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RESEARCH ARTICLE

Effect of Oocyte Diameter on *in vitro* Embryo Production in Dogs [1]

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Summary

In vitro embryo production has not been confidently applied to the dog successfully. Up to date, only one blactocyst have been achieved by *in vitro* culture. Therefore, the aim of the present study was to examine the effects of different oocyte diameters (≤100 µm and >100 µm) 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 (experiment I), effects of two different oocyte diameters on IVM of dog oocytes were investigated. The nuclear maturation rates were evaluated by aceto-orcein staining method at the end of the IVM. At the second step (experiment II), *in vitro* matured oocytes were fertilized with fresh spermatozoa for 24 h and *in vitro* cultured for 7 d. At the end of the IVC period, embryonic development was assessed by microscopic observation at 24 h intervals and fixed for staining by aceto-orcein staining method after 7 days. In comparison relating in IVM and IVF rates, larger oocytes have higher maturation (P<0.05) and cleavage (P<0.01) rates than the smaller ones. Unfortunately, none of the oocyte was reached to morula or blactocyst stage in both groups. In conclusion, it is demonstrated that the oocyte diameter may be a helpful selection criteria for dog in vitro embryo production.

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

Köpeklerde Oosit Çapının *in vitro* Embriyo Üretimi Üzerine Etkisi

Özet

Köpeklerde, *in vitro* embriyo üretimi amacıyla henüz başarılı bir yöntem bulunmamaktadır. Günümüze kadar yapılan in vitro çalışmalarda, sadece bir adet blastosist elde edildiği bildirilmiştir. Sunulan çalışmada, köpeklerde farklı oosit çaplarının (≤100 µm ve >100 µm) *in vitro* maturasyon (İVM), in vitro fertilizasyon (İVF) ve in vitro kültür (İVK) sonrası embriyonik gelişim üzerine etkisinin araştırılmasını amaçlandı. Çalışma iki aşamada gerçekleşti. İlk aşamada (Deney I), farklı oosit çaplarının İVM üzerine etkisi araştırıldı. Bu aşamanın sonunda oositlerin olgunlaşma durumları, aseto-orsein boyama metoduyla belirlendi. Çalışmanın ikinci aşamasında ise (Deney II), 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. İVM-İVF sonuçları karşılaştırmasında, büyük çapa sahip olan oositlerin, küçük olanlara göre daha yüksek oranda olgunlaşabildiği ve daha fazla oranda bölünmeler gösterebildiği saptandı (P<0.01). Her iki grupta hiçbir oosit morula ya da blastosist aşamasına kadar ulaşmadı. Sonuç olarak, in vitro köpek embriyosu üretmek amacıyla seçilecek oositler için çapın yardımcı bir ölçüt olabileceği söylenebilir.

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

INTRODUCTION

The reproductive physiology of the domestic dog is unique from that of other mammalian species. The ovulated immature dog oocyte completes its nuclear maturation 48-72 days after the LH peak *in vivo* [1]. Canine spermatozoa are also able to penetrate the zona pellucida and vitellus *in vitro* and *in vivo*, irrespective of the oocyte

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maturation stage [2]. A better understanding of these processes is required before *in vitro* maturation (IVM) and fertilization (IVF) of canine oocytes can be implemented in future gamete salvage programs for endangered canine species [3]. Until now, no offspring have been produced in the dog by transferring embryos derived from *in vitro* matured oocytes. Canine oocytes seem to be quite refractory to the different treatments that have been tested and their maturation and fertilization rates are still remain low [4]. It is known that the meiotic competence and fertilization of oocytes are affected by the oocyte size in other mammalian species [5], but there are inadequate literatures on the meiotic competence [3,6] and fertilization of canine oocytes of known diameters.

The present study was carried out to examined the effects of two different diameters (≤100 µm and >100 µm) on IVM and IVF and further embryonic development of dog oocytes.

MATERIAL and METHODS

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

Ovaries were collected from 57 mixed breed bitches (1-7 years of age) at the time of routine ovariohysterectomy. Bitches were at various stages of the estrous cycle. The ovaries were placed in Dulbecco's phosphate buffered saline (PBS) at 4°C and brought to the laboratory within 2-3 h. After waiting at room temperature at 10-15 min, the ovaries were minced and rinsed in a washing medium (heparin supplemented hepes modified TCM 199) at 38°C in order to obtain cumulus oocyte complexes (COCs). The oocytes with darkly pigmented ooplasm and complete surrounded by at least one layer of cumulus cells were classified as Grade I and selected for IVM [7]. The diameter of the oocytes was measured with a video micrometer (Olympus DP20, Shinjuku-ku, Tokyo, Japan) on a screen connected to a VCR camera on a stereo microscope. The diameter of oocyte was measured to exclude the zona pellucida at two different sizes (≤100 and >100 µm) and recorded. The measured COCs (≤100 and >100 µm) were transferred into different four-well petri dishes (NUNC®, Denmark) containing 500 µL maturation medium under mineral oil in each well at 38°C for 96 h. Incubation atmosphere for IVM was humidified 5% CO_2, 5% O_2, and 90% N_2. The *in vitro* maturation medium was: Synthetic Oviduct Fluid (mSOF; 270 mOsm, pH: 7.2) [8] supplemented with 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 10 µg/ml FSH (Sigma F-2293) + 10 µg/ml LH (Sigma L-5269) + 20 µg/ml β- Estradiol (water soluble, Sigma-Aldrich, E 4389) and 20 ng/ml Epidermal Growth Factor (EGF; Sigma, E 4127).

The sperm-rich fraction of the ejaculate (second fraction) was collected by manual stimulation from a mature fertile Turkish Shepherd Dog (Kangal). Semen were transferred into a 15 ml conical tube onto a two-layers discontinuous gradient formed by layering 1ml of the 45% Percoll solution on top of 1ml of 90% at bottom, 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 [9]. Each 40-60 oocyte that had been previously matured for 96 h placed in 500 µL culture medium and co-incubated with the sperm suspension in 38°C humidified 5% CO_2, 5% O_2, and 90% N_2 atmosphere for 24 h.

After IVF, oocytes/embryos were transferred into four-well petri dishes (NUNC®, Denmark) containing 500 µL culture medium under mineral oil in each well for IVC at 38°C for 7 days. Incubation atmosphere for IVC was at the same environmental conditions used for IVM and IVF. The culture media were changed in every 48 h. *In vitro* embryonic development was assessed by microscopic observation at 24 h intervals for 7 days.

Experiment I

A total of 1240 oocytes used in this experiment. *In vitro* matured 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 3-5 minutes at room temperature. Oocytes were fixed for 2-4 days in acetic acid/ethanol fixative (1/3, v/v). Nuclear structures were visualized after aceto-orcein (2% orcein in 45% acetic acid) staining. 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).

Experiment II

A total of 1589 oocytes used in this experiment. The collected oocytes were matured for 96 h and fertilized *in vitro* for 24 h. The cleavage situation of embryos were visualized every 24 h by inverted microscope beyond the IVC period. At 7th day after insemination, oocytes/embryos was denuded using small glass pipettes, and

stained after fixation and evaluated by phase contrast microscope by the previous method [6].

Chi-square test was applied in SPSS 13.0 program to compare the developmental stages of oocytes and embryos *in vitro*.

RESULTS

According to findings, larger oocytes (>100 µm) were have higher maturation rates than the smaller (≤100 µm) ones (P<0.05, *Table 1*). In addition, the larger (>100 µm) oocytes were cleaved more successfully than the smaller (≤100 µm) oocytes. However, morula or blactocyst stage embryos were not found in both of two different size groups *(Table 2)*.

>100 µm in diameter had higher rates of maturation than those smaller. In addition, it is stated that canine oocytes are able to resume meiosis *in vitro*, but they must be at least ≥100 µm in diameter to be able to resume meiosis and nuclear maturation [11]. There is a clear relationship between oocyte diameter and meiotic competence following *in vitro* culture [5,6], but no relationship between oocyte diameter and sperm penetration [3].

Although our results indicate that canine oocytes are able to fertilize and cleave irrespective of the oocyte diameter, greater proportions of larger oocytes matured (P<0.05) and cleaved (P<0.01) than the smaller ones. It is demonstrated that few oocytes recovered from small antral (<1 mm) follicles have the capacity to complete *in vitro* nuclear maturation compared to a high proportion

Table 1. *The comparison of in vitro maturation results of two different diameter of oocytes*
Tablo 1. *İn vitro olgunlaştırma sonuçlarının iki farklı oosit çapına göre karşılaştırılması*

Oocyte Diameter (µm)	Oocyte Number	GV (%)	GVBD (%)	M I (%)	M II (%)	M I+M II (%)	UDNM (%)
> 100	488	52 (10.7)	124 (25.4)^{c}	176 (36.1)	22 (4.5)	198 (40.6)^{b}	114 (23.4)
≤ 100	752	72 (9.6)	250 (33.2)^{d}	237 (31.5)	25 (3.3)	262 (34.8)^{a}	168 (22.3)

GV: *germinal vesicle,* ***GVBD:*** *germinal vesicle breakdown,* ***M I:*** *metaphase I,* ***M II:*** *metaphase II,* ***UDNM:*** *undetermined nuclear material*
*Values with different superscripts in the same column are significantly different (**ab:** P<0.05; **cd:** P<0.01)*

Table 2. *The comparison of in vitro fertilization and development of canine embryos in two different diameter of oocytes at 7 days*
Tablo 2. *İki farklı çapa göre köpek oositlerinin in vitro fertilizasyonu ve embriyoların 7 günlük gelişimlerinin karşılaştırılması*

Oocyte Diameter (µm)	Oocyte Number	2 cell (%)	2-4 Cell (%)	4-8 Cell (%)	8-16 Cell (%)	Morula-Blastocyst (%)	Total Cleaved Embriyo (%)
> 100	659	15 (2.28) ^{a}	4 (0.61)	11 (1.67) ^{a}	2 (0.30)	-	32 (4.86) ^{c}
≤ 100	930	6 (0.64) ^{b}	2 (0.21)	2 (0.21) ^{b}	0 (0.0)	-	10 (1.07) ^{d}

*Values with different superscripts in the same column are significantly different (**ab:** P<0.05; **cd:** P<0.01)*

DISCUSSION

It is indicated that the freshly ovulated dog oocyte measured without the ZP is 58.5-107.7 µm [10]. Although, it is known that the meiotic competence and fertilization of oocytes are affected by the oocyte size in other mammalian species [5], there is a paucity of literature on the meiotic competence and fertilization of canine oocytes of known diameters [3]. The first experimental evidence for a possible effect of canine oocyte diameter on acquisition of meiotic competence was provided in a study by Hewitt and England in 1998 [6]. They showed that large oocytes collected from larger (>2 mm) ones [12]. This suggests that although the oocytes recovered from small antral follicles have achieved their optimal size, they have not acquired functional capability. It is suspected that the oocyte requires intracellular modification during subsequent stages of follicle development before it can be fully capable of completing nuclear maturation, fertilization and forming an embryo [13]. We found a positive relationship between the oocyte diameters and *in vitro* cleavage rates (P<0.01). According to our findings, it could be said that oocyte diameter may be a helpful selection criteria of oocytes for *in vitro* embryo production in dogs. It was observed that 32 and 10 oocytes were cleaved in the

larger and smaller diameter groups respectively; but all cleaved oocytes were arrested at 2-16 cell stages. An *in vitro* block to development has been described for many species in which IVF has been attempted [4]. The stress-related protein $p66^{shc}$, which involved in the onset of senescence in somatic cells, is responsible for the early embryonic arrest [14]. It is stated that arrested embryos enter a senescence-like state. The scarcity of reports in literature of attempts 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 [4].

It is concluded that the oocytes size may be a helpful selection criteria for dog embryo production *in vitro*. Although oocytes size does not seem to be the sole factor limiting *in vitro* maturation success, more studies requires to improve the IVM, IVF, cleavage and further embryonic development of dog embryos.

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