의학석사 학위논문

Comparison of Follicle Isolation Methods for Mouse *in vitro* Ovarian Follicle Culture

난포 분리 방법이 생쥐 난포 체외배양에 미치는 영향에 관한 연구

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김 은 정

ABSTRACT

Objective: Ovarian tissue cryopreservation can be considered as a fertility preservation option for pre-pubertal girls or single woman. However, transplantation of cryopreserved ovarian tissue carries a critical risk which is the possibility of re-implantation of malignant cell to fully cured cancer survivors. Ovarian follicle *in vitro* culture is a promising fertility preservation option to avoid risk of re-introduction of malignant cell. However, *in vitro* ovarian follicle culture protocol is still in an experimental phase. For the success of *in vitro* ovarian follicle culture, proper ovarian follicle isolation is preceded before beginning *in vitro* culture. Based on this background, the objective of this study is to compare four different follicle isolation methods from ovarian tissue and evaluate the effect of follicle isolation on further *in vitro* follicle culture and oocyte competency.

Materials and methods: This study was conducted by using 11 to 14 days-old female BDF-1 mouse. Ovaries were dissected and randomly divided into 4 groups according to follicle isolation method; 1) mechanical isolation using 30G syringe needle (mechanical, MCH), 2) mechanical isolation using cell dissociation kit (mincing, MNC), 3) enzymatically digestion using collagenase type I (COL) and 4) enzymatically digestion using liberase (LIB). Follicles classified as early secondary follicle, 100~110 μm of diameter, were immediately cultured under culture media, which was consisted of 10 mIU/ml FSH, 1% ITS, 1% PS and 5% FBS in

glutaMAX-αMEM. The media was refreshed every 4 days and concentrations of estradiol, progesterone and testosterone in spent media were measured by ELISA on day 4, 8 and 10. On day 10, ovulation was induced by adding 1.5 IU/ml hCG and 5 ng/ml EGF in the media. Ovulated oocyte was checked after 18 hours. Follicular diameter was measured every 2 days. On day 10, follicular survival rate, pseudo-antrum formation rate were examined. The 18 hours after ovulation induction, the cumulus oocyte complexes (COCs) rate and the number of mature oocyte (MII oocyte) was counted. The diameter of retrieved MII oocyte was measured. To evaluate competence of mature oocyte derived from *in vitro* follicle culture, spindle and chromosome alignment and mitochondrial activity in mature oocyte were measured by immunofluorescence staining.

Results: The yields of follicles per one ovary were significantly higher in COL and LIB group. Follicular viability after isolation is significantly lower in MCH group, 80.57%, compared with the other groups (90.27%, 89.35% and 92.49%, MNC, COL and LIB group respectively). On day 10, follicular diameter was significantly greater in MCH and MNC group, 496.84 ± 11.56 μm and 488.53 ± 12.74 μm respectively, than COL and LIB group, 430.41 ± 11.65 μm and 430.03 ± 12.00 μm respectively. After 10 days of *in vitro* culture, follicle survival rate was significantly higher in MCH and MNC group, 88.7% and 92.1% respectively, compared with COL and LIB group, 70.4% and 71.2% respectively. The pseudoantrum formation rate was significantly higher in MNC group, 83.0%, than MCH, COL and LIB

group, 78.0%, 72.7% and 61.1% respectively. The COCs rate was statistically higher in MCH group, 69.1%, than MNC group, 52.5%. Then enzymatic digestion groups, COL and LIB, showed significantly lower COCs rate, 35.8% and 28.0% than MCH and MNC groups. The MNC group had made a result that significantly improved the mature oocyte rate, 73.6%, than MCH, COL and LIB group, 59.6%, 43.8% and 42.2% respectively. The estradiol level on day 10 in MCH group, 1.44 ± 0.37 ng/ml, was significantly higher than COL group, 0.53 ± 0.04 ng/ml. The progesterone and testosterone level on day 10, there was no significant difference among the experimental groups. The diameter of MCH and MNC group, 70.21 ± 0.54 μm and 69.33 ± 0.58 μm respectively, showed no significant difference compared with *in vivo* control group, 72.03 ± 0.77 μm. Normal meiotic spindle and chromosome rate is significantly higher in MCH and MNC group, 85.1% and 91.7%, than COL and LIB group, 42.9% and 50.0%. The MNC group showed the highest level of mitochondrial activity, 980.30 ± 69.01, among the other groups, 489.32 ± 25.78, 824.01 ± 27.32, 722.11 ± 25.31 and 405.56 ± 78.24 that control, MCH, COL and LIB group respectively.

Conclusion: MNC method showed significantly improved rate of follicle diameter, survival, pseudoantrum formation, a mature oocyte after *in vitro* culture. Furthermore, a normal meiotic spindle and chromosome rate was significantly higher than COL and LIB group. Based on results of this study, MNC method can be alternative method for MCH method that laborious and time consuming. The

enzymatic digestion group, COL and LIB, were inappropriate method for successful *in vitro* follicle culture on the basis of the result of present study. However, the fertilization ability of retrieved oocyte, pre-implantation and post-implantation competency of embryo from *in vitro* follicle culture should be evaluated in further study.

Keywords: Fertility preservation / Ovarian follicle / Ovarian follicle *in vitro* culture / Follicle isolation

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LIST OF ABBREVIATIONS

ANOVA: One-way analysis of variance

BSA: Bovine serum albumin

COCs: Cumulus oocyte complexes

DAPI: 4',6'-diamidino-2-phenylindole

EGF: Epidermal growth factor

ELISA: Enzyme-linked immunosorbent assay

FBS: Fetal bovine serum

FSH: Follicle-stimulating hormone

hCG: human chorionic gonadotropin

ITS: Insulin, transferrin and selenium

L-15: Leibovitz-15

MII: Metaphase II

PBS: Phosphate buffered saline

PS: Penicillin and streptomycin

INTRODUCTION

As a result of great exertion for improvement of cancer diagnostic and treatment, the survival rate of cancer patients has been significantly increased (1). However, cancer treatment, such as radiotherapy and/or chemotherapy, carries irreversible damage to fertility potential of cancer patients (2, 3). In case of female patients, radiation and/or chemotherapy can destroy follicle pool in ovary and develop a premature ovarian failure (4). Therefore, preserving fertility of patients is important before conducting cancer therapy.

Options of fertility preservation for female patient are focused on cryopreservation of oocytes, embryos and ovarian tissue. The oocyte and embryo cryopreservation need to delay cancer therapy for triggering and reach puberty. On the other hands, ovarian tissue cryopreservation has advantage which can apply to pre-pubertal and single female cancer patients and help immediate cancer treatment with unnecessary delay for triggering. Until now, over 30 live births have been reported after ovarian tissue cryopreservation (5). However, ovarian tissue cryopreservation has critical demerit that potential of re-introduction of malignant cell in following transplantation of preserved ovarian tissue to full recovery patient (6, 7). To overcome this demerit, ovarian follicle *in vitro* culture has been proposed after ovarian tissue cryopreservation (8).

The ovarian follicle culture is final fertility preservation option that not only reducing the risk of re-introduction of cancer cell but able to conduct for pre-

pubertal girl patients and single female cancer patients (9, 10). The study for establishment of *in vitro* follicle culture and cryopreservation has been processed in mouse (11, 12), bovine (13), porcine (14), sheep (15) and baboon (16, 17) model including human as well (18). Eppig and O'brien had reported the first live birth in 1996 by *in vitro* primordial follicle growth and IVF (19). The first step for ovarian follicle *in vitro* culture is follicle isolation from cryopreserved ovarian tissue. However, optimal follicle isolation method is still controversial.

Isolating intact and morphologically normal follicle is a crucial step for further follicular development in *in vitro* (20). In rodent model, various ovarian follicle isolation methods had been proposed. Many groups had reported a mechanical isolation method that using fine syringe needle (12, 21, 22). The mechanical isolation method could preserve natural follicular morphology which has intact theca cell layer and normal basement membrane. However, mechanical isolation method is laborious process and spent a lot of time to collect proper ovarian follicle for *in vitro* culture. On the other hands, the enzyme digestion method has been reported by Eppig & Schroeder, Eppig & O'Brien and Newton (11, 15, 19). While, enzyme digestion method could offer high yield and easy to perform on dense ovarian tissue cortex, damage to theca cell layer in ovarian follicle is inevitable. Using cell dissociation kit for isolating ovarian follicle, a mincing method, had been suggested by Jewgenow in domestic cat model (23). Mincing method is conceptually applicable to mechanical method. Follicle isolation by using mincing method could be conducted in short time-simple process and maintain advantage of

mechanical isolation method that can preserve natural follicular morphology equally.

The objective of this study is to evaluate the effect of isolation method on further *in vitro* follicle culture by comparing 4 different follicle isolation method. As a mechanical isolation method, mechanical method that using fine syringe needle and mincing method that using commercial cell dissociation kit and for the enzyme digestion method, collagenase and liberase were selected to assess the efficiency for further *in vitro* follicle culture. In this study, yield and viability of follicle after isolation, follicle development during *in vitro* culture and oocyte competency were evaluated.

MATERIALS AND METHODS

Experimental scheme and animals

The concise flow of this study is shown in Figure 1. This study was conducted by using 11 to 14 days of female BDF-1 (F1 hybrid, C57BL/6 [♀] X DBA6 [♂]) mouse. The BDF-1 mice were purchased from Orient Co. (Seong-nam, Korea). Animals were treated under light and temperature controlled condition. (11L:13D) Animal protocol of this study was approved by Institutional Animal Care and Use Committee of Seoul National University Bundang Hospital. All animals were sacrificed by cervical dislocation.

Ovarian follicle isolation

Bilateral ovary of mouse were collected from the ovarian bursa and transferred in L-15 medium to laboratory. Collected ovaries were randomly divided into 4 different isolation method groups, mechanical (MCH), mincing (MNC), collagenase (COL) and liberase (LIB).

i. MCH group: Ovarian tissues were mechanically punctured by using 30 gauge syringe needle in washing media, which consisted of 5% heat-inactivated fetal bovine serum (FBS; Gibco, Paisley, UK) in phosphate buffered saline (PBS; Welgene, Gyeongsan, Korea).

ii. MNC group: Ovarian tissues were placed on cell dissociation kit (Sigma-aldrich, Missouri, US) and finely minced with glass pestle in washing media. Mesh pore size of cell dissociation kit that used in this study was 40 (wire diameter; 0.254 mm and opening size; 380μm).

iii. COL group: Follicles were isolated by culture of ovarian tissue with 1 mg/ml of collagenase type Ⅰ without serum in 37℃ and 5% CO_2 for 20 minutes. Every 10 minutes, incubated ovaries were gently pipetted to facilitate proteolytic digestion. After incubation, enzyme activity was inhibited with adding serum. The treatment concentration of collagenase type I was determined according to previous study of other groups (20, 24).

iv. LIB group: 0.28 Wünch units/ml of libarase were treated to collected ovarian tissue in 37℃ and 5% CO_2 for 20 minutes. Every 10 minutes, preantral follicles were isolated repeatedly pipetting up and down in liberase to facilitate proteolytic digestion. After incubation, enzyme activity was inhibited with adding serum. The concentration of liberase is determined based on previous research by Vanacker J et al. (25).

Isolated follicles were washed in washing media to get rid of debris. For this study, intact preantral follicles were used. An intact preantral follicle is defined that has round follicular structure containing centrally located visible oocyte and more than one granulosa cell layer without antral cavity. The size of ovarian follicle is 100-

110 μm of diameter.

Viability assessment

The isolated follicles were assessed for viability to evaluate the damage on follicle during the isolation procedure. Calcein AM/ethidium homodimer-1 (CaAM/EthD-1) staining was performed by incubating isolated follicle at 37℃ in dark for 50 min with 2 μmol/L calcein AM and 5 μmol/L ethidium homodimer-1 (Live/Dead® Viability/Cytotoxicity kit; Invitrogen, Massachusetts, USA). Live/dead stained follicles were observed using a fluorescence microscope at 100X magnification. Live cells were stained green, and dead cells were stained red.

In vitro ovarian follicle culture

Isolated follicles were *in vitro* cultured on 96 well plate for 10 days in 75 μl of follicle culture media which consisted of 5% FBS, 1% ITS (5 μg/ml insulin, 5 μg/ml transferrin and 5 ng/ml selenium; Sigma-aldrich, Missouri, US), 1% penicillin and streptomycin (PS; Gibco, Paisley, UK) and 10 mIU/ml follicle stimulating hormone (FSH; Merk-sereno, Darmstadt, Germany) in glutaMAX α-minimal essential medium (α-MEM; Gibco, Paisley, UK). Follicle diameter was measured every other day. Every 4 days, 30 μl of follicle culture media was removed and refreshed. On day 10 of culture, 1.5 IU/ml of human chorionic

gonadotropin (hCG; Sigma-aldrich, Missouri, US) and 5 ng/ml of epidermal growth factor (EGF; Sigma-aldrich, Missouri, US) was added to culture media in order to oocyte maturation and ovulation. Also, survival rate and pseuodantrum formation rate were counted according to follicular morphologies. For survival rate, follicle was considered as survived when the oocyte, granulosa cell and stromal cell are visible and not degraded. Pseudoantrum formation was confirmed when antral-like cavity surrounded oocyte was observed. An ovulated oocyte was retrieved 18 hours after hCG administration. The COCs rate was counted if COCs were mucified from cultured follicle. MII oocyte rate was also measured.

Measuring hormone levels

The culture media was collected during media change. To evaluate the hormone level that secreted by follicle, concentrations of estradiol, progesterone, and testosterone in culture medium were measured. Estradiol, progesterone, and testosterone levels were measured by enzyme-linked immunosorbent assay (ELISA, estradiol and testosterone; Calbiotech, Spring valley, CA, progesterone; DRG Instruments GmBH, Marburg, Germany) according to the manufacturer's protocol. The optical density was read at 450 nm using a microtiter plate reader (Molecular Devices, Sunnyvale, CA).

Meiotic spindle and chromosome evaluation

Mature oocytes were fixed in 4% paraformaldehyde (Sigma-aldrich, Missouri, US) for overnight at 4℃ after collection. After washing 3 times in 0.3% bovine serum albumin (BSA; Sigma-aldrich, Missouri, US) in PBS for 5 min, the mature oocytes were moved to 0.5% triton X-100 for permeabilization. After washing in same procedure, mature oocytes were transferred to 3% BSA in PBS for 1 hour at room temperature. After blocking, mature oocytes were incubated with β-tubulin polyclonal antibody (Cell signaling, Miami, US) diluted 1:100 in 0.3% BSA in PBS for overnight at 4℃. After washing, the microtubules in mature oocytes were stained with fluorescein isothiocyanate (FITC) conjugated with anti-rabbit IgG (Abcam, Cambridge, UK) diluted 1:1000 in 0.3% BSA in PBS for 1 hour at room temperature in dark. After washing, mature oocyte were mounted with Vectorshield mounting medium (Vector laboratories, Burlingame, US), containing 0.5 μg of 4',6-diamidino-2-phenylindole (DAPI). The observation of spindle and chromosome in mature oocytes was performed under x400 magnification using a fluorescence microscope (CARL ZEISS, Jena, Germany) and represent image were photographed using confocal microscope (CARL ZEISS, Jena, Germany). The wave length for detection of DAPI and FITC were 330-380 nm and 450-590 nm, respectively. The typical configurations of spindle in mature oocyte that barrel shape were considered as normal. If spindle aggregated or has untypical configuration, the spindle was considered as abnormal (26).

Measurement of mitochondrial activity

Live mature oocytes were stained in 100 nM MitoTracker® Red CMXRos (Life Technologies, CA, USA) for 20 min in 37℃. After washing in 5% FBS in PBS, oocytes were transferred in hoechst 33342 for 10 min in RT in order to nuclear staining. After nuclear staining, oocytes were washed in 5% FBS in PBS. Mitochondrial activity in oocyte was measured under confocal microscope. The mature oocytes for control group were collected by 5 IU hCG injection to 5 weeks-old female BDF-1 mouse post 48 hours of 5 IU PMSG injection. The *in vivo* mature oocytes were collected from oviducts that dissected after 18 hours of hCG injection. *In vivo* mature oocytes were stained in same protocol with experimental groups.

Statistical Analysis

The statistical analyses were conducted using SPSS 12.0 (IBM, NY, USA) and GraphPad Prism 6.0 (GraphPad Software Inc., CA, USA). The proportion and normality were analyzed by chi-square or ANOVA. Difference were considered significant when p value was lower than 0.05.

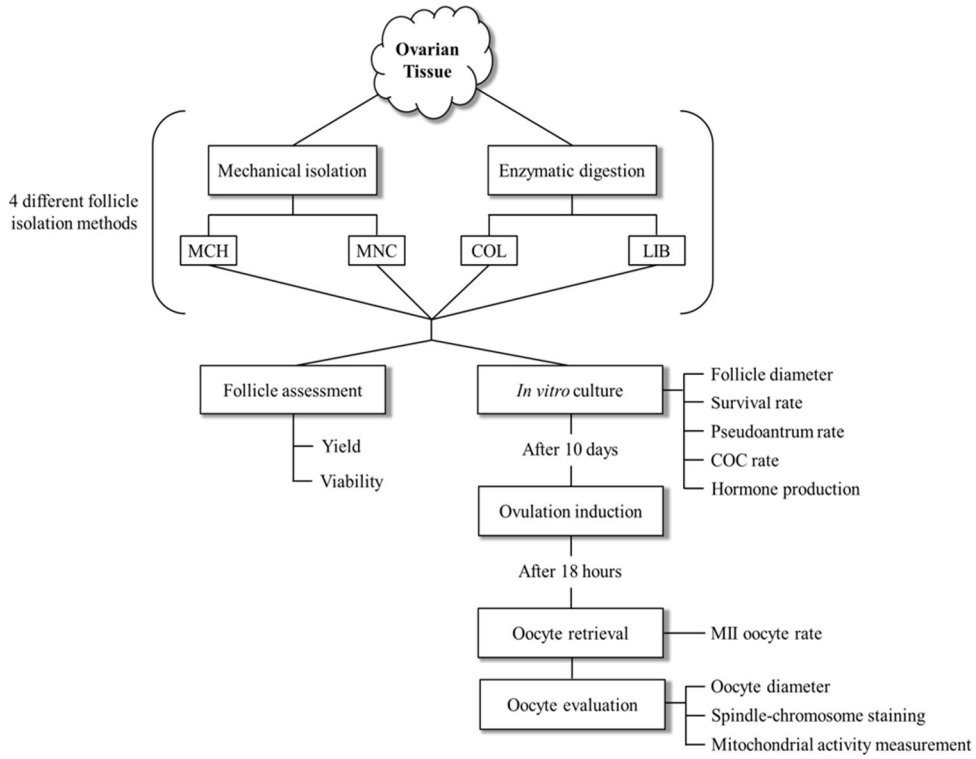

Figure 1. Experimental scheme for this study

The experiment had conducted by following the procedure as shown in an upper diagram. Ovarian follicles were isolated by using 4 different isolation methods. Then, follicles were analyzed before and after *in vitro* culture. After 10 days of culture, ovulation was induced and oocytes were retrieved. (MCH; mechanical, MNC; mincing, COL; collagenase, LIB; liberase, COCs; cumulus oocyte complexes and MII; metaphase II)

RESULTS

Follicle yield and viability after isolation

MCH, MNC, COL and LIB groups yield immediately after follicle isolation were 26.56 ± 3.6, 28.10 ± 2.57, 41.90 ± 2.56 and 43.10 ± 1.10 follicles per one ovary which have intact granulosa cell and proper morphology to *in vitro* culture, respectively. (Figure 2) The COL and LIB group showed significantly higher follicle yield per one ovary than MCH and MNC groups.

Follicle viability immediately after isolation in MNC, COL and LIB group was significantly higher (90.27%, 89.35% and 92.49%, respectively) than MCH group (80.57%). (Figure 3)

Follicle *in vitro* growth

Follicle diameter was gradually increased in all experimental groups. (Figure 4 and 5) On day 0, follicular diameter of all experimental groups showed no significant difference (104.58 ± 0.67 μm, 104.54 ± 0.64 μm, 102.54 ± 0.57 μm and 103.24 ± 0.52 μm, respectively). In early phase of culture period, day 2, follicular diameter of enzyme digestion group (161.41 ± 3.38 μm and 137.79 ± 4.17 μm, COL and LIB respectively) was significantly greater than MCH and MNC group (121.07 ± 1.77 μm and 120.80 ± 1.43 μm respectively). However, in the last phase of *in vitro* culture, day 10, MCH and MNC groups (496.84 ± 11.56 μm and 488.53

± 12.73 μm respectively) had showed the greater follicular diameter compared with enzyme digestion groups, COL and LIB group (430.41 ± 11.65 μm and 430.03 ± 12.00 μm, respectively).

On day 10 of *in vitro* growth, follicle survival rate had demonstrated statistically higher in MCH and MNC group, 88.7% and 92.1% respectively, than COL and LIB group, 70.4% and 71.2% respectively. (Table 1)

Follicle *in vitro* maturation

The pseudoantrum formation rate and COCs rate following ovulation induction 18 hours later were considered as the evaluation factor of follicular maturation. The pseudoantrum formation rate of MCH, MNC, COL and LIB group were 78.0%, 83.0%, 72.7% and 61.1%, respectively (Table 1). The MNC group showed statically higher rate than COL and LIB group.

The COCs rate was significantly higher in MCH group, 69.1% than MNC, COL and LIB group (52.5%, 35.8% and 28.0% respectively). Furthermore, MCN group also showed statistical difference between COL and LIB group. (Table 1)

Hormone production during *in vitro* culture

The estradiol concentration in spent media has generally increased for 10 days of culture. (Figure 6-A) The concentration of estradiol in spent media in MCH, MNC,

COL and LIB group was 0.04 ± 0.00 ng/ml, 0.05 ± 0.00 ng/ml, 0.05 ± 0.00 ng/ml and 0.06 ± 0.01 ng/ml respectively, on day 4 and 0.31 ± 0.08 ng/ml, 0.54 ± 0.10 ng/ml, 0.87 ± 0.26 ng/ml and 0.53 ± 0.03 ng/ml respectively, on day 8. On day 10, 1.44 ± 0.37 ng/ml, 0.66 ± 0.30 ng/ml, 0.53 ± 0.04 ng/ml and 0.73 ± 0.08 ng/ml of estradiol levels were measured in MCH, MNC, COL and LIB group, respectively. On day 4, LIB group showed significantly higher level of estradiol than MCH. However, on day 10, the estradiol level of MCH group was measured statistically higher than COL group.

In terms of progesterone, the progesterone concentration in spent media also has increased progressively to day 10 (Figure 6-B). On day 4, 0.24 ± 0.02 ng/ml, 0.24 ± 0.03 ng/ml, 0.30 ± 0.04 ng/ml and 0.36 ± 0.03 ng/ml of progesterone was measured in MCH, MNC, COL and LIB group, respectively. On day 4, the progesterone level of LIB was significantly higher than MNC group. The concentration of progesterone in spent media in MCH, MNC, COL and LIB group was 0.84 ± 0.11 ng/ml, 1.70 ± 0.58 ng/ml, 2.93 ± 0.61 ng/ml and 2.42 ± 0.54 ng/ml respectively, on day 8 and 1.44 ± 0.80 ng/ml, 2.30 ± 0.62 ng/ml, 2.82 ± 0.16 ng/ml and 2.70 ± 0.54 ng/ml respectively, on day 10. The COL group showed significant higher level of progesterone on day 8 than MCH. However, on day 10, there was no significant difference among the groups.

The concentration of testosterone in spent media in MCH, MNC, COL and LIB group was 0.37 ± 0.01 ng/ml, 0.40 ± 0.01 ng/ml, 0.59 ± 0.05 ng/ml and 0.52 ± 0.02 ng/ml respectively, on day 4 and 0.64 ± 0.11 ng/ml, 0.41 ± 0.06 ng/ml, 0.60 ± 0.10 ng/ml and 0.69 ± 0.04 ng/ml respectively, on day 8 (Figure 6-C). On day 10,

testosterone level in spent media of MCH, MNC, COL and LIB group was 0.65 ± 0.10 ng/ml, 0.49 ± 0.03 ng/ml, 0.49 ± 0.03 ng/ml and 0.69 ± 0.05 ng/ml, respectively. On day 4, COL and LIB group showed significantly higher testosterone level than MCH and MNC group. However, on day 8 and 10, there was no significant difference among the groups.

Mature oocyte retrieval and assessment

Mature oocytes were retrieved 18 hours after hCG administration. MNC group had presented the significantly highest rate of mature oocyte, 73.6%, compared with MCH, COL and LIB group, 59.6%, 43.8% and 42.2% respectively. (Table 1, Figure 7)

The diameter of MCH and MNC group, 70.21 ± 0.54 μm and 69.33 ± 0.58 μm respectively, showed no significant difference compared with *in vivo* control group, 72.03 ± 0.77 μm (Figure 8). However, diameter of mature oocyte derived from *in vitro* follicle culture in COL and LIB group, 67.57 ± 0.97 μm and 67.42 ± 0.92 μm respectively, were significantly lower than *in vivo* control group.

Normal meiotic spindle and chromosome alignment rate is significantly higher in MCH and MNC group, 85.1% and 91.7%, than COL and LIB group, 42.9% and 50.0% (Figure 9 and 10).

In MNC group, mitochondrial activity in oocyte, 980.30 ± 69.01, was significantly higher than *in vivo* control, COL and LIB group, 489.32 ± 25.78,

722.11 ± 25.31 and 405.56 ± 78.24 respectively (Figure 11 and 12). The mitochondrial activity in MCH group was 824.01 ± 27.32 that significantly higher than *in vivo* control and LIB group.

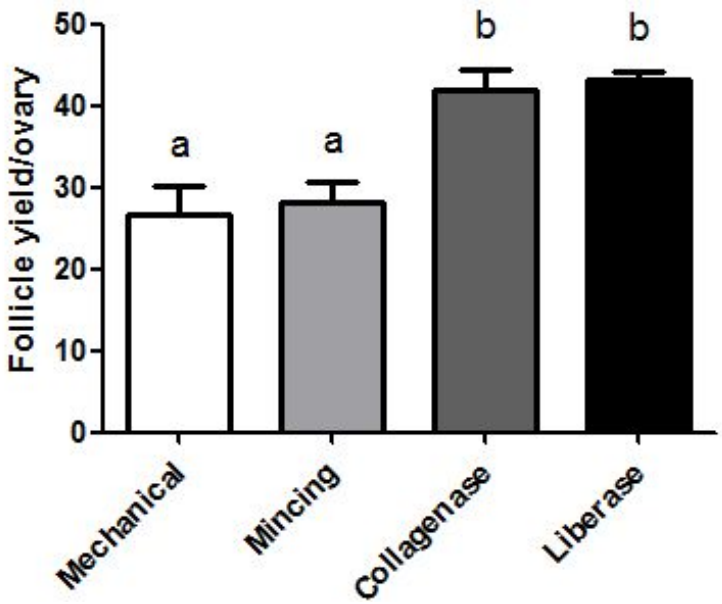

Figure 2. Follicle yield per one ovary after isolation according to isolation method

The X axis shows the 4 different methods for follicle isolation and the Y axis shows yield of follicle per one ovary. Results are presented as mean ± SEM. This study was replicated 3 times.

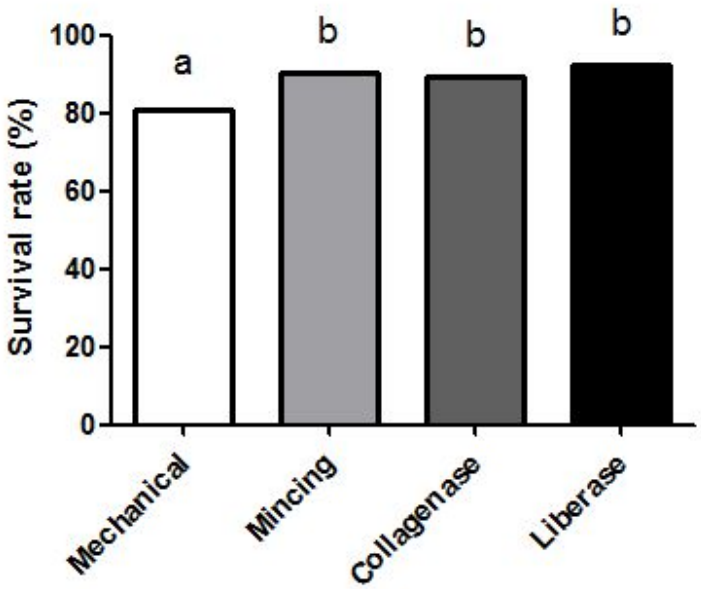

Figure 3. Follicle survival rate after isolation according to isolation method

The X axis shows the 4 different methods for follicle isolation and the Y axis shows survival rate of follicle after isolation. The different footnotes indicate the statistically significant differences between the groups ($P < 0.05$). This study was replicated 3 times.

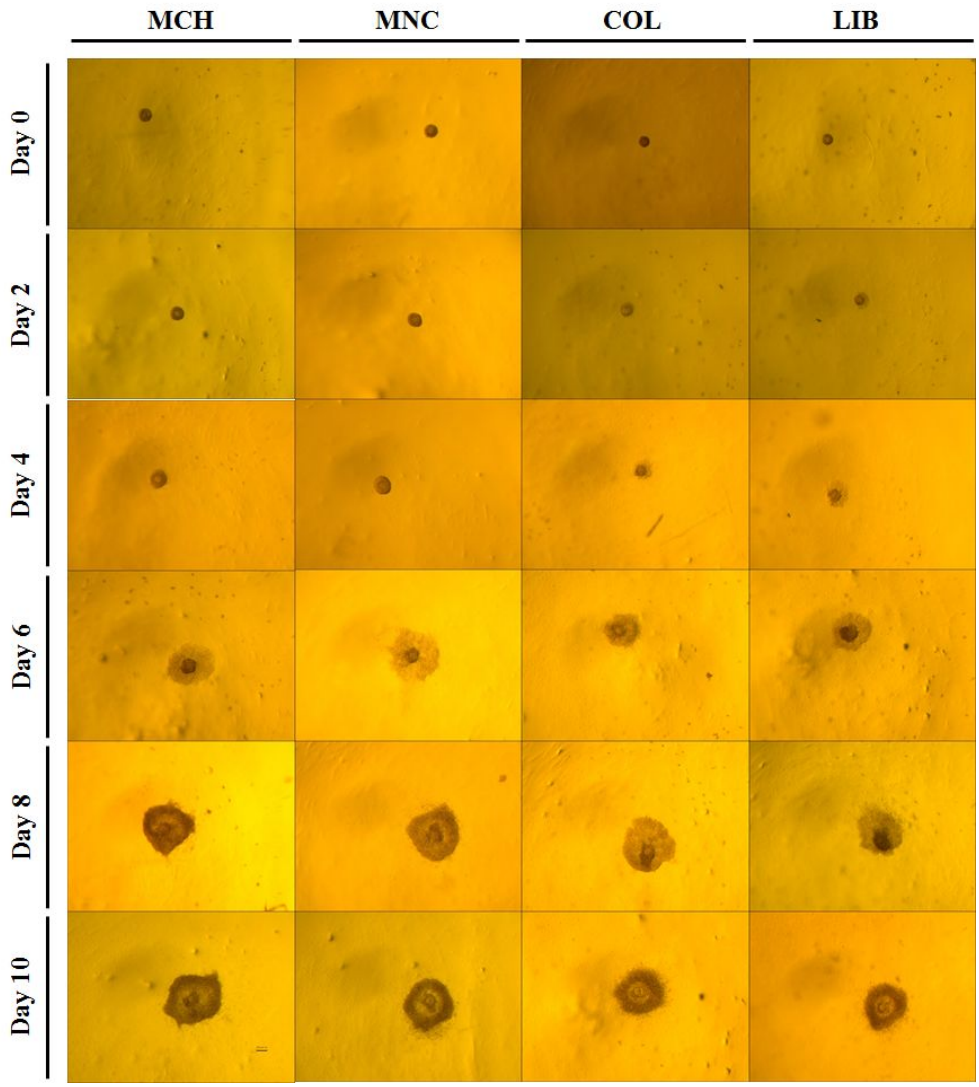

Figure 4. Representative images of *in vitro* growth on day 0, 2, 4, 6, 8 and 10 of isolated follicle according to isolation method (X40, scale bar = 100 μm)

The X axis shows the 4 different follicle isolation methods and the Y axis shows the day of *in vitro* culture (MCH; mechanical, MNC; mincing, COL; collagenase and LIB; liberase)

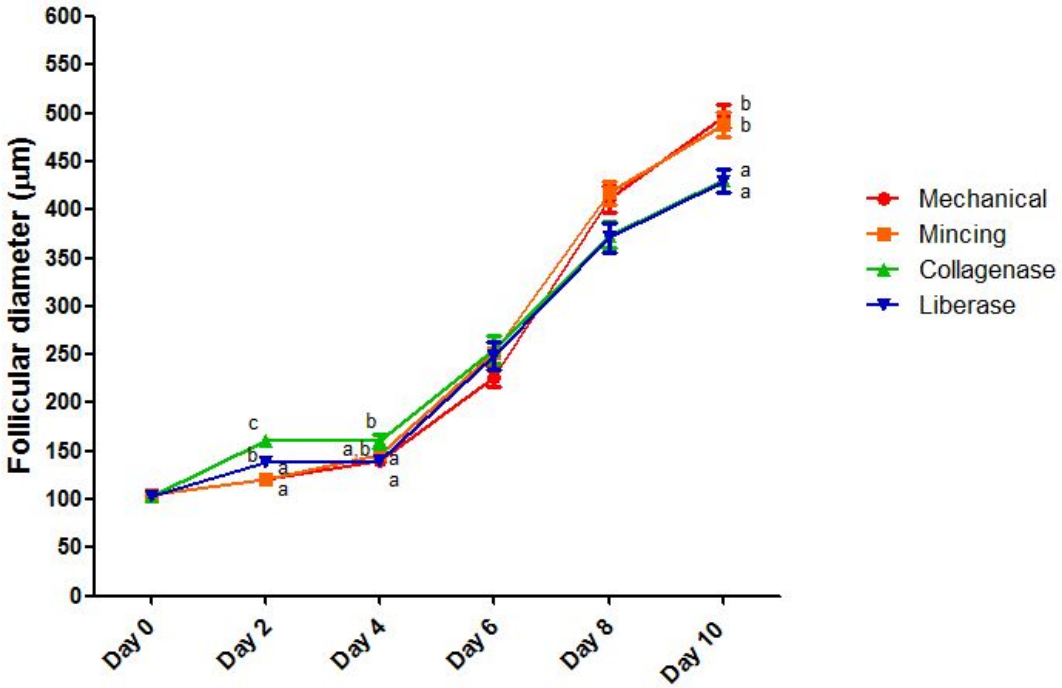

Figure 5. *In vitro* follicle growth curve according to follicle isolation method

The X axis shows day of *in vitro* culture and the Y axis shows follicular diameter (μm). The different footnotes indicate the statistically significant differences between the groups ($P < 0.05$). This study was replicated 3 times.

Group	No. of ovary	Total	Survive (%)	Psuedo-antrum formation (%)	COCs (%)	MII oocyte (%)
Mechanical	39	416	369 (88.7)[a]	288 (78.0)[a,b]	255 (69.1)[a]	152 (59.6)[a]
Mincing	39	416	383 (92.1)[a]	318 (83.0)[a]	201 (52.5)[b]	148 (73.6)[b]
Collagenase	39	416	293 (70.4)[b]	213 (72.7)[b]	105 (35.8)[c]	46 (43.8)[c]
Liberase	39	416	296 (71.2)[b]	181 (61.1)[c]	83 (28.0)[d]	35 (42.2)[c]

Table 1. Follicle survival rate, pseudoantrum formation rate, COCs rate and mature oocyte rate after *in vitro* follicle culture according to follicle isolation method

The different footnotes indicate the statistically significant differences between the groups ($P < 0.05$). The MII oocyte rate was calculated based on the number of COCs from *in vitro* follicle culture. (COCs; cumulus oocyte complexes and MII; metaphase II) This study was replicated 13 times.

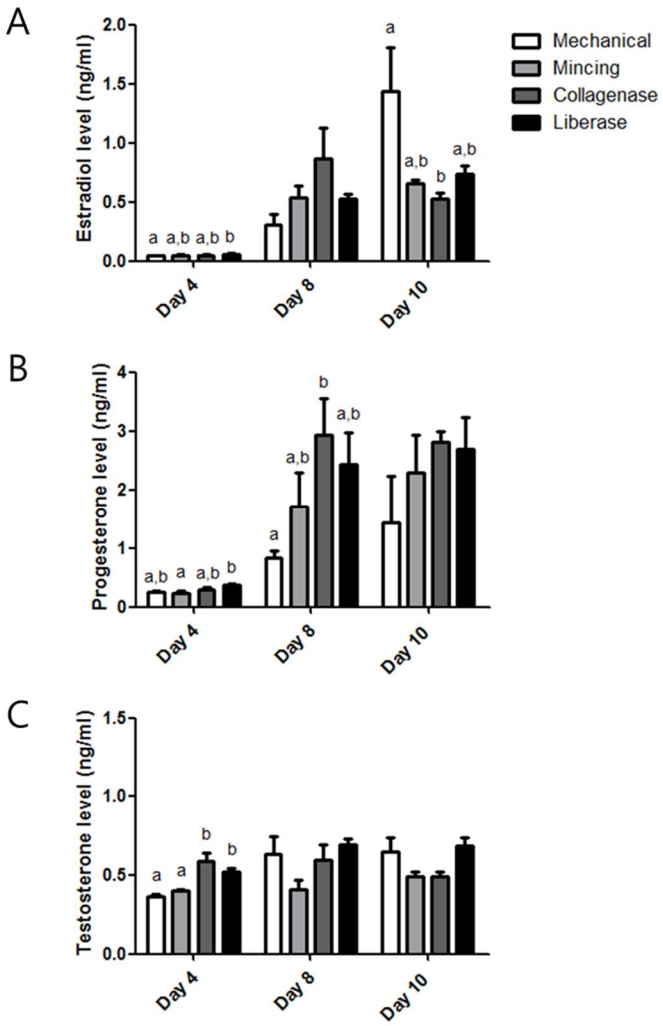

Figure 6. The concentration of hormone in the spent culture media on day 4, 8 and 10

The X axis shows the culture period and the Y axis shows hormone level (A; estradiol, B; progesterone, and C; testosterone, ng/ml) in spent culture media. Results are presented as mean ± SEM. The number of sample for each group was 7 spent of media.

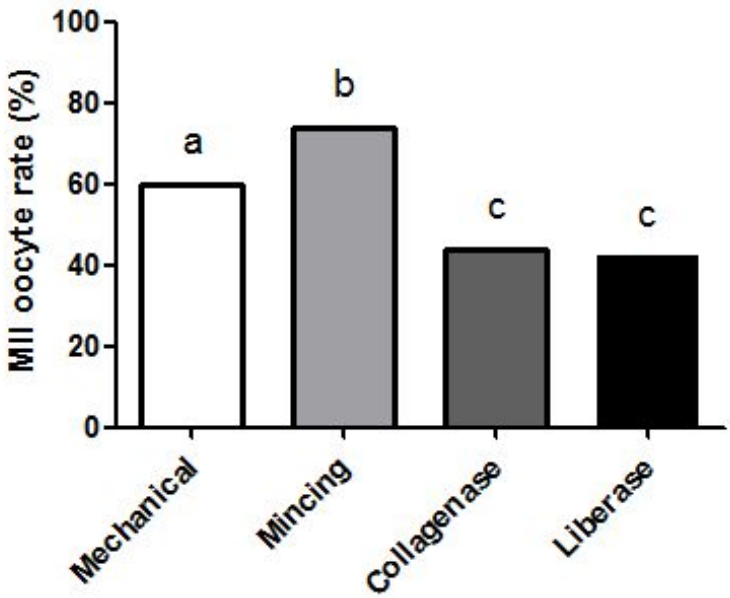

Figure 7. Mature oocyte rate 18 hours after ovulation induction according to isolation method

The X axis shows the 4 different methods for follicle isolation and the Y axis shows a mature oocyte rate 18 hours after ovulation induction. (MII; metaphase II) The different footnotes indicate the statistically significant differences between the groups ($P < 0.05$). This study was replicated 13 times.

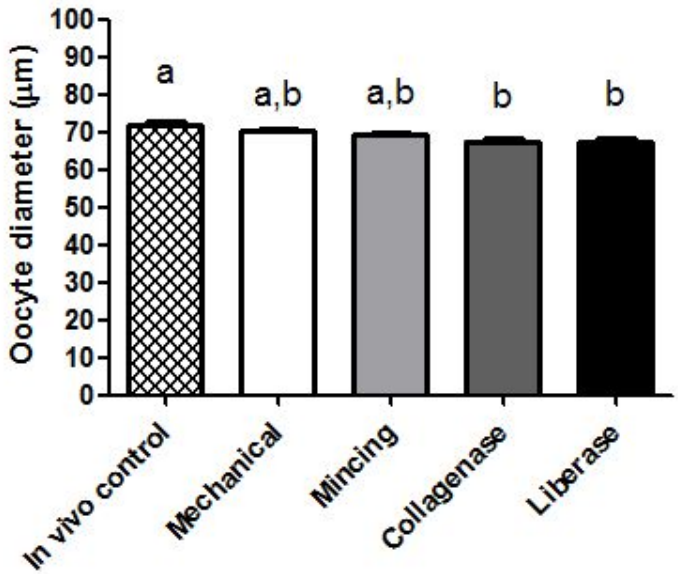

Figure 8. Oocyte diameter according to control and experimental groups

The X axis shows the control and 4 different methods for follicle isolation and the Y axis shows oocyte diameter. The different footnotes indicate the statistically significant differences between the groups ($P < 0.05$). This study was replicated 5 times.

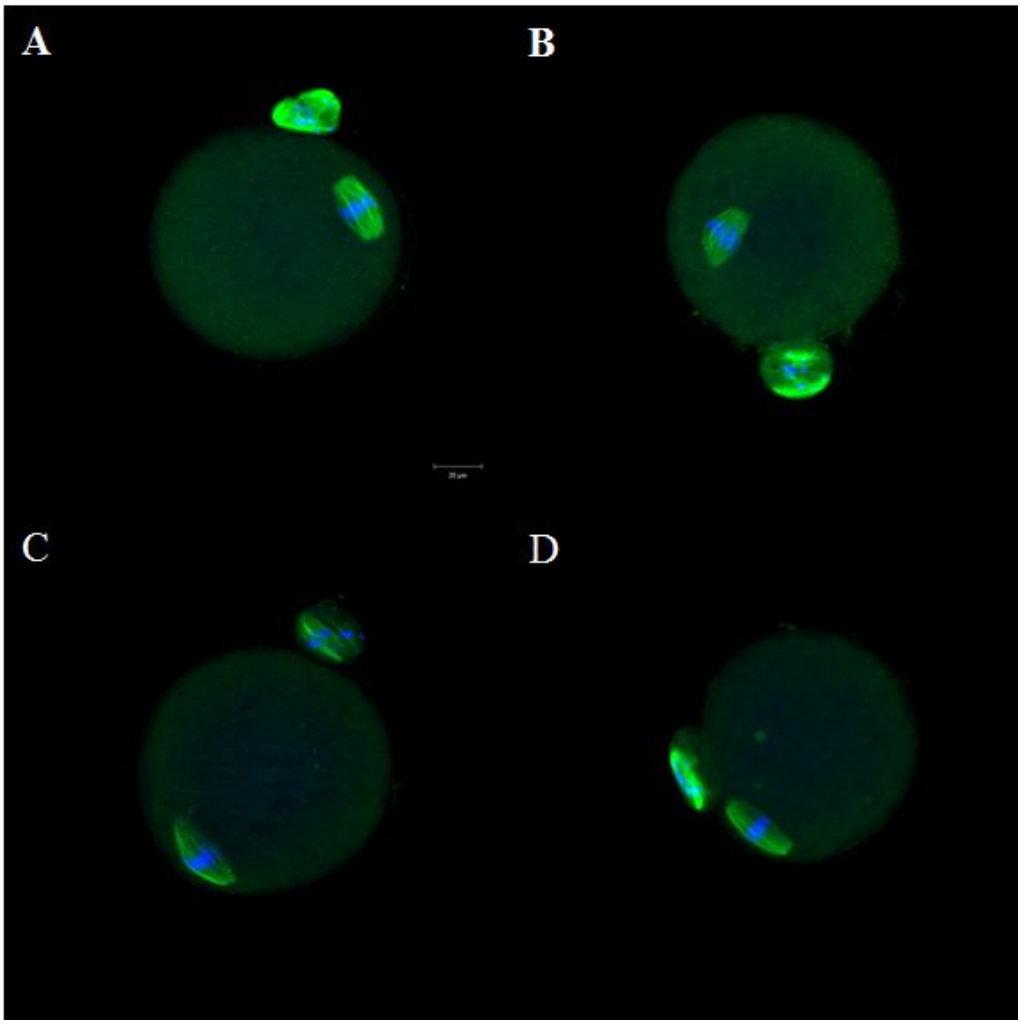

Figure 9. Representative images of meiotic spindle organization (green) and chromosome alignment (blue) immunofluorescence staining of a mature oocyte retrieved from *in vitro* follicle culture (X400, scale bar = 20 µm) (A) mechanical group, (B) mincing group, (C) collagenase group and (D) liberase group.

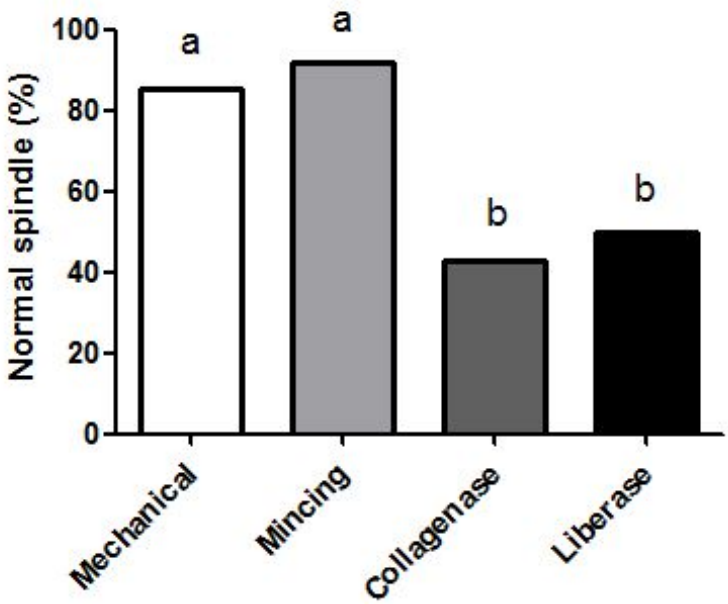

Figure 10. Normal meiotic spindle ratio of a mature oocyte retried form *in vitro* follicle culture

The X axis shows the 4 different methods for follicle isolation and the Y axis shows normal meiotic spindle rate of a mature oocyte 18 hours after ovulation induction. The different footnotes indicate the statistically significant differences between the groups ($P < 0.05$). This study was replicated 4 times.

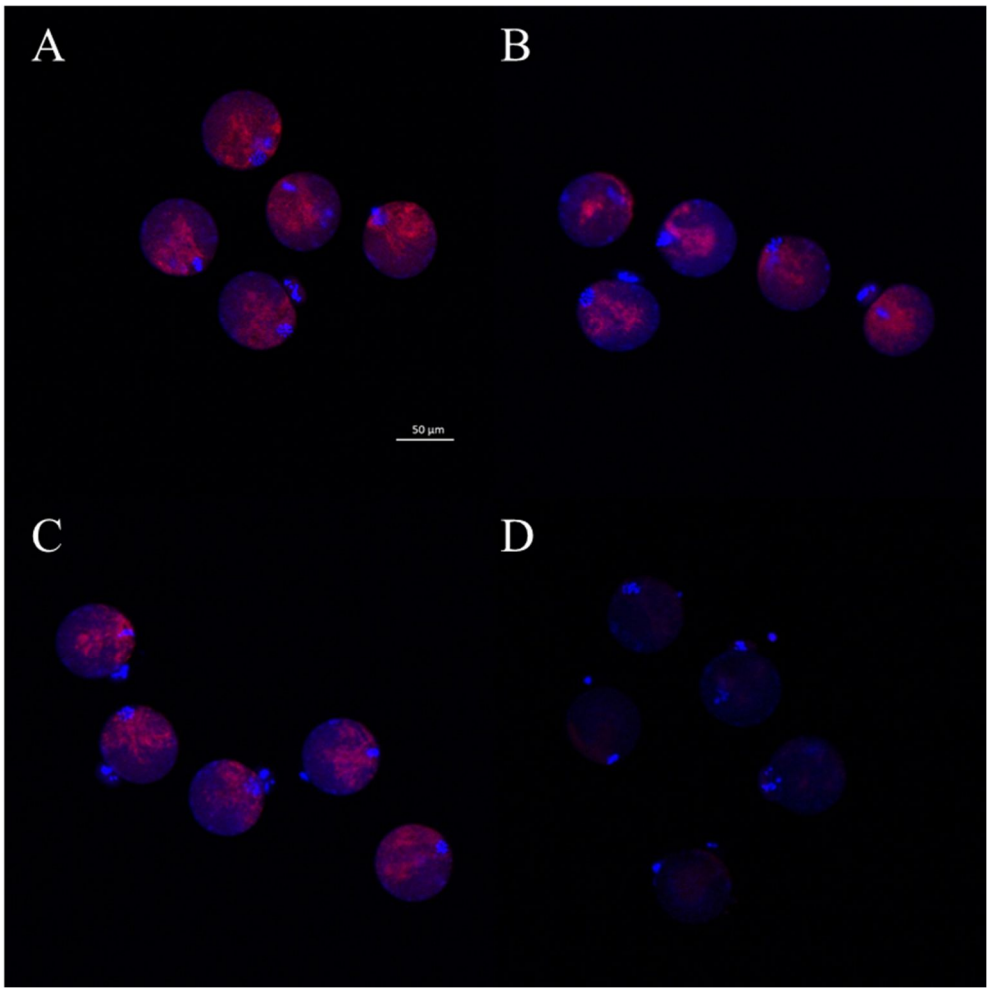

Figure 11. Representative images of mitochondrial activity (red) and chromosome alignment (blue) immunofluorescence staining of a mature oocyte retrieved from *in vitro* follicle culture (X200, scale bar = 50 µm) (A) mechanical group, (B) mincing group, (C) collagenase group and (D) liberase group.

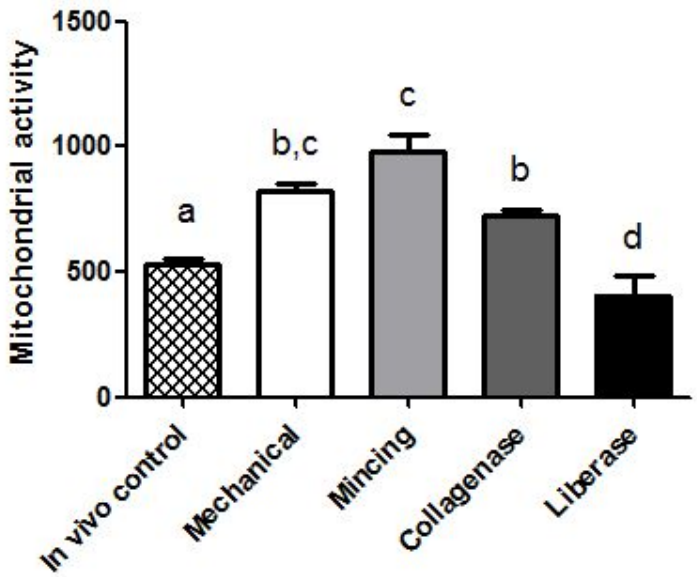

Figure 12. Mitochondrial activity in mature oocyte retrieved from follicle culture

The X axis shows the control and 4 different methods for follicle isolation and the Y axis shows mitochondrial activity. The different footnotes indicate the statistically significant differences between the groups ($P < 0.05$). This study was replicated 3 times.

DISCUSSION

The *in vitro* follicle culture is the essential study in order to not only understand the physiological aspect of oocyte growth, maturation and granulosa cell proliferation in ovary but optimize the fertility preservation procedure using ovarian follicle for cancer survivors (20, 27, 28). For the successful *in vitro* follicle culture, numerous factors, such as *in vitro* culture system, atmosphere and/or temperature condition, composition of media, follicle isolation method and follicle selection criteria, should be considered (8). The selection of proper and adequate follicle before beginning of *in vitro* follicle culture is the critical mater that determines the favorable progress of *in vitro* follicle culture. In the present study, the effects of 4 different follicle isolation methods, MCH, MNC, COL and LIB, on subsequent *in vitro* follicle culture were compared.

In last decades, MCH method, as known as manual micro-dissection, has been widely used for follicle isolation because it can preserve the proper follicular basement membrane and thecal cell layer after isolation (12, 22, 29). However, MCH method is a time consuming and laborious procedure (30). Moreover, ovarian tissues and follicles were prolonged exposure in unconditioned atmosphere during micro-dissection in manually. In contrast of this, the enzymatic digestion, such as using collagenase type I, deoxyribonuclease (DNase) and/or liberase, yields plenty of follicles in short time. However, enzymatic digestion methods have possibility that could destroy appropriate follicular morphology and damage to

basement membrane and thecal cell layer (20, 31).

In the present study, MNC method which is using cell dissociation kit was used for ovarian follicle isolation. The cell dissociation kit was generally used as tissue grinder in order to homogenize the large tissue (32). On the basis of result, MNC method can be an alternative way for follicle isolation before *in vitro* follicle culture. The MNC method is a simple method and also takes short time with avoiding serious damage on follicular natural morphology.

Many research groups reported that follicle yield of enzymatic digestion method for follicle isolation is the greater than mechanical method (30, 31, 33). The similar result of follicle yield in this study was observed. In a process of follicle isolation, reducing exposure time in unconditioned atmosphere is the issue for consideration. In present study, follicle viability immediately after isolation of MCH method is significantly lower than other groups, MNC, COL and LIB group. We assumed that extended time for isolation is the primary cause of significant reduction of viability after follicle isolation in MCH group.

The present study showed the significant greater size after 10 days of *in vitro* culture in MCH and MNC group than COL and LIB group. *In vivo*, the basement membrane in ovarian follicle plays a crucial role for preservation of follicular spherical morphology and its surface expands enormously during growth (30,000-fold in mice) (34). On the background of this, it could explain the higher growth in MCH and MNC group than COL and LIB group. We presumed that the expansion

of basement membrane probably supports the follicular growth and development competence during follicle culture. In present study, enzyme digestion groups (COL and LIB) showed significantly greater follicular diameter on day 2 of *in vitro* culture. In 1993, Gosden et al. reported that granulosa cells of follicles that isolated by enzyme digestion method quickly attach to the plastic surface and spread away from oocyte in early phase of *in vitro* culture (34). We presumed that similar follicle growth was observed in present study. The follicle survival rate after follicle culture showed significantly lower in enzymatic digestion group. The damage on basement membrane in early phase of culture impacts on follicle growth and development and therefore enzymatic digestion groups results poor survival rate at the end of culture duration (20, 31). The MNC group shows significantly improved rate of pseudoantrum formation and mature oocyte than MCH group. The result of the present study suggest that the shortening time into *in vitro* culture medium after ovarian tissue dissection and isolation of MNC method can results the better rate of pseudoantrum formation and mature oocyte than MCH method. On the other hands, the damage on basement membrane during enzymatic isolation could also become the cause of poorer *in vitro* growth in COL and LIB group (20, 31). Furthermore, pseudoantrum formation rate and COCs rate are also significantly lower than MCH and MNC group. In contrast to follicle yield result in the present study, the calculated yield rate of MII oocyte was 14.67%, 13.50%, 2.82% and 2.08% in MCH, MNC, COL and LIB group, respectively. This result means that although initial follicle yield per ovary is greater in enzyme groups than MCH and MNC group, the final MII oocyte yield is much higher in MCH and MIN

group than enzyme digestion groups.

The biosynthesis and regulation of steroid hormone during follicular growth is the important factor in proliferation of granulosa cell. Also, the monitoring progesterone level during *in vitro* follicle culture is widely used to evaluation proper growth and maturation of follicles that cultured *in vitro* (35, 36). In present study, estradiol and progesterone level were gradually increased during *in vitro* culture period. On day 10, MCH group showed significantly the higher level of estradiol compared with COL group. Thus, MCH method is better for estradiol synthesis rather than COL method. In terms of MNC or LIB method, there were no significant differences to synthesis the estradiol than MCH or COL group. In terms of progesterone, there is no difference on production on day 10.

Estradiol production is dependent on the presence of androgen precursors secreted by the theca cell and aromatized to estradiol by the granulosa cell (20). The androgens exert a direct stimulatory action on follicular development, especially during the preantral-early antral stage transition (37). In this study, on day 4 that early stage of *in vitro* culture, COL and LIB group showed significantly increased the level of testosterone than MCH and MNC group. These increasing in the steroid concentration in COL and LIB groups could be explained the contamination of theca-like cell during enzyme digestion described by H. Motohashi (33). We initially expected that the testosterone level would be increased during *in vitro* culture duration. In fact, the level of testosterone remained stable throughout the entire culture period. Similarly, Demeestere. I. et al. already reported that stable

testosterone secretion during *in vitro* culture (20).

It was reported that the diameter of oocyte that retrieved from follicle *in vitro* culture was smaller than *in vivo* grown mature oocyte (38, 39). However, diameter of oocyte that collected from MCH and MNC group has no significant difference with *in vivo* control in the present study. On the other hands, the oocyte diameter of COL and LIB group were statistically smaller compared with *in vivo* control. Thus, mechanical isolation method showed better results in terms of oocyte diameter than enzyme isolation method.

The meiotic maturation of oocyte is a crucial process for maximize competency of maternal factor for early embryo genesis (40). For the estimation of oocyte potential for pre- and/or post-implantation embryo development after fertilization, spindle and chromosome localization is widely used method (41, 42). The normal meiotic spindle rate was significantly higher in MCH and MNC groups than COL and LIB groups. The present study provides the evidence that oocyte maturation of follicle which isolated in enzymatic method is inferior to follicle from MCH and MNC method.

Mitochondrial activity is a crucial factor in oocyte maturation, fertilization and embryonic development (43, 44). In MNC group, mitochondrial activity was significantly higher than *in vivo* control, COL and LIB group. In 2009, Wang et al. reported that the stronger MitoTracker signals were observed in the *in vitro* matured oocytes than control (45). The elevated MitoTracker signals suggest that *in*

vitro follicle culture system significantly boosted mitochondrial biogenesis mtDNA replication. Although, mtDNA analysis was not performed in present study, mtDNA copy number is greater in *in vitro* cultured oocyte thant control oocyte in several studies (45, 46). We presumed that increased mtDNA copy number causes inhanced mitochondrial activity and function in order to overcome hypoxic and *in vitro* growth condition during *in vitro* culture period. Based on data, the oocyte derived from MNC group might have the best potential for fertilization and embryonic development.

Based on the result of this study, MCH and MNC methods could be considered as an effective follicle isolation method than enzymatic digestion method, COL and LIB groups. However, as descripted above, MCH method is laborious and time consuming to collect a proper follicle for *in vitro* culture. Therefore, MNC method could be alternative method for *in vitro* culture.

In the present study, effect of follicle isolation method on *in vitro* fertilization and embryo development were not evaluated. Therefore, in further study, research for the fertility potential of retrieved oocyte, pre-implantation and post-implantation competences of embryos were required. In addition, this study was conducted using mouse model. Given that the difference between species, researchers should consider for other animal study. Nevertheless of this, the present study clearly demonstrated that the harmful effect of enzyme digestion method on *in vitro* follicle culture. Therefore, in case of large animal such as bovine, caprine and baboon that enzyme digestion method is widely used for follicle isolation. Enzyme

use should be minimized and combination with mechanical isolation should be considered.

The present study is the first report for evaluation the effect of cell dissociation kit (mincing method) for mouse ovarian follicle isolation and further *in vitro* follicle culture compared with mechanical isolation and enzyme digestion. The COL and LIB groups showed significantly poorer results in in *in vitro* follicle growth, survival rate after culture, COCs rate, MII oocyte rate and normal spindle rate compared with MCH and MNC groups. In addition, follicle survival, MII oocyte rate and mitochondrial activity of MNC group was significantly superior to MCH group. Therefore, the results of present study demonstrated that MNC method have a solid evidence to be alternative method for follicle isolation.

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국 문 초 록

목적: 난소 조직 동결은 사춘기 이전의 여성 소아암환자 혹은 배우자가 없는 여성 암환자 모두에게 적용할 수 있는 가임력 보존 방법 중에 하나이다. 그러나 동결 보존된 난소 조직을 체내 이식하는 방법은 암이 완치된 환자에게 종양세포를 다시 이식할 수 있는 치명적인 단점을 가지고 있다. 난포 체외 배양 방법은 이러한 종양세포 재이식의 가능성을 배제할 수 있는 가임력 보존의 방법이다. 그러나 난포 체외 배양 방법은 현재까지 실험적인 수준에 남아있는 실정이다. 성공적인 난포 체외 배양을 위해서는 체외 배양을 하기에 앞서 적절한 난포 분리법이 선행되어야 한다. 이러한 이유를 토대로, 본 연구의 목적은 서로 다른 네 가지의 난포 분리법이 추후 체외 배양과 난자 성숙에 미치는 영향을 알아보고자 하였다.

방법: 본 연구에서는 11일에서 14일령의 암컷 BDF-1 생쥐를 사용하였다. 난소 조직을 절제한 후 난소는 서로 다른 4가지 방법의 난포 분리법에 따라 4군으로 무작위 배분하였다. (MCH군: 30G 주사기 바늘을 이용하여 난포 분리, MNC군: cell dissociation kit를 이용하여 난포 분리, COL: collagenase type 1을 사용하여 난포를 효소분해, LIB: liberase를

이용하여 난포를 효소분해) 난포는 지름이 100~110 μm의 초기 이차 난포 (early secondary follicle) 만을 선별적으로 수득하였다. 수득한 난포는 10 mIU/ml FSH, 1% ITS, 1% PS 그리고 5% FBS가 들어있는 glutaMAX-αMEM을 체외 배양액으로 하여 10일동안 체외 배양 하였다. 배양액은 4일에 한 번 전체 양의 반씩 교체하였고 사용된 배양액에서 estradiol, progestrone 그리고 testosterone 농도를 측정하였다. 10일째 되는 날 1.5 IU/ml hCG와 5 ng/ml EGF를 첨가하여 배란을 유도하였다. 난자 확인은 배란 유도 18시간 후에 실시하였고 체외 배양기간 동안 이틀에 한번씩 난포의 지름을 측정하였다. 또한 체외 배양 10일째에 난포의 생존율과 가동형성(pseudoantrum formation)율을 확인하였다. 배란 유도 18시간 후엔 축적 난모세포 결합(cumulus oocyte complex)율과 성숙난자의 수를 확인하였고 그 지름을 측정하였다. 얻어진 난자 내 mitochondrial activity를 면역형광염색을 통해 확인하였다. 또한 감수분열능 확인을 위하여 난자 내 방추사와 염색체를 면역형광염색을 실시하여 확인하였다.

결과: 하나의 난소에서 얻을 수 있는 난포의 수율은 COL과 LIB군에서 높은 결과를 보였다. 그러나 난포 분리 직후의 생존율은 MNC, COL 그리고 LIB군에 비하여 (90.27%, 89.35% 그리고 92.49%) MCH군에서 유

의하게 낮았다 (80.57%). 체외 배양 후 10일 째 난포의 지름은 MCH군과 MNC군에서 각각 496.84 ± 11.56 μm 그리고 488.53 ± 12.74 μm로 측정되어 각각 430.41 ± 11.65 μm 그리고 430.03 ± 12.00 μm로 측정된 COL군과 LIB군보다 유의하게 컸다. 10일째의 난포 생존율 또한 MCH군과 MNC군에서 각각 88.7%와 92.1%로 각각 70.4% 그리고 71.2%인 COL군과 LIB과 비교하여 통계적으로 유의한 차이를 보였다. MNC군에서는 83.0%의 가동형성율을 보여 그 외 다른 군들과 유의한 차이를 보였다 (MNC, COL 그리고 LIB군 각각 78.0%, 72.7% 그리고 61.1%). 배란유도 18시간 후 축적 난모세포결합율은 MCH군에서 69.1%로 52.5%인 MNC군과 유의한 차이를 보였으며 COL군과 LIB군은 각각 35.8%, 28.0%로 MCH군 및 MNC군보다 유의하게 낮았다. 또한 MNC군에서는 월등한 성숙난자율을 보였으며 (73.6%) 이는 각각 59.6%, 43.8% 그리고 42.2%인 MCH군, COL군 그리고 LIB군과 통계적 유의차를 보였다. 배양 후 10일 째에 MCH군에서 estradiol의 농도는 1.44 ± 0.37 ng/ml로 0.53 ± 0.04 ng/ml인 COL group보다 유의하게 높았다. 반면에 배양 후 10일 째 progesterone과 testosterone의 농도는 각 군당 유의한 차이를 보이지 않았다. MCH군과 MNC군에서 얻은 난자의 지름은 각각 70.21 ± 0.54 μm 와 69.33 ± 0.58 μm로, 72.03 ± 0.77 μm인 *in vivo* control군과 유의한 차이를 보이지 않았다. 그리고 난자 내 정상 방추사 및 염색체 비율은 MCH군과 MNC군에서 85.1%와 91.7%로 각각

42.9%와 50.0%인 COL군과 LIB군보다 유의하게 높았다. 난자 내 mitochondrial activity는 MNC군에서 980.30 ± 69.01로 MCH, COL, LIB 및 control 군의 mitochondrial activity의 수치인 489.32 ± 25.78, 824.01 ± 27.32, 722.11 ± 25.31 그리고 405.56 ± 78.24보다 유의하게 높았다.

결론: MNC법은 체외 배양 후 난포의 지름, 생존율, 가동형성율, 성숙난자율에서 유의하게 향상된 결과를 보였다. 아울러 정상 방추사 및 염색체 비율 또한 COL법과 LIB법에 비하여 높은 결과를 보였다. 이러한 결과를 토대로, MNC 방법은 많은 노동력과 시간이 소모되는 MCH의 대안으로 고려될 수 있다. 반면 COL법 및 LIB법과 같은 난포 효소분해 방법은 본 연구의 결과로 비추어 볼 때 효율적인 난포 체외 배양에 적절치 않은 것으로 나타났다. 그러나 향후 난포 체외 배양 후 수득된 난자의 수정능이나 착상 전후의 배아의 발달능에 관한 연구가 더 필요할 것으로 사료된다.

주요어: 가임력 보존 / 난포 / 난포 체외 배양 / 난포 분리

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