



# Adverse Effect of Superovulation Treatment on Maturation, Function and Ultrastructural Integrity of Murine Oocytes

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Regular monitoring on experimental animal management found the fluctuation of ART outcome, which showed a necessity to explore whether superovulation treatment is responsible for such unexpected outcome. This study was subsequently conducted to examine whether superovulation treatment can preserve ultrastructural integrity and developmental competence of oocytes following oocyte activation and embryo culture. A randomized study using mouse model was designed and in vitro development (experiment 1), ultrastructural morphology (experiment 2) and functional integrity of the oocytes (experiment 3) retrieved after PMSG/hCG injection (superovulation group) or not (natural ovulation; control group) were evaluated. In experiment 1, more oocytes were retrieved following superovulation than following natural ovulation, but natural ovulation yielded higher ( $p < 0.0563$ ) maturation rate than superovulation. The capacity of mature oocytes to form pronucleus and to develop into blastocysts in vitro was similar. In experiment 2, a notable ( $p < 0.0186$ ) increase in mitochondrial deformity, characterized by the formation of vacuolated mitochondria, was detected in the superovulation group. Multivesicular body formation was also increased, whereas early endosome formation was significantly decreased. No obvious changes in other microorganelles, however, were detected, which included the formation and distribution of mitochondria, cortical granules,

microvilli, and smooth and rough endoplasmic reticulum. In experiment 3, significant decreases in mitochondrial activity, ATP production and dextran uptake were detected in the superovulation group. In conclusion, superovulation treatment may change both maturational status and functional and ultrastructural integrity of oocytes. Superovulation effect on preimplantation development can be discussed.

**Keywords:** artificial reproductive Technology (ART), development, gonadotrophins, microorganelle function, oocyte maturation

## INTRODUCTION

Superovulation treatment has been used for retrieving large numbers of mature oocytes (Fowler and Edwards, 1957; Nagy, 2003), which triggers enormous improvements in artificial reproductive technology (ART) of both human and domestic animal species. The safety of superovulation treatment has been confirmed, which generally preserves oocyte morphology and functional integrity (Munoz et al., 1994; Nagy, 2003). The procedure of superovulation treatment has continuously been optimized (Combelles and Albertini, 2003; Edwards and Gates, 1959; Fowler and Edwards,

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1960; Hamberger and Wikland, 1993; Legge and Sellens, 1994; Miller and Armstrong, 1981; Steward et al., 2014; Templeton and Morris, 1998), which leads to successful in vitro fertilization (IVF) and embryo transfer (ET) program (Hamberger and Wikland, 1993; Templeton and Morris, 1998).

In several reports, however, it has been reported that superovulation causes perturbation of maternal and paternal imprinted methylation (Fortier et al., 2008; Market-Velker et al., 2010), reduced intrafollicular estradiol and progesterone levels (Assey et al., 1994) and delayed embryonic and fetal development (Van der Auwera and D'Hooghe, 2001). Injection of hormones at supra-physiological levels may cause ultrastructure deformities of oocytes, which leads precocious maturation with functional aberrations (Hasegawa et al., 2015; Moor et al., 1985; Yun et al., 1989). As a matter of fact, occasional fluctuation of ART outcome may be experienced even after employing the optimized superovulation protocol in field trials, and various environmental factors including seasonal variation and miscellaneous changes in oocyte quality may be responsible for such perturbation.

Consequently, we examined how superovulation treatment influences oocyte maturity and development, and their ultrastructural and function integrity. Mature (metaphase II stage) oocytes were provided following superovulation treatment with pregnant mare's serum gonadotropin (PMSG)/human chorionic gonadotropin (hCG) or not (natural ovulation). Initial observation on retrieval, maturation, pronuclear formation of oocytes and preimplantation development of activated oocytes was monitored. Next, morphological observation of microorganelles such as mitochondria, multivesicular bodies was undertaken using transmission electron microscope (TEM) analysis. Since mitochondria have an important role in various cellular processes (Balaban et al., 2005; Salminen et al., 2014) and since they have particular patterns of distribution during development (Van Blerkom, 1991; Van Blerkom and Runner, 1984), adenosine triphosphate (ATP) production by mitochondria (Kulkarni et al., 1998; Mancini et al., 1997) and early endocytosis (Moore et al., 1997; West et al., 2004) were compared between the superovulation and natural ovulation groups.

## MATERIALS AND METHODS

### Experimental design

F1 hybrid mice of the non-block B6CBAF1 strain were hyperstimulated with PMSG, and superovulated oocytes following hCG injection were retrieved. As a control treatment, oocytes were retrieved after natural ovulation. In experiment 1, oocyte retrieval, maturation and developmental competence were monitored. Both superovulated and naturally ovulated oocytes were either activated parthenogenetically or fertilized in vitro and were subsequently cultured for 120 h. Number of oocytes retrieved and developed to the metaphase II (mature), pronuclear, 2-cell, 4-cell, morula and blastocyst stages was monitored. In experiment 2, ultrastructural normality of oocytes were monitored with TEM, which includes mitochondria, cortical granules, multivesicular bodies, lipid droplets, microvilli, and the endoplasmic reticulum

system. In experiment 3, ATP generation representing mitochondrial function and endocytosis in mature oocytes retrieved from superovulation or natural ovulation were evaluated by JC-1 staining and dextran uptake, respectively.

### Experimental animals

B6CBAF1 hybrid mice were employed as experimental animals. Parental C57BL6 female and CBA/caj male mice were purchased from Jackson Laboratory. Mature oocytes were harvested from 8-week-old female mice after experimental treatments. All animals were maintained under conditions of controlled lightening (14 h of light/10 h of darkness), temperature (20-22°C), and humidity (40-60%). All animal management, breeding, and euthanasic procedures were performed according to the standard protocols of Seoul National University. The experimental protocols were approved by the Institutional Animal Care and Use Committee (approval number SNU-091028-4). Additionally, experimental samples were managed appropriately, and quality control of the laboratory facility and equipment were conducted.

### Collection of mature oocytes

Naturally ovulated oocytes were collected by oviduct flushing of F1 female mice in estrus 16 h after they were mated with a vasectomized male mice. Vaginal smears were performed to check the estrous cycle stage of the female donors. The flushing medium was M2 medium (Sigma-Aldrich, St Louis, MO). For ovarian hyperstimulation, 5 IU of PMSG (Folligon™; Intervet International, Boxmeer, the Netherlands) was injected intraperitoneally and ovulation was induced by intraperitoneal injection of 5 IU of hCG (Pregny™; Organon, Oss, the Netherlands) 48 h later (Jang et al., 2007). Oocytes were recovered 16 h post-hCG. After hormonal treatments females were sacrificed by cervical dislocation. Oocyte maturation at the metaphase II stage was verified by extrusion of the first polar body in the perivitelline space. For this analysis, oocytes were released from cumulus cells through incubation in M2 medium supplemented with hyaluronidase (Sigma-Aldrich; 200 IU/ml) for 5 min at 37°C.

### Parthenogenetic activation and IVF of oocytes

To activate oocytes parthenogenetically, oocytes released from cumulus cells were cultured in calcium-free potassium simplex optimized medium (KSOM) supplemented with 10 mM SrCl<sub>2</sub> and 5 µg/ml cytochalasin B for 4 h. For IVF, cumulus-enclosed oocytes retrieved after either natural ovulation or ovarian hyperstimulation were fertilized through incubation with epididymal semen in KSOM for 4-6 h (Byers et al., 2006). Oocytes that had been activated parthenogenetically or inseminated in vitro were then cultured constantly in 5 µl droplets of modified Chatot, Ziomek, and Bacister (CZB) medium for a further 120 h at 37°C under an atmosphere of 5% CO<sub>2</sub> in air. Pronucleus formation, cleavage, and development to the 4-cell, morula, and blastocyst stages were monitored under an IX70 inverted microscope (Olympus, Japan) at 6, 24, 48, 72 and 120 h after activation or fertilization.

### TEM analysis

Mature oocytes retrieved after experimental treatment were incubated in modified Karnovsky's solution overnight and were subsequently fixed through incubation with osmium tetroxide for 2 h. The fixed oocytes were dehydrated in a graded ethanol solution. Then, en bloc staining was conducted overnight. The specimens were then embedded in Spurr resin. Ultra-thin sections (60 nm thick) were prepared with an ultramicrotome. The sections were then mounted on grids and stained with uranyl acetate and Reynolds' lead citrate (Kim, 2008). They were subsequently examined with a LIBRA 120 energy-filtering TEM (Carl Zeiss, Germany). Images of ooplasm (area: 100  $\mu\text{m}^2$ ) at magnifications of 6,000 to 25,000 were used.

The area of section being observed was randomly chosen, which resulted randomly distributed from the middle to the peripheral areas. The area of section was selected randomly, so all levels had comparative value between experimental groups. Total number of each cellular organelle in single TEM image was calculated and based on the scale bar being designated, number of each cellular organelles per unit area were counted. At least ten images were used for undertaking numerical analysis.

### Evaluation of the ratio of activated to less-activated mitochondria by confocal microscopy

We used the potential-sensitive fluorescent dye JC-1 (Molecular Probes, USA) to examine the activity of mitochondria in the ovulated oocytes. JC-1 was dissolved to a stock concentration of 0.2 mM in DMSO and diluted to a final concentration of 2  $\mu\text{M}$  in M2 medium. Oocytes were exposed to JC-1 at 37.5°C under an atmosphere containing 5% CO<sub>2</sub> in a humidified incubator for 1 h and then washed three times in M2 medium to remove any surface fluorescence. Stained oocytes were transferred to a confocal dish containing M2 medium. A laser scanning confocal microscope was used to examine mitochondrial activity. The excitation laser line was set to 488 nm, and the emission wavelengths were separated by a 530 nm dichroic mirror, with further filtering through a 515-530 nm band-pass filter (green emission) or a 585 nm long-pass filter (red emission) filter. Fluorescence intensity ratios were measured from the confocal images using NIS-Elements BR 3.0 imaging software (Nikon, Japan). Mitochondria with high membrane potentials (activated) were visualized in red, whereas those with low membrane potentials were in green.

### Quantification of ATP synthesis

ATP levels in ovulated oocytes were measured using a Bioluminescent Somatic Cell Assay Kit (FL-ASC; Sigma-Aldrich), which makes use of the luciferin-luciferase reaction. Briefly, oocytes (18 oocytes from natural ovulation and 39 oocytes from hyperstimulation) were placed into 100  $\mu\text{l}$  of ice-cold somatic cell reagent (FL-SAR; Sigma-Aldrich) for 5 min. They were then incubated with 100  $\mu\text{l}$  of diluted ice-cold assay mix [FL-AAM reagent, diluted 1:25 in ATP Assay Mix Dilution Buffer (FL-AAB reagent; Sigma-Aldrich)] for an additional 5 min. The solution was then transferred to an AutoLumat LB 953 Luminometer (EG&G Berthold) for the measurement of

luminescence. A 10-point standard curve (0-10 pmol/tube) was constructed for every 20 oocytes, and ATP content was calculated using the formula from linear regression of the standard curve.

### Endocytosis assay

To assess dextran uptake, ovulated oocytes were retrieved, and the zona pellucida was removed using acid Tyrode solution. For the analysis of endocytic activity in ovulated oocytes, each group was incubated with FITC-dextran (10,000 MW; Molecular Probes) for 30 min at 37°C. As a positive control, oocytes were cooled to 4°C prior to incubation with dextran at 4°C for 30 min. Oocytes were washed three times and immediately transferred to a confocal dish containing M2 medium, which was kept on ice at all times. To obtain confocal images, we used a krypton-argon mixed-gas laser. The excitation laser line was set to 494 nm, and the emission wavelength was set to 521 nm using a filter. Fluorescence intensity ratios were measured from the confocal images using NIS-Elements BR 3.0 imaging software (Nikon).

### Statistical analysis

All experiments were replicated more than three times, and the data obtained were subjected to statistical analysis. Not all oocytes retrieved from each experiment were provided because the superovulation treatment yielded different number of oocytes retrieved. To compensate this discrepancy, randomly selected oocytes were allotted to several treatment groups. A generalized linear model (PROC-GLM) created using Statistical Analysis System (SAS) software version 9.1 (SAS Institute, USA) was used to analyze the data. When a significant model effect was detected, comparisons among groups were subsequently conducted using the least-squares or Duncan methods. A p-value of less than 0.05 indicated a significant difference.

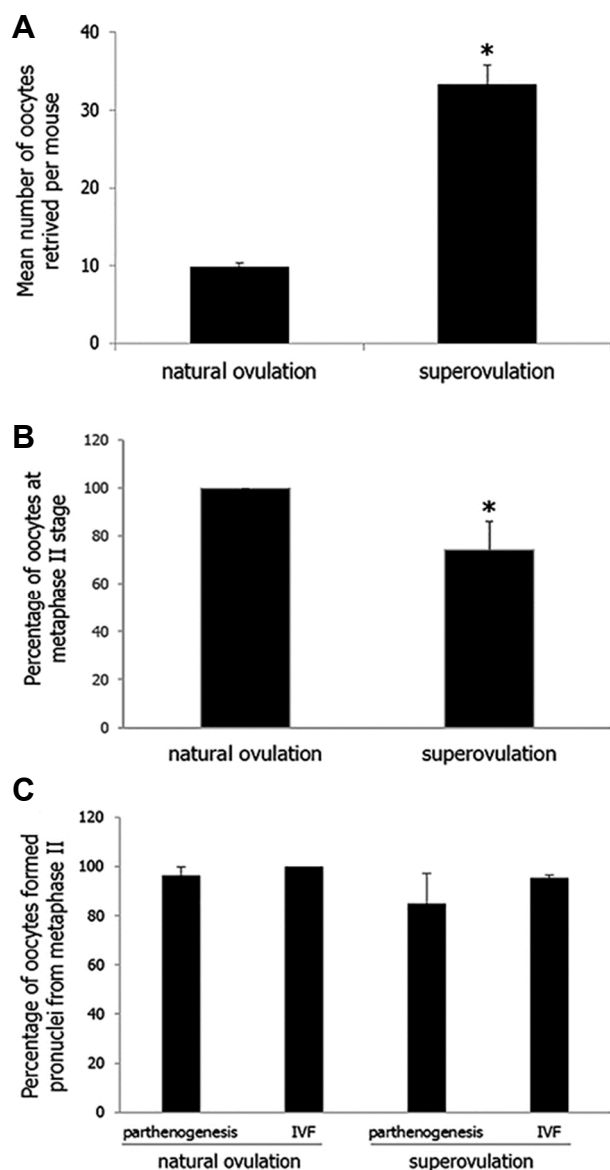
## RESULTS

### Experiment 1

As shown in Fig. 1, more (32.6 vs. 9.8 oocytes/head;  $p < 0.0001$ ) oocytes were retrieved after superovulation treatment than after natural ovulation. However, significant ( $p < 0.0563$ ) decrease in maturation rate was detected in the superovulation group, compared with the control (100% vs. 74%). Both IVF and parthenogenesis of mature oocytes triggered pronuclear formation of oocytes (85-100%), which did not differ significantly ( $p < 0.4863$ ) among groups. As shown in Table 1, the model effect of superovulation on preimplantation development was not ( $p > 0.4$ ) detected. The developments to the 2-cell, 4-cell, morula and blastocyst stages were 94 to 100%, 87 to 96%, 87 to 93% and 72 to 82%, respectively.

### Experiment 2

Total 11 mice were euthanized for observing oocyte ultrastructure and, 3 and 8 mice were allotted into the superovulation and the natural cycle group, respectively. The number of oocytes examined was 110 for the superovulation and 109 for the natural cycle groups. However, only 10 images



**Fig. 1. Retrieval, maturation and activation of oocytes derived from B6CBAF1 either superovulated or not (natural mating).** For numerical comparison, number of oocytes retrieved per mouse either superovulated or not (A), percentage of metaphase II stage oocytes to retrieved oocytes (B), percentage of pronuclear stage oocytes to metaphase II stage oocytes (C) were counted. Statistical comparison employing PLOC-GLM model was conducted with numerical values and data are presented as mean  $\pm$  SE. Model effects ( $p$  value) of each parameter were less than 0.0001 (A), 0.0563 (B) and 0.4863 (C). Asterisk indicated statistical significance with other treatments ( $p < 0.0563$ ).

of different oocytes in each group were randomly selected and subsequently employed for the analysis. As shown in Fig. 2A2, a significant increase in mitochondrial deformity was detected ( $p = 0.0186$ ). The percentage of mitochondria that were vacuolated was significantly increased after superovu-

lation treatment (39% vs. 30%), and normal mitochondria with well-developed cristae and without vacuoles were concomitantly observed. The overall number of mitochondria per mature oocyte did not differ significantly ( $p > 0.8999$ ) between the two groups (20.1 vs. 20.8). There were more multivesicular bodies in the superovulation group than in the natural ovulation group (1.44 vs. 4.27;  $p = 0.0221$ ; Fig. 2D). Apart from these microorganelles, no other deformities were found (Figs. 2B and 2C). Cortical granules were found in the area beneath the plasma membrane. Whereas the granules were located just beneath the oolemma in naturally ovulated oocytes, they were located a little farther underneath the oolemma in the superovulation group (0.4-0.6  $\mu$ m beneath the oolemma). The perivitelline spaces were slightly larger in the superovulation group than in the natural ovulation group. Well-developed smooth endoplasmic reticulum was observed in both groups.

### Experiment 3

Nine mice were provided for JC-1 staining (96 oocytes) and ATP measurement (57 oocytes). As shown in Fig. 4, activated mitochondria in the superovulation group (Fig. 4D2) were distributed in a semi-peripheral pattern as compared with the control group (Fig. 4C2). There was a significant difference in mitochondrial activity between two groups ( $p = 0.0179$ ; Fig. 4E). Moreover, the ratio of activated mitochondria to inactivated mitochondria was lower after superovulation (1.17 vs. 0.97). As shown in Fig. 4F, superovulation treatment had a significant effect on ATP synthesis activity ( $p = 0.0002$ ), which was lower in non-stimulated oocytes than in hyperstimulated oocytes (3.66 vs. 0.74 pmol/oocyte). Dextran uptake was measured in 87 oocytes. As shown in Fig. 5, dextran uptake, measured as a marker of early endocytosis, was lower in the hyperstimulation group compared with the control group (65.14 vs. 5.32;  $p = 0.004$ ), although there was an increase in the number of multivesicular bodies, indicating that late endocytosis was increased.

### DISCUSSION

The results of this study demonstrated that compared with natural ovulation, the superovulation treatment with PMSG and hCG decreases the proportion of mature oocytes to retrieved oocytes. In some oocytes being superovulated, ultrastructural alterations in mitochondria and lysosomal complexes, characterized by increased numbers of vacuolated mitochondria and multivesicular bodies were observed. ATP synthesis became lower in the superovulation group, which might be related to the mitochondrial deformity, and early endocytosis was also influenced by the superovulation. However, the observation of these experimentations do not reduce the feasibility of superovulation in ART program activation and development. While superovulation treatment has become the basic methodology of human and animal ART programs, it has been reported that decreased quality of superovulated oocytes before and after IVF resulted the reduced fecundity. Increased preimplantation mortality after superovulation has been reported in mice (Beaumont and Smith, 1975; Ertzeid and Storeng, 1992; Kalthur et al., 2015)

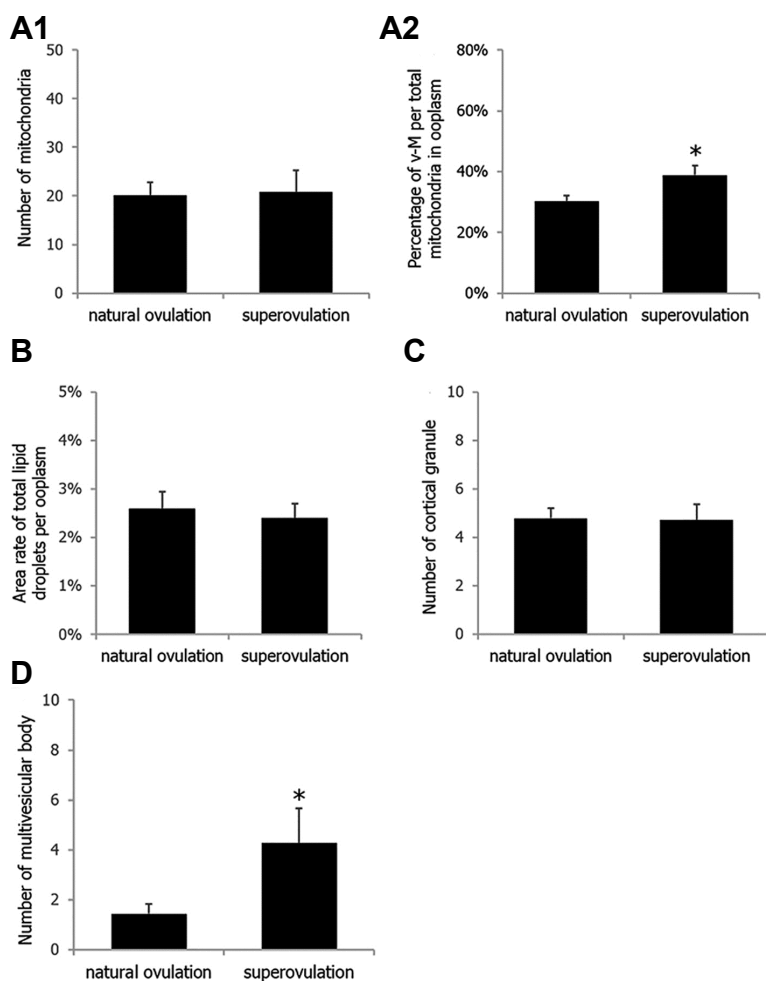
**Table 1.** Developmental competence of B6CBAF1 oocytes retrieved after natural ovulation or ovarian hyperstimulation with PMSG and hCG

Treatments	Methods of oocyte activation	No. of oocytes activated	No. (%) <sup>a</sup> of oocytes developing to			
			2-cell	4-cell	Morula	Blastocyst
None (natural mating)	Partheno genesis	31	29 (94)	27 (87)	27 (87)	23 (74)
	IVF	28	28 (100)	27 (96)	26 (93)	23 (82)
Hyperstimulation	Partheno genesis	61	60 (98)	56 (92)	54 (89)	44 (72)
	IVF	86	82 (95)	80 (93)	78 (91)	65 (76)

Model effects of treatment that were indicated as p value were 0.4169, 0.5993, 0.8299 and 0.7066 in the number of oocytes developing to the 2- cell, 4-cell, morula and blastocyst stages, respectively.

<sup>a</sup>Percentage of the number of oocytes cultured.

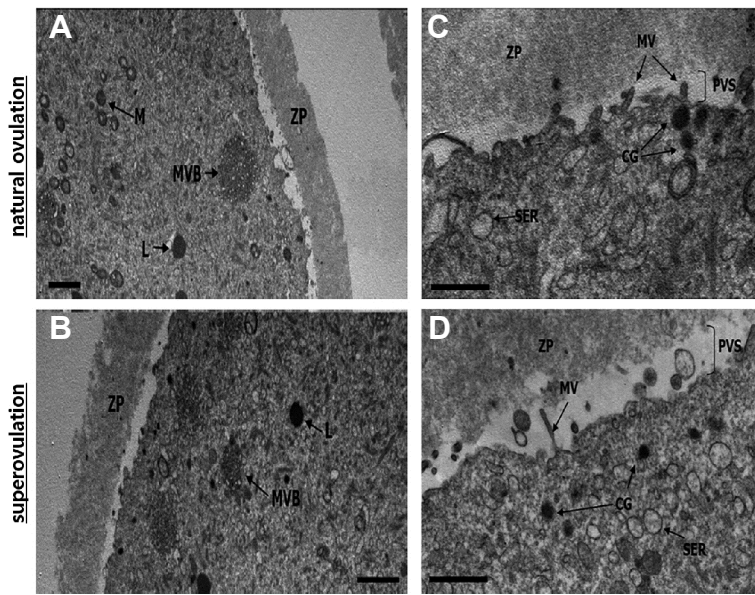
PMSG, pregnant mare serum gonadotrophin; hCG, human chorionic gonadotrophin; IVF, *in vitro* fertilization.



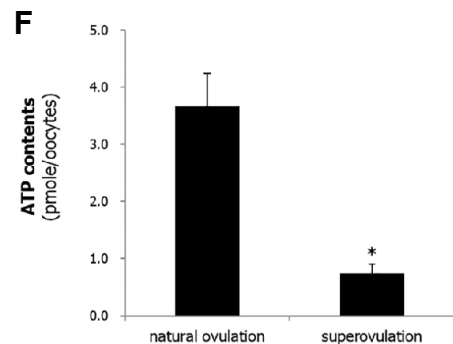
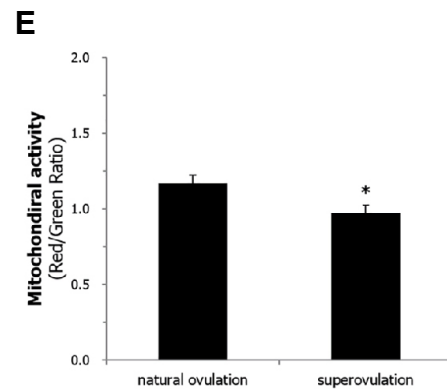
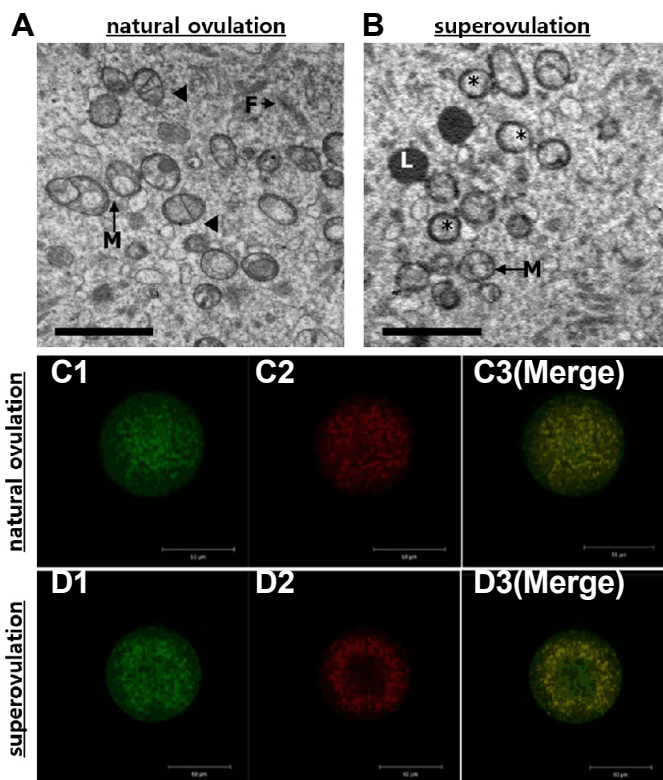
**Fig. 2.** Differences in the number of microorganelles in mature (metaphase II stage) oocytes retrieved after superovulation treatment or natural ovulation. For numerical comparison, number of mitochondria per 100 μm<sup>2</sup> of ooplasm (A1), percentage of vacuolated-mitochondria per total mitochondria in 100 μm<sup>2</sup> of ooplasm (A2), percentage of total lipid droplets per 100 μm<sup>2</sup> of ooplasm (B), number of cortical granules per 10 μm of oolemma (C), and number of multivesicular bodies per 200 μm<sup>2</sup> of ooplasm (D) were counted. Statistical comparison employing PLOC-GLM model was conducted with numerical values and data are presented as mean ± SE. \*Statistical significance in A2 (p = 0.0186) and D (p = 0.0221)

and rats (Miller and Armstrong, 1981). Ovarian hyperstimulation can increase chromosomal abnormalities in murine embryos (Elbling and Colot, 1985; Huffman et al., 2015; Luckett and Mukherjee, 1986; Ozturk et al., 2016), and mi-

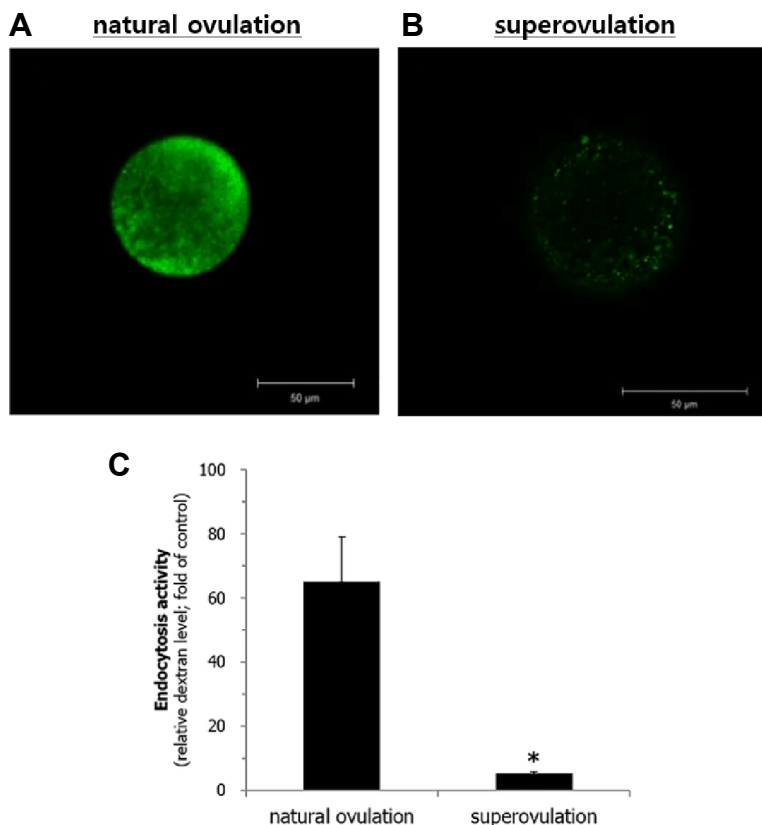
tochondrial deformity further induced oocyte aneuploidy (Plachot, 2003; Takeuchi et al., 2005). Gonadotrophin-induced superovulation induces the proliferation of ovarian surface epithelial cells, which results cell transformation into



**Fig. 3. Ultrastructure of mature (metaphase II stage) oocytes retrieved after superovulation treatment or natural ovulation.** Transmission electron microscope was employed for monitoring morphologic analysis. Micrograph A (natural ovulation) and B (superovulation) show multivesicular bodies. Micrograph C (natural ovulation) and D (superovulation) show the morphology and distribution of cortical granules, microvilli and smooth endoplasmic reticulum. L: lipid; M: Mitochondria; MVB: Multivesicular body; ZP: Zona pellucida; CG: Cortical granule; MV: Microvilli; PVS: Perivitelline space; SER: smooth endoplasmic reticulum; ZP: Zona pellucida. Magnification =  $\times 6,000$  (A),  $\times 8,000$  (B) and  $\times 25,000$  (C, D). Scale bars =  $2 \mu\text{m}$  (A, B) and  $1 \mu\text{m}$  (C, D).



**Fig. 4. Deformity and functional activity of mitochondria in mature (metaphase II stage) oocytes retrieved after superovulation treatment or natural ovulation.** (A) (natural ovulation) and (B) (superovulation) show morphological difference of mitochondria. Confocal images of mitochondria in oocytes retrieved from natural ovulation (C) or superovulation after JC-1 staining (D). (E) Statistical comparison of mitochondrial activity (mean  $\pm$  SE) with using of confocal images. Significant ( $p = 0.0179$ ) difference between two treatments. (F) Quantitative comparison of ATP generation (mean  $\pm$  SE) in oocytes retrieved from after superovulation or retrieved from natural ovulation. Significantly ( $p = 0.0002$ ) different. Arrowhead, Well-developed mitochondrial cristae; asterisk, vacuolated mitochondria; F, Filament; L, Lipid droplet; M, Mitochondria. Magnification =  $\times 12,000$ . Scale bars =  $2 \mu\text{m}$  (A, B)



**Fig. 5. Uptake of dextran-FITC by oocytes retrieved after natural ovulation (A) or after superovulation treatment (B).** Confocal image was employed for monitoring dextran uptake as the representative of endocytosis (mean  $\pm$  SE). Intensity of signal in confocal images was quantitatively compared (C) and significant ( $p = 0.004$ ) difference was detected. \*Statistical significance.

cancer cells (Burdette et al., 2006). Nevertheless, lots of oocytes retrieved after superovulation treatment have superb capacity of maturation and absolute number of degeneration-fated oocytes take an opportunity to mature by superovulation, which overwhelms adverse effect of superovulation. As a matter of fact, no significant difference on developmental competence of oocytes between the natural cycle and the superovulation groups confirmed normality of mature oocytes retrieved after superovulation (Table 1). The results of these data can contribute not only to obtaining toxicological data, but also to improving ART efficiency by decreasing potential adverse effect of superovulation.

Number of toxicological data could be retrieved from the results of this study. Even under this limited condition of evaluation on ultrastructural morphology, not only structural changes but also functional alterations were detectable after superovulation. Semi-peripheral ring-like distribution of metachromatic organelles of superovulated oocytes showed structural and functional changes of oocytes simultaneously (Fig. 4). These changes were prominent in mitochondria and multivesicular bodies. Significant decrease in ATP generation observed in this study may adversely affect embryonic development via altering chromosomal segregation, meiotic spindle adjunction (Takeuchi et al., 2005), microtubule polymerization (Eichenlaub-Ritter et al., 2004; Plachot, 2003) and calcium oscillations during oocyte activation and embryogenesis (Calarco, 1995). On the other hand, mitochondrial deformity induces oocyte aneuploidy (Field et al., 1999) via

predivision or nondisjunction of chromatids (Guerin et al., 2001).

Superovulation treatment also alters endocytotic function of the oocytes, which is critical for metabolic coupling and synthetic activity (Chi et al., 2011; Dobrowolski and De Robertis, 2011; Eden et al., 2009). As a results above, early endocytosis represented by dextran uptake were extremely down-regulated but multivesicular bodies in ultrastructure found more in the superovulation group (Figs. 2 and 5). Increased influx of excessive gonadotropin may result the presence of multivesicular bodies, which changes lots of metabolic pathways (Katzmann et al., 2002). Increased endocytosis at the late stage followed by increased multivesicular bodies may couple with decreasing of metabolic uptake, which cause severe deficiencies of metabolic substrates and cytological stress. In this study, the ring-like distribution of organelles was found in JC-1 staining, which could be related with those stresses. Decreased activities of mitochondria at this stage represented by low endocytosis and mitochondrial membrane potential could be one of cause for those stress for oocyte (Figs. 4 and 5). Mitochondrial damages observed in ultrastructural level connoted developmental disadvantages (Ghavami et al., 2015), which may increase the susceptibility of oocytes to physiological or environmental stress leading to fluctuation of ART outcome.

Various exogenous causes such as temperature, photoperiod and/or other environmental variables may combine with the effect of superovulation treatment, which may result the

fluctuation of ART outcome. As a matter of fact, most of studies on superovulation in humans were designed to evaluate the optimal gonadotrophin dosage for retrieving excellent quality of oocytes (Hohmann et al., 2003). Otherwise, relationship between hormonal treatment and aneuploidy rate of embryos (Baart et al., 2007) were examined. Comparing superovulation treatment with natural ovulation in human, however, no obvious difference were found in embryo development, (Gras et al., 1992; Ziebe et al., 2004) and aneuploidy rate (Labarta et al., 2012; Qin et al., 2013). Optimization of microenvironment can decrease adverse effect of superovulation, which further improve ART outcome.

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