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RESEARCH ARTICLE

Effect of Epidermal Growth Factor on In Vitro Maturation of Cat Oocytes Recovered from Ovaries at Follicular and Luteal Stages

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Summary

This study aimed to determine the effect of different concentrations of Epidermal growth factor (EGF) on in vitro maturation (IVM) of cat oocytes collected from ovaries at follicular and luteal phases of reproductive cycle. A total of 612 cumulus oocytes complexes (COCs) were obtained from 42 queens at either follicular or luteal phases. Oocytes were cultured in: (1) TCM 199 (no EGF); (2) TCM 199 plus 10 ng/ml EGF; (3) TCM 199 plus 50 ng/ml EGF, in 5% CO_2 aerobic condition for 48 hours. According to the chromosomal analyses, each oocyte was placed into the following categories: germinal vesicle (GV), metaphase I (M I), metaphase II (M II) and degenerate. In the luteal phase, the number of oocytes that remained at the GV stage was higher in the EGF 50 group (41.7%; $P<0.05$). In the follicular phase, the number of oocytes in M II stage was higher in EGF 10 group (37.9%; $P<0.05$). The percentage of matured oocytes that reached M II stage was higher in luteal phase ($P<0.05$) than in follicular phase for all EGF treated groups. This concluded that, addition of low concentration of EGF (10 ng/ml) to maturation media has a positive effect on the oocytes in comparison with control group (0 ng/ml). In contrast, a high concentration of EGF (50 ng/ml) has a negative result on IVM of cat oocytes and oocytes collected from ovaries on luteal phase are more advisable than follicular phase.

Keywords: *Cat, In vitro maturation, Epidermal growth factor, Sexual cycles*

Folliküler ve Luteal Dönemdeki Kedi Ovaryumlarından Alınan Oositlerin In Vitro Maturasyonunda Epidermal Büyüme Faktörünün Etkisi

Özet

Bu çalışmada, seksüel siklusun folliküler ve luteal fazında bulunan kedi ovaryumlarından toplanan oositlerin nükleer maturasyonu üzerinde IVM vasatına çeşitli konsantrasyonlarda katılan EGF'nin etkilerinin belirlenmesi amaçlandı. Çalışmada folliküler ve luteal dönemde bulunan toplam 42 kediden alınan 612 kumulus oosit kompleks kullanıldı. Oositler, (1) TCM 199 (EGF 0); (2) TCM 199 + 10 ng/ml EGF; (3) TCM 199 + 50 ng/ml EGF olarak 3 gruba ayrılıp IVM vasatına alındı ve %5 CO_2'li ortamda 48 saat süreyle mature edildi. Kromozomal analizlere göre her bir oosit germinal vezikül (GV), Metafaz I-II (M I-II) ve dejenere olarak adlandırıldı. EGF 50 grubunda, luteal dönemdeki ovaryumlardan alınan oositlerin yüksek oranda (%41.7) GV aşamasında kaldığı gözlendi ($P<0.05$). EGF 10 grubunda folliküler dönemdeki oositlerin önemli oranda (%37.9) M II aşamasına ulaştığı saptandı ($P<0.05$). Tüm EGF gruplarında luteal dönemdeki ovaryumlardan alınan oositlerin folliküler döneme göre yüksek oranda M II aşamasına ulaştığı görüldü ($P<0.05$). Sonuç olarak sunulan çalışmada IVM vasatına katılan düşük dozda EGF'nin (10 ng/ml) EGF katılmayan gruba kıyasla maturasyonu olumlu yönde etkilediği gözlendi. Ancak maturasyon vasatına yüksek miktarda (50 ng/ml) katıldığında ise kedi oositlerinin IVM'si üzerinde olumsuz etkilerinin olduğu ve luteal dönemdeki ovaryumlardan toplanan oositlerin folliküler döneme göre IVM için daha uygun olduğu kanısına varıldı.

Anahtar sözcükler: *Kedi, In vitro maturasyon, Epidermal büyüme faktörü, Seksüel siklus*

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INTRODUCTION

Domestic cat is an important experimental model for assisted reproductive technologies; especially development of reliable methods for IVM and in vitro fertilization (IVF) of oocytes collected from ovaries can be subsequently used to preserve endangered species [1]. Nonetheless, there are several studies were performed to determine the IVM of domestic cat oocytes of using a different media and in vitro culture (IVC) conditions [2-5].

The control of oocyte growth and maturation in vivo conditions has a complex mechanism which includes the regulation and modulation of meiosis with growth factors which mediated via the granulosa and/or cumulus cells [6,7]. Peptide growth factors such as EGF have an important role in ovarian folliculogenesis. These are locally produced in ovarium, and regulate cell proliferation, differentiation and steroidogenesis in ovarium [8].

The first report of EGF in cat was given by Göritz et al.[9]. The binding of EGF activates the intrinsic tyrosine kinase of EGF receptor (EGF-R) and which result in EGF-R autophosphorylation and subsequent tyrosine phosphorylation of numerous substrates within the cell. The determination of EGF presence in feline ovaries was performed by localization and measurement of it and detection of EGF-binding sites. Primary, secondary and tertiary follicular granulosea cells and interstitial gland cells have specific binding sites for EGF. Depending on these binding sites, EGF plays a crucial role in ovarian folliculogenesis, acts as a mitogenic factor with granulosa cell proliferation, but in antral follicles, granulosa cell differentiation and maturation of oocytes can took place [10]. Merlo et al.[11] confirmed that EGF regulates the maturation of cat oocytes.

EGF is frequently on IVM of mammalian oocytes. Although various doses were used in oocytes of different species, there is no commonly accepted optimal dose. It is reported that addition of 50 ng/ml EGF into IVM medium for equine oocytes [12], 100 ng/ml [13] and 30 ng/ml for bovine oocytes [14], 20 ng/ml on buffalo oocytes [15] and 10 ng/ml on rabbit [16] oocytes have positive results. Researches on optimal dose still going on, despite addition of different amounts (5, 10, 25, 30, 50 ng/ml) of EGF on cat oocytes [11,17-19].

The objective of this study was to determine the effect of reproductive cycle stage and supplementation of an IVM medium with varying concentrations of EGF on nuclear maturation of cat oocytes.

MATERIAL and METHODS

Recovery and culture of oocytes

Ovaries were collected from sexually mature cats at both follicular and luteal phases of the reproductive cycle following routine ovariohysterectomy operation at the Department of Obstetrics and Gynaecology, Faculty of Veterinary Medicine, University of Ankara. Overall, 612 cumulus-oocyte complex (COCs) were collected from 42 pairs of ovaries. Ovaries were placed in m-PBS supplemented with 100 IU/ml penicillin, 100 mg/ml streptomycin. The reproductive cycle stage of ovarian pairs was determined as follicular (one or more mature follicles) or luteal (one or more corpora lutea), and ovaries were sliced with a scalpel blade to release COCs in a 90 mm in diameter culture dishes containing m-PBS. Cumulus oocyte complexes were identified in grades I and II oocytes were used [20] in this study.

The COCs washed three times in IVM medium, composed of HEPES buffered TCM 199 supplemented with 6 mg/ml bovine serum albumin, 0.2 mM Na pyruvate and 100 IU/ml penicillin, 100 mg/ml streptomycin. Oocytes were cultured in: (1) TCM 199 (no EGF); (2) TCM 199 plus 10 ng/ml EGF; (3) TCM 199 plus 50 ng/ml EGF, then cultured in 100 ml droplets of IVM medium (10-15 COCs/droplet) at humidified atmosphere of 5% CO_2 aerobic condition for 48 h. All chemicals were obtained from Sigma Aldrich (St. Louis, MO, USA).

Assessment of meiotic status of oocytes

After maturation for 48 h, the oocytes were prepared for cytogenetic analyses. Cumulus cells were removed with pipetation, and the denuded oocytes were subsequently fixed with acetic acid: ethanol (1:3) for 24 h. The fixed oocytes were stained using 1% aceto-orcein solution for 5 min and then destained with acetic acid-glycerol. Each oocyte was identified and observed with light microscopy to evaluate meiotic status. According to the chromosomal analyses, each oocyte was placed into the following categories: germinal vesicle breakdown (GV), metaphase I (M I), metaphase II (M II) and degenerate.

Statistical analyses

All data on nuclear status after IVM was analyzed using a Chi-square test (SPSS 14.00 pocket programme, Licence No: 9869264) $P<0.05$ was considered significant.

RESULTS

From a total of 612 COCs obtained from 42 queens at the follicular and luteal stages. The percentage of matured oocytes that reached M II stage was found higher in luteal phase (P<0.05) than in follicular phase for all EGF treatment groups. The number of degenerated oocytes in the luteal phase with EGF 0, 10 and 50 ng/ml groups were 8 (7.7%), 6 (6.7%), and 9 (8.3%) respectively; in the follicular phase with EGF 0, 10, 50 groups were 11 (11.7%), 13 (13%), and 10 (8.4%) respectively. However, the degenerated oocytes were not used in statistical analyzes because of their fewer number.

In the luteal phase, the number of oocytes that remained at the GV stage was higher in the EGF 50 group (41.7%) compared with EGF 0 (25.3%) and EGF 10 (26.0%) groups. There were no significant differences in the number of oocytes at the GV stage between EGF 0 and EGF 10 groups in the luteal phase. Although the percentage of oocytes at the M II stage was higher in the EGF 10 group (44.2%), it was not significantly different from the EGF 0 (35.8%) and EGF 50 (36.5%) groups (P>0.05).

In the follicular phase, the percentage of oocytes that remained at the GV stage was higher in the EGF 0 group (36.1%) in comparison with in EGF 10 (27.6%) and EGF 50 (29.0%) groups, but was not significantly different from that groups (P>0.05). The number of oocytes in M II stage was higher in EGF 10 group (37.9%) than in EGF 0 (24.1%) and EGF 50 (33.6%) groups (P<0.05).

Table 1. *Effect of reproductive cycle stage and supplementation of an IVM medium with varying concentrations of EGF on nuclear maturation in the cat oocytes.*

Tablo 1. *Kedi oositlerinde seksüel siklus dönemi ve IVM vasatına değişik oranlarda EGF katılmasının nükleer maturasyona etkisi*

Developmental stage of oocytes	Groups	Luteal Stage (%)	Follicular Stage (%)
Germinal Vesicle	EGF 0	24 (25.3)	30 (36.1)
	EGF 10	20 (26.0)	24 (27.6)
	EGF 50	40 (41.7)*	31 (29.0)
Metaphase I	EGF 0	37 (38.9)	33 (39.8)
	EGF 10	23 (29.9)	30 (34.5)
	EGF 50	21 (21.9)	40 (37.6)
Metaphase II	EGF 0	34 (35.8)	20 (24.1)
	EGF 10	34 (44.2)	33 (37.9)*
	EGF 50	35 (36.5)	36 (33.6)

**: P<0.05*

DISCUSSION

In vitro maturation of cat oocytes depends on a different factors, such as the stage of the oestrous cycle [21,22], the quality of the COCs [20,23], and the time of culture and supplementation [3,24-26]. Therefore, it is necessary to optimize culture systems, taking in account all factors essentially to complete oocyte maturation in vitro [12].

There is no definite opinion about the optimum time for IVM of cat oocytes [5], but Johnston et al.[2] reported that the optimum maturation time was 52 h, Wolfe and Wildt [3] reported that it was 32 h in cats. Freistedt et al.[27] reported that 24-28 h incubation time was not enough when TCM-199 medium was used for maturation. Similarly, Nagano et al.[28] found that oocytes reached M II stage on 30th, 36th, and 48th h of maturation were higher than matured for 24 h. Thereof, cat oocytes were matured longer time (48 h) as specified in Evecen et al.[29] than a three study [3,27,30] to have a maximum oocytes reached M II stage in this study.

Even though EGF is frequently being added to IVM of cat oocytes, a little information has been available concerning the effect of reproductive status of the ovaries. Accordingly, the effects of EGF on nuclear maturation of oocytes collected from different reproductive status (follicle and luteal) were investigated in this study. The stage of reproductive cycle affects the success of IVM of oocytes [2]. In present study, the percentage of matured oocytes that reached M II stage was higher in luteal phase than in follicular stage for all EGF treated groups. It is concluded that the oocytes collected from ovaries on luteal stage were more advisable than follicular stage for IVM. Freistedt et al.[27] found that development of potential oocytes recovered from ovaries in the luteal phase was fairly good, whereas oocytes obtained from inactive or freshly ovulated ovaries had reduced cleavage rates after IVF similar to these results. These researchers reported that this case is probably due to endocrine and/or paracrine variations on ovaries in follicular stage. Roth et al.[31] found that hormonal imbalance due to corpus hemorrhagica in follicular stage could affect negatively. Similarly, Katska-Ksiazkiewicz et al.[5] found that in vitro cleavage and development was decreased in oocytes from follicular ovaries as compared to that of oocytes from luteal or inactive ovaries.

The addition of growth factor to the maturation media can enhance IVM of oocytes [10,18,24]. However,

the optimum dose of EGF could not be detected in cats as in other species. In the present study, addition of 10 ng/ml of EGF to maturation medium in follicular phase increased the number of oocytes reached M II stage significantly (P<0.05); also the same dose of EGF increased the number of matured oocytes in luteal phase, but not significantly important. Two studies, which did not investigate the follicular and luteal stage of reproductive cycle explained that 10 ng/ml of EGF increased the number of oocytes reached M II, 10-25 ng/ml EGF affected positively the cytoplasmic maturation [11], but there is no report indicating the effect of EGF in vitro maturation of cat oocytes [18].

In this study, the criteria of cytoplasmic maturation of IVF and IVC stages were not inspected. There was no significant difference in the M II stage after addition of different doses of EGF (5, 10, and 30 ng/ml) to maturation media. However, oocytes matured in medium supplemented with 30 ng/ml of EGF, developed to morula and blastocyst stage were found significantly higher than other doses [17]. Goud et al.[19] found that positive effect of EGF on human cumulus-intact oocytes was strongly evidence at fertilization; relatively the number of oocytes under normal fertilization in the EGF-supplemented group was higher than the unsupplemented oocytes. Therefore, in this study the supplementation of 10 ng/ml EGF to maturation media could has a positive effect on IVF and IVC stage.

In oocytes of different species, high concentrations of EGF affects maturation negatively. Abeydeera et al.[32] reported that the use of 40 ng/ml EGF in maturation medium reduced maturation rates of pig oocytes compared to lower doses than 40 ng/ml the IVM of pig oocytes. Due to this result, authors suggested that use of high concentrations of EGF might lead to a reduction in the number of cell receptors of the oocytes and hence called this condition as “down regulation”. Similarly Polat and Salmanoğlu [13] reported that the proportion of first polar body expulsion in the bovine oocytes was significantly lower in 1000 ng/ml group in comparison to lower (1, 10, 100 ng/ml) doses of EGF. In the present study, oocyte number reached the M II stage was lower in 50 ng/ml EGF group than in 10 ng/ml EGF group. Because of this, it was thought that use of 50 ng/ml EGF was a high dose for IVM of cat oocytes, which resulted in agreement with data obtained by Merlo et al.[11]. Jewgenow 8 described an inhibition at estradiol production by high concentration of EGF (>10 ng/ml), which enhances atresia of follicles. In addition, excessive use of EGF during IVM may exert a detrimental rather than a positive effect on cytoplasmic maturation of cat oocytes [11].

In conclusion, addition of EGF (10 ng/ml) into the IVM medium (TCM 199) affected more positive than control group. However, a high dose of EGF (50 ng/ml) in IVM medium reduced the maturation rates of cat oocytes. In addition, oocytes collected from ovaries in luteal phase were more advisable rather than oocytes collected in follicular phase. Further experiments should be conducted to investigate the duration of maturation, different doses and, the stimulatory mechanism of EGF on cat oocyte maturation.

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