의학석사 학위논문

생쥐 전동난포 체외배양 시 난소 자궁내막종액의 첨가가 난포 발달, 난자 성숙 및 난포세포의 기능에 미치는 영향

Effect of Supplementation of Ovarian Endometriotic Fluids on Follicular Development, Oocyte Maturation and Follicular Cell Function in in vitro Mouse Preantral Follicle Culture

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의학과 산부인과학 전공

김 슬 기

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Effect of Supplementation of Ovarian Endometriotic Fluids on Follicular Development, Oocyte Maturation and Follicular Cell Function in in vitro Mouse Preantral Follicle Culture

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The Department of Obstetrics & Gynecology

Seoul National University College of Medicine

Seul Ki Kim

ABSTRACT

Objective: Several lines of clinical evidence suggest that the presence of an ovarian endometrioma causes lower ovarian reserve and poor IVF outcomes. However, the exact mechanism underlying decreased ovarian function in women with endometrioma is mostly unknown. The aim of the present study was to investigate the potential detrimental effect of human endometriotic fluids (EF) on in vitro growth of mouse preantral follicles.

Methods: Preantral follicles (isolated from ovaries of 7- to 8-week-old mice) were cultured in growth medium for 10 days and then in maturation medium for 2 days. During in vitro culture, EF supernatants (0%, 2.5%, 5%, and 10%) were supplemented. Meiotic spindle integrity of MII oocytes was analyzed. Hormone (17β-estradiol and AMH) levels in the final spent media were measured

by ELISA.

Results: The survival rates of follicles at day 10 were significantly lower in three EF-supplemented groups (56.1%, 30.6%, and 6.2%; 83.6% in non-supplemented group). The production of total oocytes per initiated follicle was also significantly lower in the 3 EF-supplemented groups (34.7%, 18.4% and 4.1%; 68.1% in the non-supplemented group). Proportions of the oocyte with normal spindles were significantly lower in the 3 EF-supplemented groups (10%, 0% and 0%; 52% in the non-supplemented group). In the final spent media, the level of 17β-estradiol was significantly lower only in the 10% EF-supplemented group, and the level of AMH was significantly lower in three EF-supplemented groups, when compared with the non-supplemented group.

Conclusion: During in vitro culture of mouse preantral follicles, the

survival rate, oocyte acquisition, spindle integrity of MII oocytes and AMH production were greatly affected by EF supplementation. These findings suggest a possibility of detrimental effects of endometriotic cyst on folliculogenesis in adjacent ovarian tissues.

Keywords: endometrioma / endometriosis / preantral follicle / in vitro maturation / folliculogenesis

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INTRODUCTION

Ovarian endometrioma is a common presentation of endometriosis that occurs in 17–44% of patients with endometriosis and is frequently associated with pelvic pain and infertility (1). Ovarian endometrioma is an ovarian cyst lined with endometrial tissue histologically and functionally similar to the eutopic endometrium and in which the internal fluid is thought to arise from the accumulation of menstrual debris deriving from the shedding of active implants inside the cyst (2).

Several lines of clinical evidence suggest that the presence of an ovarian endometrioma causes damage to the surrounding otherwise healthy ovarian tissue. Women with ovarian endometrioma have a lower ovarian reserve even before surgical excision (3, 4). In the ovary affected by an

endometrioma, the incidence of spontaneous ovulation is half that of ovaries not affected by an endometrioma (5). However, a similar rate of spontaneous ovulation has also been reported between ovary affected and not affected by an endometrioma (6).

It has been demonstrated that in women with bilateral endometriomas undergoing an IVF cycle, responsiveness to ovarian stimulation and number of oocytes retrieved were significantly reduced compared with age-matched unexposed control subjects (7). However, the exact mechanism underlying decreased ovarian function in women with endometrioma is mostly unknown.

In tissues surrounding endometrioma, increased expression of 8-hydroxy-2' -deoxyguanosine (as an oxidative marker) (8), increased tissue fibrosis (9, 10), and less follicle numbers (9-13) were observed compared to tissues surrounding other

types of cysts. These results support the idea that endometrioma greatly affects the morphological and functional characteristics of surrounding normal ovarian tissue (2).

Endometriomas contain high concentrations of toxic substances such as reactive oxygen species (ROS), inflammatory molecules, proteolytic enzymes and free iron (2, 14-18). It is still unknown whether the endometrioma contents are able to diffuse through the cyst wall. However, the endometrioma is not surrounded by a true capsule and the wall of the cyst is thin and composed of the ovarian cortex itself and connective tissue (12, 19, 20). Therefore, it is thought that endometriotic cyst fluids can cross the lining cyst wall and then adversely affect folliculogenesis in nearby ovarian tissues and follicles (21-23).

The aim of the present study was to investigate the potential detrimental effect of endometriotic fluids (EF) on in vitro growth of preantral follicles in a mouse model.

MATERIALS AND METHODS

Experimental design

From a single patient, EF was obtained by aspiration at the time of a laparoscopic cystectomy. The EF was kept refrigerated at −80° C and warmed to 37° C before the experiment. After centrifugation, supernatants were collected and EF supernatants (0%, 2.5%, 5%, and 10%) were supplemented into growth and maturation medium during in vitro culture of mouse preantral follicle.

In vitro growth of preantral follicle

Commercially available BDF1 mice (Orient Co., Seoul, Korea) were fed ad libitum and were kept under a 12−hour light/12−hour dark cycle at 23° C. The experimental protocols and animal handling procedures were reviewed and approved by

the Animal Care and Use Committee of Seoul National University Bundang Hospital. After one week of the adaptation, 7- to 8-week-old mice were sacrificed by cervical dislocation and the bilateral ovaries were collected in a 1 mL L-15 medium (WelGENE, Daegu, Korea) supplemented with 0.4% bovine serum albumin (BSA, Sigma, St. Louis, MO, USA). Determination of female mouse age was based on a previous study from our center (24).

Intact preantral follicles were mechanically isolated from fresh ovaries. A total of 409 preantral follicles were cultured individually and randomly assigned to four groups. Preantral follicle culture medium was composed of α-Minimum Essential Medium (αMEM; WelGENE), 5% fetal bovine serum (FBS, Gibco, Paisley, UK), and 10 mIU/mL recombinant FSH (rFSH; Serono, Geneva, Switzerland), 1% insulin-transferrin-selenium mixture (Sigma-Aldrich, St. Louis, MO, USA), and 1%

penicillin-streptomycin mixture (Sigma-Aldrich). Individual follicles were cultured in a 96-well plate (BD BioCoat; BD Falcon, Franklin Lakes, NJ, USA) (a single follicle in 75 μL of medium without mineral oil)) and maintained at 37° C in 5% CO_2 for 10 days. Every four days, the medium was exchanged and follicle survival and formation of the antrum were assessed using an inverted Leica DM IRB microscope with transmitted light and phase objectives (Leica, Bannockburn, IL, USA). Follicles were designated as dead if the oocyte was no longer surrounded by a granulosa cell layer or if the granulosa cells had become dark and fragmented and the follicle decreased in size.

Maturation of antral follicle and oocyte retrieval

After 10 days' culture, the follicles were transferred to maturation medium composed of αMEM, 5% FBS, 10 mIU/mL

rFSH, 1.5 IU/mL hCG (Serono), and 5 ng/mL recombinant mouse epidermal growth factor (rmEGF, Sigma), 1% insulin-transferrin-selenium mixture, 1% penicillin-streptomycin mixture for 16 hours at 37° C in 5% CO_2. Spontaneous rupture of the follicles was assessed, and if no rupture was present, oocytes were released from the follicles (Figure 1). All oocytes were denuded of the surrounding cumulus cells by treatment with 0.3% hyaluronidase (Sigma) and gentle aspiration through a polished drawn glass pipette. The oocytes were considered to have reached the germinal vesicle (GV) stage if the GV was visible; if not, they were recorded as having GV breakdown (GVBD). If a polar body was present in the perivitelline space, the oocytes were classified as metaphase II (MII). Fragmented or shrunken oocytes were classified as degenerated. The percentage of MII oocytes per total oocyte, or per survived follicle, or per isolated follicle

was calculated.

Meiotic spindle integrity

Spindle integrity was assessed using previously described methods (25). The MII oocytes were washed three times with 1% BSA in PBS for 5 minutes and then fixed with 4% paraformaldehyde for 1 hour at room temperature (RT). After washing twice with 1% BSA in PBS, permeabilization was performed with 0.25% Triton X-100 in PBS for 10 minutes at RT. After washing twice with 1% BSA in PBS, blocking was performed with 3% BSA in PBS for 1 hour at RT and then washed twice with 1% BSA in PBS. A primary antibody for a-tubulin (Cell Signaling, Danvers, Massachusetts, USA) diluted in 1% BSA (1:100) was added and incubated overnight at 4° C. After washing three times with 1% BSA in PBS, a secondary antibody (Alexa fluor 488 goat anti-rabbit immunoglobulin G;

Invitrogen, Carlsbad, CA, USA) (diluted in 1% BSA [1:100]) was added for 1 hour at RT in the dark. After washing three times with 1% BSA in PBS, the oocytes were air dried on a silane-coated slide (DAKO, Glostrup, Denmark). The slide was counterstained with DAPI and examined using a confocal microscope (Carl Zeiss, LSM 710). A typical barrel-shaped microtubule structure between both poles with centrally aligned chromosomes (MII) was considered normal (Figure 2).

Measurement of hormones in final spent media

In each experimental set, the final spent media from 5-8 follicles were pooled and then frozen at -80° C. After thawing, 17β-estradiol and anti-Müllerian hormone (AMH) were measured by commercially available enzyme-linked immunosorbent assay (ELISA) kits [17β-estradiol (Enzo life sciences, Farmingdale, NY, USA), AMH (Anshlabs, Webster,

TX, USA)]. The limits of sensitivity for 17β-estradiol and AMH were 10 pg/mL and 0.01 ng/mL, respectively.

Statistical analysis

Fisher's exact test was used to compare the proportions among the groups. Hormone levels in the final spent media were compared by Kruskal-Wallis and Mann-Whitney U analysis. Statistical analyses were performed using SPSS 22.0 (SPSS Inc., Chicago, IL, USA) and P value <0.05 were always considered significant.

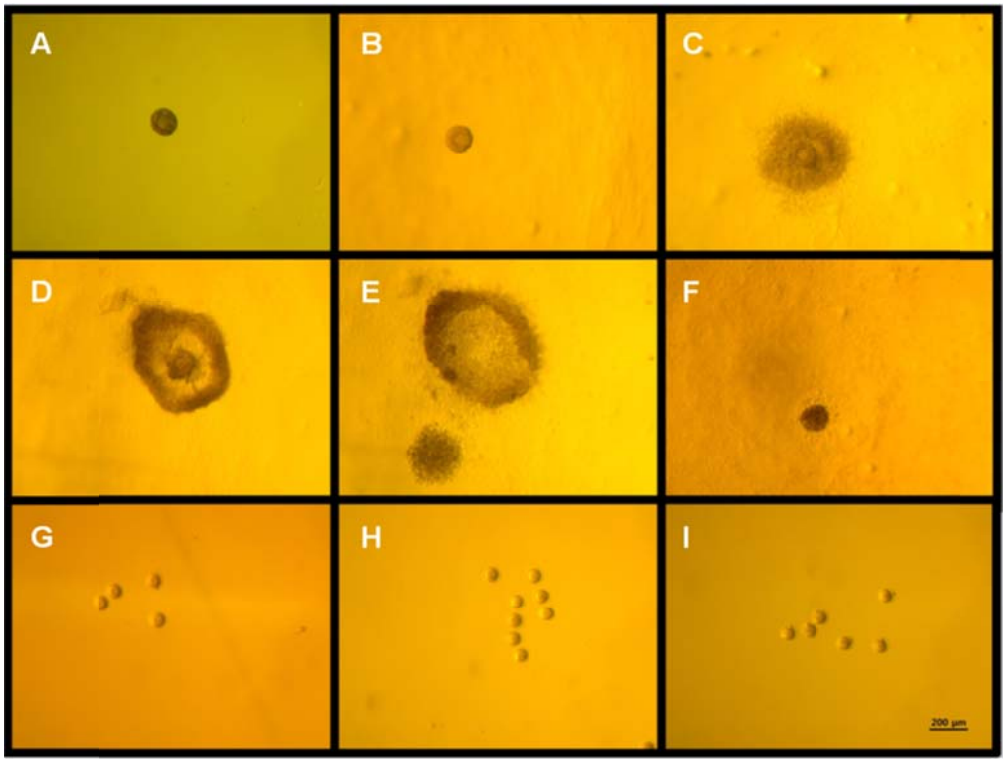

Figure 1. Microphotographs showing in vitro growth of mouse preantral follicles and the resultant oocytes (50X)

(A) follicle at day 0, (B) growing follicle at day 4, (C) growing follicle at day 8, (D) antral follicle at day 10, (E) ovulated follicle at day 11, (F) dead follicle at day 8, (G) a group of germinal vesicle oocytes, (H) a group of metaphase I oocytes, and (I) a group of metaphase II oocytes

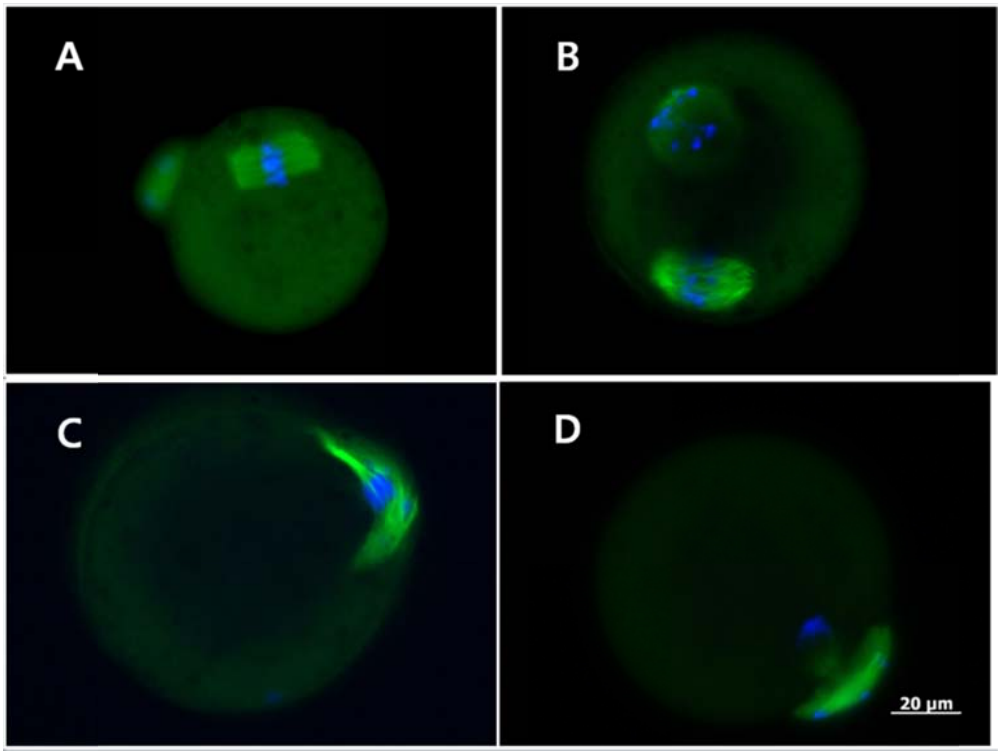

Figure 2. Representative confocal microphotographs showing the meiotic spindle organization and chromosome alignment in mature mouse oocytes (1,000X)

(A and B) normal metaphase II (C and D) abnormal metaphase II

RESULTS

The outcomes of in vitro growth of preantral follicles and the percentage of MII oocytes with normal spindles are listed in Table 1. All EF-supplemented groups exhibited a dose-dependent decrease in the survival rate at 10 days (56.1%, 30.6%, and 6.2%, respectively) compared with the non-supplemented group (83.6%). Dose-dependent decreases were also found in the antrum formation per initiated follicle (79.3%, 45.9%, 22.4%, and 4.1%), the ovulation rate per initiated follicle (72.4%, 35.7%, 18.4%, and 4.1%), the oocyte acquisition per initiated follicle (68.1%, 34.7%, 18.4%, and 4.1%) and the percentage of MII oocytes (21.6%, 10.2%, 3.1%, and 1.0%).

There were also significant decreases in the percentage of antrum formation from surviving follicles in all EF-

supplemented groups. However, the ovulation rate per antral follicle was similar in all groups. Proportions of oocytes with normal spindles were significantly lower in three EF-supplemented groups.

The median levels of 17β-estradiol and AMH in the final spent media are presented in Table 2. The 10% EF-supplemented group showed a significantly lower 17β-estradiol level (84.7 pg/mL) when compared with the non-supplemented group (122.5 pg/mL), and three EF-supplemented groups showed significantly lower AMH levels compared with the non-supplemented group.

Table 1. Outcomes of in vitro growth of preantral follicles under supplementation of endometriotic cyst fluids supernatants

Concentration of EF supernatants	0%	2.5%	5%	10%
No. of repeats	7	6	6	6
No. of preantral follicles isolated	116	98	98	97
No. of follicles surviving at day 10	97	55	30	6
(% per isolated follicle)	(83.6) [a]	(56.1) [b]	(30.6) [c]	(6.2) [d]
No. of follicles with antrum formation	92	45	22	4
(% per surviving follicle)	(94.8) [a]	(81.8) [b]	(73.3) [b]	(66.7) [b]
(% per isolated follicle)	(79.3) [a]	(45.9) [b]	(22.4) [c]	(4.1) [d]
No. of follicles with spontaneous rupture	84	35	18	4
(% per antral follicle)	(91.3) [a]	(77.8) [b]	(81.8) [a,b]	(100) [a,b]
(% per surviving follicle)	(86.6) [a]	(63.6) [b]	(60) [b]	(66.7) [a,b]
(% per isolated follicle)	(72.4) [a]	(35.7) [b]	(18.4) [c]	(4.1) [d]
No. of total oocytes	79	34	18	4
(% per surviving follicle)	(81.4) [a]	(61.8) [b]	(60) [b]	(66.7) [a,b]
(% per isolated follicle)	(68.1) [a]	(34.7) [b]	(18.4) [c]	(4.1) [d]
No. of degenerated oocytes	4	1	6	1
No. of GV oocytes	28	9	6	2

No. of GVBD oocytes	23	14	3	0
No. of MII oocytes	25	10	3	1
(% per total oocyte)	(31.6) [a]	(29.4) [a]	(16.7) [a]	(25) [a]
(% per surviving follicle)	(25.8) [a]	(18.2) [a]	(10) [a]	(16.7) [a]
(% per isolated follicle)	(21.6) [a]	(10.2) [b]	(3.1) [c]	(1.0) [c]
No. of MII oocytes with normal spindle (%)	13 (52) [a]	1 (10) [b]	0 (0) [b]	0 (0) [b]

Data are compared by Fisher's exact test.

Different superscripts mean a statistical significance within the same row.

GV: germinal vesicle
GVBD: germinal vesicle breakdown

Table 2. Hormone level in the final spent media after in vitro growth of preantral follicles under supplementation of endometriotic cyst fluids supernatants

Concentration of EF supernatants	0%	2.5%	5%	10%
No. of samples	11	10	10	10
17β-estradiol (pg/mL)	122.5 [a] [104.4 – 4,536]	161.7 [a] [90.4 – 656.2]	103.4 [a,b] [81.9 – 346.9]	84.7 [b] [48.8 – 189.2]
Anti-müllerian hormone (ng/mL)	13.9 [a] [12.2 – 16.7]	6.6 [b] [5.4 – 8.9]	5.7 [b] [5.1 – 8.1]	6.0 [b] [5.2 – 8.5]

Median [95% confidence interval].

Data are compared by the Kruskal-Wallis test.

Different superscripts mean a statistical significance within the same row.

DISCUSSION

The present study demonstrated for the first time that supplementation of human EF supernatants could negatively affect in vitro folliculogenesis in a mouse model. Supplementation of human EF supernatants profoundly lowered the survival of follicles and reduced antrum formation in the surviving follicles. When these results are applied to the situation of a human ovary with an endometriotic cyst, EF could possibly diffuse around the ovarian tissue and negatively affect the development of neighboring follicles.

In our study, the ovulation rate per antral follicle was not decreased in the EF-supplemented groups compared to the non-supplemented group. This suggests that once the preantral follicle grows to the antral follicle stage, exposure to EF does not have an adverse effect on the ovulation capability.

Our finding supports the results from a recent report by Maggiore et al (6), which prospectively evaluated the rate of spontaneous ovulation in women with unilateral endometrioma. They concluded that ovarian endometriotic cysts did not impair spontaneous ovulation in the affected ovary. However, Benaglia et al. reported a reduced spontaneous ovulation rate in the endometrioma-affected ovary (5). The chemical compositions of EF and the levels of diffusion to surrounding ovarian tissue may vary in different cysts, thus the effect on ovulation is likely to differ depending on the chemical and physical properties of the endometriotic cysts.

The acquisition of total oocyte per initiated follicle was significantly lower in the EF-supplemented groups. This was mainly due to a significantly lowered follicular survival rate during 10-days of culture. In fact, acquisition of total oocyte per surviving follicle in EF-supplemented groups was not

inferior to the non-supplemented group.

In the present study, immunofluorescent spindle staining of MII oocytes was performed to evaluate the chromosomal and spindle integrity of MII oocytes resulting from in vitro growth of preantral follicles. In EF-supplemented groups, high percentages of MII oocytes with abnormal spindle morphology were observed. Therefore, there is a possibility that MII oocytes obtained from ovaries with an endometriotic cyst are chromosomally abnormal. However, in humans, it is still unknown whether a chromosomal abnormality is high in the oocytes or embryos obtained from ovaries with an endometriotic cyst.

Even in the non-supplemented control group, approximately half of the MII oocytes showed abnormal spindle morphology. This incidence was high, when considering a report from our center in which all of MII oocytes in routine mouse IVF model

showed normal spindle morphology (25). It can be postulated that 12-day's long-term culture of preantral follicles in vitro might affect chromosomal and spindle integrity of in vitro-produced MII oocytes.

In the final spent media, the level of 17β-estradiol was significantly lower only in the 10% EF-supplemented group, but the AMH level was considerably lower in all EF-supplemented groups. These findings indicate that EF supplementation lowered follicular survival as well as follicular function. Poor follicular function, as indicated by a low level of AMH, may lead to poor fertilization potential of retrieved MII oocytes (26). In addition, our results are consistent with previous reports in which decreased serum AMH levels were observed in women with endometrioma even before ovarian surgery (3, 4).

In our experiment, the final spent media were mixtures of

spent media from both dead and live follicles. The lower level of AMH in the final spent media in the EF−supplemented group might be principally due to decreased production of AMH from dead follicles. This circumstance may be similar in women with endometrioma; decreased serum AMH level in women with endometrioma might be due to decreased production of AMH from dead or damaged follicles adjacent to ovarian endometrioma. In a further experiment, measurement of hormonal levels in spent media from each dead and live follicle will clarify this idea.

In conclusion, during in vitro culture of mouse preantral follicles, follicular survival rate, oocyte acquisition from the follicle, spindle integrity of MII oocyte, and AMH production from follicle were greatly affected by EF supplementation. These findings suggest the possibility of a detrimental effect

of endometriotic cyst on the folliculogenesis in the adjacent ovarian tissues.

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국 문 초 록

목적: 난소 자궁내막종은 난소의 기능을 감소시키고 체외수정시술 결과를 저하시킨다고 알려져 있으나 그 정확한 기전은 알려져 있지 않다. 본 연구에서는 난소 자궁내막종액이 생쥐 전동난포의 발달에 미치는 영향을 평가하고자 하였다.

방법: 7-8 주령 생쥐로부터 얻은 전동난포를 12 일간 체외배양하면서 난소 자궁내막종액의 상층액을 각각 다른 농도로 (0%, 2.5%, 5%, 10%) 배양액에 첨가하였다. 난포의 생존율, 동난포 형성율, 배란율을 확인하고, 획득한 난자에서 성숙난자율을 산정하였으며, 성숙난자 내 방추체 염색을 통하여 정상 방추체 여부를 확인하였다. 체외배양 후 최종적으로 남은 배양액 내의 17β-estradiol 과 AMH 농도를 ELISA 를 이용하여 측정하였다.

결과: 난포의 생존율은 자궁내막종액을 첨가한 3군 모두에서

유의하게 감소하였다 (첨가군: 56.1%, 30.6%, 6.2% ; 비첨가 대조군: 83.6%). 성숙난자 획득율도 자궁내막종액 첨가군 모두에서 유의하게 감소하였다 (첨가군: 10.2%, 3.1%, 1% ; 비첨가 대조군 21.6%). 정상 방추체를 가진 성숙난자의 비율도 자궁내막종액 첨가군 모두에서 유의하게 감소하였다 (첨가군: 10%, 0%, 0% ; 비첨가 대조군: 52%). 체외배양 후 최종적으로 남은 배양액 내의 17β-estradiol 농도는 10% 자궁내막종액 첨가군에서만 유의하게 감소하였고, AMH 농도는 첨가군 모두에서 유의하게 감소하였다.

결론: 생쥐 전동난포 체외배양 시 난소 자궁내막종액의 첨가는 난포의 생존율, 성숙난자 획득율, 정상 방추체를 가진 성숙난자의 비율을 감소시켰으며 난포세포의 AMH 생성을 감소시켰다. 이러한 결과들은 자궁내막종이 인접한 난소 조직에서 난포 발달, 난자 성장, 난포세포의 기능에 악영향을 줄 수 있음을 시사한다.

주요어: 자궁내막종 / 자궁내막증 / 전동난포 / 체외배양 / 난포

발달

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