBD, MI, MII and MI+MII) than the oocytes in the TCM 199 medium (P<0.001).

In vitro fertilization and IVC results are summarized in *Table 2*. A total of 300 oocytes in mSOF and 250 oocytes in TCM 199 medium were inseminated. After the IVF and IVC period, although only one two cell embryo found in TCM 199 group, there were 2 2 cell, 2 3-4 cell, 2 5-8 cell, 1 9-16 cell and 1 morula stages embryos in mSOF medium group (P=0.037, *Table 2* and *Fig. 1*).

Table 1. *In vitro maturation results of dog oocytes cultured in two different media*
Tablo 1. *İki farklı medyumda kültüre edilen köpek oositlerinin in vitro olgunlaştırma sonuçları*

Medium Group	Oocyte Number	GV (%)	GVBD (%)	M I (%)	M II (%)	M I+M II (%)	UDNM (%)
mSOF	381	20 (5.2)[a]	110 (28.9)[b]	126 (33.1)[b]	35 (9.2)[b]	161 (42.3)[b]	90 (23.6)
TCM 199	394	169 (42.9)[b]	50 (12.7)[a]	47 (11.9)[a]	13 (3.3)[a]	60 (15.2)[a]	115 (29.2)

Values with different superscripts in the same column are significantly different (P<0.001)
GV: *germinal vesicle,* **GVBD:** *germinal vesicle breakdown,* **M I:** *metaphase I,* **M II:** *metaphase II,* **UDNM:** *undetermined nuclear material*

Table 2. *In vitro fertilization and development of canine embryos in two different media*
Tablo 2. *İn vitro fertilizasyon ve köpek embriyolarının iki farklı medyumdaki in vitro gelişimleri*

Medium Group	No of Oocytes Inseminated	2 Cell (%)	2-4 Cell (%)	4-8 Cell (%)	8-16 Cell (%)	Morula (%)	Total Cleaved Embriyo* (%)
mSOF	300	2 (0.7)	2 (0.7)	2 (0.7)	1 (0.3)	1 (0.3)	8 (2.7) [b]
TCM 199	250	1 (0.4)	-	-	-	-	1 (0.4) [a]

***** *Statistical analysis were done only for total values column*
Values with different superscripts in the same column are significantly different (P<0.05)

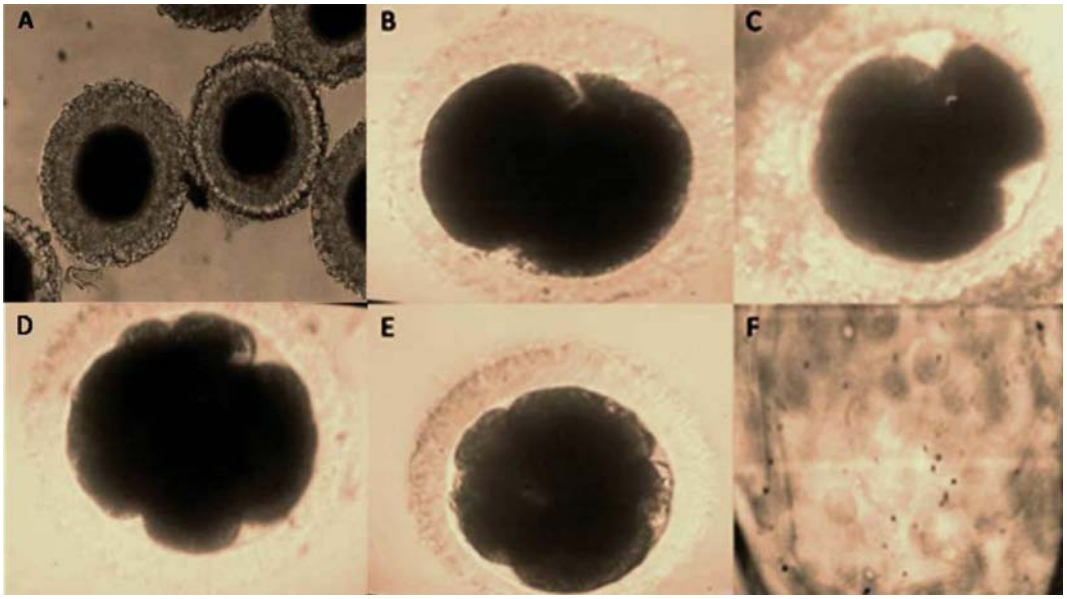

Fig 1. Photomicrographs (x 200) of primer oocytes and *in vitro* derived dog embryos at various stage of development. ***(A)*** primer oocytes, ***(B)*** 2-cell, ***(C)*** 4-cell, ***(D)*** 8-16 cell, ***(E)*** morula and ***(F)*** morula fixed and stained by aceto orcein at 7 days post insemination (x400)

Şekil 1. Primer oositler ile *in vitro* üretilmiş farklı gelişim dönemlerindeki kopek embriyolarının mikroskobik fotoğrafları (x 200). ***(A)*** primer oositler, ***(B)*** 2-hücreli, ***(C)*** 4- hücreli, ***(D)*** 8-16 hücreli, ***(E)*** morula ve ***(F)*** İVF sonrası 7. Günde fikze edilip aseto-orseinle boyanmış morula (x400)

DISCUSSION

Only 15-20% of ovarian dog oocytes could have been developed to the M II stage, and 30-40% IVF rates seem satisfactory. However, serious limitations are encountered in embryonic development (IVC) rate of fertilized oocytes and reaching morula-blastocyst stage is almost impossible [2]. Until to date, only two morulas [6,7] and one blactocyst [8] have been achieved, and no offspring have been produced in the dog or other canids by transferring embryos derived from *in vitro* matured oocytes [2]. For these reasons, the most important finding of our study was the development of one embryo to the morula stage which was the third morula obtained so far.

The influence of various factors on the *in vitro* maturation of canine oocytes such as age of oocyte donors [15], reproductive statuses [16], follicle diameter [2], oocyte diameter [17], ovary transport temperature [18] and *in vitro* maturation period [6] has been evaluated. However, the rates of meiotic completion, IVF and IVC rates of canine oocytes have still been very low [11]. *In vitro* culture systems developed for maturing oocytes of other species have been found inadequate for the maturation of dog oocytes [5]. It is difficult to identify the components lacking in the medium, or which compounds may be detrimental or suppressive [19]. Different media and/or various amount of protein, vitamin, hormone or growth factor addition to the medium were used to increase the IVM, IVF and IVC rates [5,11]. However, it is still not known how supplements added to medium affect dog embryo development *in vitro*. It is generally accepted that the process of keeping the medium as simple as possible is the most reliable manner of knowing to what oocytes are being subjected. But, despite the complexity TCM 199 has emerged as a commonly used medium for various species, including canids [20].

Some researchers stated that no difference was found in nuclear maturation of canine oocytes cultured in SOF versus TCM 199 [1,21]. Contrary, we found that the percentage of dog oocytes reaching MII was higher with mSOF than with TCM 199 ($P<0.001$). Moreover, the rate of unmatured oocytes (GV stage) was lower in the mSOF than the TCM 199 medium group ($P<0.001$). It is stated that maturation of COCs is probably the most critical *in vitro* step affecting subsequent embryo development to the blactocyst stage [22]. It is known that a parameter routinely used as a marker of oocytes maturation is the expansion of cumulus cells. Expansion and mucification are well-established mechanisms derived from hyaluronic acid and protein production through LH secretion and cAMP increase in cumulus cells [19]. It is reported that epidermal growth factor (EGF) added to the medium induced meiosis and facilitated cumulus cell expansion of dog oocytes *in vitro* [23]. However, it has been suggested

that cumulus cell expansion which is a sign of oocytes maturation in other mammals, was rare and seemed non essential for normal *in vitro* maturation and *in vitro* cleavage in dogs [19]. At the end of 72 h *in vitro* maturation period, cumulus expansions were observed in all the oocytes in both media groups of the study. However, IVM rates in the TCM 199 group were lower than those of mSOF group (P<0.001). Also, in our study, total cleavage rate was higher in mSOF than TCM 199 (P=0.037). After seven day of the IVC period, only one oocyte was cleaved in the TCM 199, while eight oocytes cleaved, and one of them developed to morula stage in mSOF medium group.

Synthetic oviduct fluid was originally formulated for use in the sheep [13] and it is not known how similar are the composition of canine oviductal content [1]. Synthetic oviduct fluid has been used successfully to improve *in vitro* development of bovine [24], ovine [13] and feline [25] embryos. Our results suggests that in spite of its complex composition and bearing some vitamins, TCM 199 medium was not as successful as mSOF medium which is simpler in composition for IVM, IVF and further embryonic development of dog oocytes. However in this study from the eight zygotes produced at mSOF medium group, four embryos were arrested at 2-8 cell stage. An *in vitro* block to development has been described for many species in which IVF has been attempted [11]. It is stated that arrested embryos enter a senescence-like state and that the stress-related protein $p66^{shc}$, which is responsible for the onset of senescence in somatic cells, is involved in the early embryonic arrest [26]. On the other hand, the maternal embryonic transition constitutes a critical phase of embryo development. In foxes and dogs, structural studies and cultivation with ^{3}H-uridine indicate that activation of the genome occurs at the 6 to 8-cell stage in foxes and the 8-cell stage in dog embryos [27]. The scarcity of reports in literature attempting to modify the culture conditions *in vitro* for IVM-derived embryos after the 8-cell stage may indicate that some difficulties have been encountered in propagating development past this stage, but too little information is available to conclude that such an *in vitro* block exists in dog oocytes [11]. Relatively low cleavage rates in the present study could be attributed to inadequate *in vitro* culture environment. In conclusion, it could be concluded that mSOF medium could be more efficient than the TCM 199 on maturation, fertilization and further developmental stages of immature dog oocytes *in vitro*. In dogs, reliable systems for *in vitro* embryo production and further development of embryos are yet to be developed and further studies are required.

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