Investigation of in vitro parameters and fertility of mouse ovary after storage on optimal temperature and duration for transportation.

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Running title: Ovarian storage during transportation

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Abstract

Study question: How do the temperature and duration of storage affect ovaries during transportation?

Summary answer: Fertility is reduced with the extension of the storage duration.

What is known already: Live birth has been reported after ovarian transport overnight on ice before freezing ovarian tissue, but there have been no basic investigations of ovarian storage conditions focused on fertility. There are no guidelines on optimal ovarian storage conditions and the maximum storage time during transportation.

Study design, size and duration: Experiments were performed using C57BL/6J mice. Ovaries of 4-week-old mice were harvested, stored at 4°C, 14°C, 37°C, or room temperature (RT) for 24 hours, and subjected to histological examination. Alternatively, ovaries were stored at 4°C for 4, 8, or 24 hours and subjected to histological examination. Then orthotopic transplantation of ovaries, stored at 4°C for 4, 8, or 24 hours, were performed in 6-week-old C57BL/6J mice, and fertility was assessed by *in vitro* fertilization and embryo transfer. Freshly harvested ovaries were used as controls for comparison with ovaries stored under the above-mentioned conditions and experiments were repeated at least 3 times.

Participants/materials, setting and methods: In experiments on the ovarian storage temperature, hematoxylin-eosin (HE) staining was performed for histological examination. In experiments on the storage duration, HE staining, the TUNEL assay, Ki-67 staining, and electron microscopy were performed, and the number of follicles was counted. Fertility was assessed from the number of eggs, fertilization rate, embryo development rate, implantation rate, and rate of live pups.

Main results and the role of chance: Histological changes were minimal after storage of ovaries at 4°C for up to 24 hours. At 4°C, there were no significant changes in the number of MII oocytes, fertilization rate, or blastocyst development rate with storage up to 24 hours. The implantation rate was 82.7±17.3% in the control group, while it was 82.2±7.7%, 14.6±14.6%, and 4.4±4.4% after storage for 4, 8, or 24 hours, respectively. After 8 or 24 hours of storage, the implantation rate was significantly lower in than in the control group ($p<0.05$). The rate of live pups was 24.8±13.2% in the control group, while it was 23.9±6.6%, 4.2±4.2%, and 4.4±4.4% after storage for 4, 8, or 24 hours, respectively. After 8 or 24 hours of storage, the rate of live pups was significantly lower than in the control group ($p<0.05$).

Limitations, reasons for caution: Further investigations are needed in mammals with ovaries of a similar size to human ovaries. Also, the assessment of fertility following transplantation of frozen and thawed ovaries is necessary.

Wider implication of the findings: The present results suggest that prolonging the ovarian storage time reduces fertility in mice. Thus, ovaries should be frozen immediately after harvesting or transported as rapidly as possible to minimize damage. To allow young cancer patients to preserve fertility, regional medical centres need adequate ovarian tissue cryopreservation techniques.
Study funding/competing interests: The authors have no competing interests to declare.

Key words: cryopreservation, fertility, in vitro fertilization, mouse, ovarian storage

Introduction

The prognosis of cancer has improved with progress in treatment, and the 5-year survival rate of female cancer patients is over 68% (Siegel *et al.*, 2014). Also, progress in reproductive technology has enabled the long-term storage of reproductive cells and tissues, allowing cancer patients to preserve fertility. In 2004, Donnez *et al.* reported the first live birth after autologous transplantation of human ovarian tissue (Donnez *et al.*, 2004). To date, at least 60 babies have been born using this technique (Donnez *et al.*, 2015; Stoop *et al.*, 2014). However, cryopreservation of ovarian tissue can only be performed by a limited number of medical centres, which means that some female cancer patients miss the chance for preservation of fertility. In Germany and Denmark, ovaries are harvested at regional hospitals because laparoscopic resection can be performed easily, and are sent to centres that can perform ovarian tissue cryopreservation (Dittrich *et al.*, 2015; Rosendahl *et al.*, 2011).

To allow ovarian tissue cryopreservation to be more widely employed for preservation of fertility, it is necessary to establish a system for the storage, transportation, and freezing of harvested ovaries. Both histological and functional investigations have been performed to determine the optimal storage conditions for ovaries (Isachenko *et al.*, 2009; Schmidt *et al.*, 2003). However, optimal storage conditions have not been determined by investigating fertility, so the influence of storage temperature and duration on fertility remains unclear.

Therefore, the present study was performed to histologically investigate the influence of storage temperature and duration on mouse ovaries, as well as to clarify the relationship between the optimal storage temperature or duration and fertility by assessing embryonic development and the rate of live pups using eggs harvested from stored ovaries.

Materials and methods

Experimental protocol

Ovary was harvested from 4-week-old C57BL/6J mice and was stored for 24 hours at 4°C, 14°C, 37°C, or room temperature (RT) in microtubes containing culture medium (Figure 1). Hematoxylin-eosin (HE) staining was performed for histological examination and freshly harvested ovaries were used as the control.

Next, the relation between fertility and the storage time was investigated. Ovaries were stored at 4°C (optimum storage temperature) for 4 hours, 8 hours, or 24 hours, with freshly harvested ovaries as the control. For histological examination, HE staining, the terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay, Ki-67

staining, and electron microscopy were performed, and the number of follicles was counted. In addition, orthotopic transplantation of stored ovaries was performed in 6-week-old C57BL/6J mice, and fertility was assessed by *in vitro* fertilization and embryo transfer. These experiments were repeated at least 3 times.

Animals

Male and female C57BL/6J mice and female ICR mice were purchased from Japan SLC, Inc. (Shizuoka, Japan). The mice were housed at a temperature of 22°C and humidity of 55% with a 12-hour light/12-hour dark cycle, and were allowed free access to food and water. Animals were handled and housed in accordance with the procedures specified by the Department of Animal Experiments at St. Marianna University School of Medicine (Kanagawa, Japan).

Ovarian resection and storage

Ovaries were harvested from 4-week-old female C57BL/6J mice and were stored in microtubes containing Dulbecco's modified Eagle's medium/Ham's F-12 (1:1 v/v), 10% foetal bovine serum (FBS), 10 mg/ml insulin, 10 mg/ml transferrin, and 50 mIU/ml follicle-stimulating hormone (Sadeu *et al.*, 2008). This medium was optimized for ovarian tissue culture on 37°C. We used this medium in all experiment to consolidate condition.

Examination of ovaries

After storage according to the experimental protocol (Figure 1), ovaries were embedded in paraffin and cut into sections 6 μm thick. The sections were stained with HE and observed under a microscope at ×40 and ×200 (Nikon Corporation, Tokyo, Japan).

Morphological investigation of follicles

The total number of primary follicles, secondary follicles, and vesicular follicles was counted in ovaries stored at 4°C for 4, 8, or 24 hours and in fresh ovaries (controls). To prevent double counting, only the follicles with nuclei were counted.

The follicles were classified into three grades (G1-3) (Gandolfi *et al.*, 2006), and the percentage of G1 follicles was calculated (Figure 2).

The number of primordial follicles was also counted and the morphology of these follicles was investigated by electron microscopy. Whole ovaries were fixed overnight at 4°C in 2% paraformaldehyde/2.5% glutaraldehyde solution (0.1 M phosphate buffer, pH 7.4), washed with phosphate buffer, and fixed with 1% osmium tetroxide solution (in 0.1 M phosphate buffer) for 2 hours at 4°C. After dehydration with ethanol, it was displaced

with propylene oxide, and polymerization was done with EPON resin. Semi-thin sections (1 μm) of the ovaries were cut and stained with toluidine blue, while thin sections (80 nm) were cut and stained with uranyl acetate and lead citrate. Observation was performed with a transmission electron microscope (JEOL JEM-1220; JEOL Ltd., Tokyo, Japan) at a magnification of ×1000 to ×10000. To assess morphological differences of primordial follicles related to storage duration, 20 primordial follicles were randomly selected and were classified as G1, G2, or G3 (Figure 3A). The sections were examined independently by 2 researchers.

Investigation of apoptosis

Apoptosis was investigated by using the TUNEL assay with an in-situ death detection kit (Roche, Mannheim, Germany) according to the manufacturer's protocol. If apoptosis of more than 30% of the cells in a follicle was observed, the follicle was classified as apoptotic (Youm *et al.*, 2014).

Investigation of cell proliferation

Immunostaining was performed to detect Ki-67, a nuclear antigen that is a marker of cell growth during the late G1, S, G2, and M phases of the cell cycle (excluding the G0 phase). After deparaffinization, the sections of ovarian tissue were autoclaved in 10 mM citric acid buffer (pH 6.5) for 20 minutes and allowed to cool. Then the sections were placed in 0.3% hydrogen peroxide in methanol for 30 minutes, washed with 50 mM Tris-HCl buffer (pH 7.5), and blocked with 1.0% BSA in 50 mM Tris-HCl buffer for 30 minutes at RT. A rat anti-Ki-67 monoclonal antibody (×100, Dako, Tokyo, Japan) was applied to each section and incubation was done overnight at 4°C. Detection was performed by using a Histostain-SP kit (Broad Spectrum, AEC; Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Each section was assessed independently by 2 researchers. Follicles containing at least one Ki-67-positive granulosa cell were defined as growing follicles (Dolmans *et al.*, 2007).

Ovarian transplantation

The ovaries of 4-week-old female C57BL/6J mice were stored at 4°C for 4, 8, or 24 hours, followed by orthotopic transplantation into 6-week-old female C57BL/6J mice from which both ovaries had been resected. Freshly removed ovaries were also transplanted in the same way as a control. A modified method of Migishima was used for transplantation (Migishima *et al.*, 2003). In brief, anaesthesia was induced by intraperitoneal injection of pentobarbital sodium (7.5 g/kg bw). A midline longitudinal

skin incision was made in the lower back, followed by bilateral small incisions was made on the fascia and muscles immediately above the ovary, through which the recipient's reproductive tract was lifted out. A small slit was made on the ovarian bursa to expose the ovary. The recipient's ovaries were removed under an operating microscope and donor's ovaries were make replacement, after which the bursal membrane was closed with suturing and placed inside the body. Then the skin incision was sutured.

In vitro fertilization, blastocyst transfer, and caesarean section

All mice were detected recovery of the oestrous cycle at 7 to 10 days after transplantation and were housed 2 to 3 weeks after transplantation for inducing primordial follicle recruitment. These mice received an intraperitoneal injection of serotropin (5 IU; ASKA Pharmaceutical Co., Ltd., Tokyo, Japan), followed by a intraperitoneal injection of gonadotropin (10 IU; ASKA Pharmaceutical) 48 hours later. At 15 h later, cumulus oocyte complexes (COCs) were collected from the ovaries and ampulla of fallopian tubes. COCs were then placed in 100-μl of TYH medium (LSI Medience Corporation, Tokyo, Japan) with sperm (3×10^5 /ml). The sperm were collected from 10-to 12-weeks-old male C57BL/6J mice and incubated in TYH medium for 10 minutes at 37°C under 5% CO^2/95% air to completed capacitation.

Fertilization was carried out for 5 to 6 hours and inseminated oocytes were placed in a 30-μl drop of KSOM medium (Merck Millipore Corporation, Tokyo, Japan) under mineral oil (Irvine Scientific Sales Company Inc., Saitama, Japan), and incubated at 37°C for 24 hours. Then two-cell embryos were selected and not inseminated oocytes were removed. After further incubation of the two-cell embryos, the number of blastocysts and morulas was scored. Then the blastocysts and morulas were transferred into the uteruses of 6-to 10-week-old pseudo-pregnant female ICR mice (9.5 ± 1.28 embryos / recipient; supplementary table 1). Caesarean section was performed at 19 days after oocyte harvesting, and the number of implantation sites and foetuses was counted.

Statistical analysis

The percentage of follicles of each stage and the number of follicles of each grade were calculated and expressed as the mean ± standard error. Kruskal-Wallis test and the Steel-Dwass test were performed. The level of significance was set at $p < 0.05$.

Ethical approval

Conduct of this study was approved by the Animal Experiments committee of St. Marianna University School of Medicine (approval no.1406014).

Results

Influence of storage temperature

When the ovaries of 4-week-old mice were stored for 24 hours at 4°C, 14°C, RT , or 37°C, no marked histological differences were noted between freshly harvested control ovaries and those stored at 4°C (Figures 4A-D). In contrast, ovaries stored at 14°C, RT, or 37°C showed changes such as interstitial oedema, gaps between the granulosa cells and theca cells, and reduced adhesion between granulosa cells (Figures 4E-J). These findings suggested that histological changes were minimal when ovaries were stored at 4°C.

Influence of storage time

Because storage at 4°C was associated with minimal histological change, ovaries were stored at this temperature for 4 hours, 8 hours, or 24 hours to assess the influence of the storage time, with freshly harvested ovaries as the control. As a result, there were no significant intergroup differences of interstitial oedema, gaps between the granulosa cells and theca cells, or adhesion between granulosa cells (Figure 5).

The number of follicles and the percentage of high grade follicles (G1) were also investigated in relation to storage time. It was found that the storage time had no significant influence on the number of follicles (Supplementary Figure 1). There was no significant difference in the combined percentage of G1 primary follicles, secondary follicles, and vesicular follicles between ovaries stored for 4 hours (67.3±3.0%) and the control ovaries (74.6±1.2%). However, the combined percentage of G1 primary follicles, secondary follicles, and vesicular follicles was significantly lower than in control ovaries after storage for 8 hours (60.5±2.7%) or 24 hours (39.4±2.3%) (Figure 2B).

In each group, 20 primordial follicles were randomly selected and graded by electron microscopy. It was found that 80.0% of the primordial follicles were G1 in the control ovaries, while no G1 primordial follicles were found after storage for 4, 8, or 24 hours. After 4 or 8 hours of storage, primordial follicles were classified as G2 because the periphery of the oocyte nucleus was irregular and there was a gap between the cytoplasm of the oocyte and the granulosa cells. After storage of ovaries for 24 hours, primordial follicles were classified as G3 because there was loss of the nuclear membrane, mitochondrial ballooning, and aggregation of chromatin (Figure 3B).

The effects of storage on cells in the follicles were also investigated by using the TUNEL assay to assess apoptosis and Ki-67 staining to assess cell proliferation. Following storage of ovaries for 8 or 24 hours, some follicular cells were TUNEL-positive, but over 30% of the cells were not TUNEL-positive after either storage time (Figure 6A).

Granulosa cells in the follicles were positive for Ki-67 after all storage times (Figure 6B). These findings suggested that growth of the follicles was maintained during storage.

Effect of storage time on fertility

Ovaries were transplanted into recipient mice after storage at 4°C for 4 hours, 8 hours, or 24 hours and embryos were obtained by *in vitro* fertilization. As a result, there were no significant differences in the number of MII oocytes, the fertilization rate, or the blastocyst development rate between the control group (fresh ovaries) and the other groups, and a longer storage time did not alter these parameters(Figures 7A-C).

The implantation rate showed no significant difference between the 4-hour storage group (82.2±7.7%) and the control group (82.7±17.3%). However, the implantation rate was significantly lower in the 8-hour storage group (14.6±14.6%) and the 24-hour storage group (4.4±4.4%) compared with the control group (Figure 7D).

There was also no significant difference of the rate of live pups between the 4-hour storage group (23.9±6.6%) and the control group (24.8±13.2%). However, this rate was significantly lower in the 8-hour storage group (4.2±4.2%) and the 24-hour storage group (4.4±4.4%) compared with the control group (Figure 7E).

Discussion

The following 2 main findings were obtained in the present study: 1) ovaries stored at 4°C for up to 24 hours show minimal histological changes and 2) the implantation rate and rate of live pups both decrease as storage at 4°C is prolonged.

Human organs are usually stored at a suprazero temperature (0-7°C) (Isachenko *et al.*, 2009). It has been reported that organs can be stored at a temperature lower than 37°C (normal body temperature) for up to 96 hours because biological metabolism, oxygen consumption, and tissue autolysis are reduced despite the absence of blood flow (Hicks *et al.*, 2006). When human ovaries were stored on ice for 4 hours, it was reported that the primordial follicles remained viable (Schmidt *et al.*, 2003). However, whether primordial follicles remain viable when human ovaries are stored with other temperature has not been investigated. Also, it has been pointed out that the optimal storage temperature may be somewhere between 0°C and 37°C (Schmidt *et al.*, 2003). Therefore, ovaries were stored at 14°C, RT, and 37°C in addition to storage at 4°C during the present study. Examination of HE-stained sections suggested that histological changes were minimal when ovaries were stored at 4°C, while there were obvious changes after storage at 14°C, RT, or 37°C. Based on these findings, a temperature of 4°C is superior to the other temperatures tested for storage up to 24 hours. It was

reported that follicular growth was well maintained without a decrease of primordial follicles after storage of ovaries at a suprazero temperature for up to 26 hours (Isachenko *et al.*, 2009), and this report is consistent with our findings in the present study. However, fertility after ovarian storage at a low temperature has not been investigated by basic research, and the relation between storage time at low temperature and fertility remains unclear. Therefore, we investigated the relationship between the storage time at 4°C and fertility, revealing that the implantation rate and rate of live pups both decreased as the storage time became longer. In Denmark, ovarian tissue cryopreservation and transplantation has been performed 18 times in 12 females during the past 10 years, with recovery of ovarian function in all 12 and birth of 3 healthy babies. The ovaries transplanted into the women who delivered the 3 babies were stored in ice for 4 to 5 hours during transportation before being frozen (Rosendahl *et al.*, 2011). In Germany, ovaries stored overnight at 4°C during transportation before being frozen were transplanted in 8 women, after which 5 of them became pregnant and 2 gave birth (Dittrich *et al.*, 2015). These reports suggest that fertility can be preserved if ovaries are stored on ice during transport before cryopreservation. However, fertility has not been compared between stored ovaries and ovaries frozen immediately after being harvested, and the relationships between fertility and the low temperature storage time has not been investigated. Even though some babies have been born after transplantation of stored ovaries, basic research should still be performed to optimize the fertility of stored ovaries for clinical application of this method (Rosendahl *et al.*, 2011). The present study showed that there were no significant differences of the number of MII oocytes, the fertilization rate, or the blastocyst development rate as the storage time became longer. However, the implantation rate and the rate of live pups both decreased as storage was prolonged. It has been reported that the implantation rate and live birth rate are lower in older women due to poor embryo quality associated with ageing (Dew *et al.*, 1998; Navot *et al.*, 1991). It has also been reported that the development rate of embryos from older women is lower, even though there are no differences of morphological grade (Yan *et al.*, 2012), and this may be related to impaired mitochondrial function and DNA damage associated with oxidative stress (Hamatani *et al.*, 2004; Steuerwald *et al.*, 2007; Van Blerkom, 2004; Van Blerkom *et al.*, 1995). Aging affects implantation and live pups rate due to impaired mitochondrial function and DNA damage, although it does not affect morphology in embryo. This study focused on preservation of ovary and some condition showed similar tendency with aging. Therefore, these results in this study suspect that certain condition in ovarian preservation causes same effect due to aging in ovary. In addition, reperfusion after organ transplantation induces oxidative stress and

inflammation that cause tissue damage (Maulik *et al.*, 1999; Seal and Gewertz, 2005). Therefore, reperfusion injury after ovarian transplantation may affect some eggs and interfere with implantation and pregnancy. In the present study, the number of morphologically normal follicles declined over time as storage was prolonged at 4°C. Such morphological changes probably reflected the effects of low-temperature storage, because it has been reported that long-term storage leads to irreversible structural changes of cell membranes and organelles (Fuller, 1987). On the other hand, our assessment of primordial follicles by electron microscopy revealed a difference in the percentage of G1 follicles between control fresh ovaries and those stored for 4 hours, although there was no difference of fertility. This appears to suggest that morphological investigation of ovarian tissues is not sufficient for predicting fertility. The results of our present experiments in mice suggested that fertility declines as the ovarian storage time becomes longer. To establish fertility preservation for female cancer patients, it will be necessary for regional medical centres to be able to perform adequate ovarian tissue cryopreservation, and it would be preferable to freeze ovaries immediately after removal or minimize the storage time during transportatino. Vitrification can be performed anywhere without special equipment by using a dedicated kit (Suzuki *et al.*, 2012), and there have been a number of live births after vitrification was employed for ovarian tissue cryopreservation (Kawamura *et al.*, 2013; Suzuki *et al.*, 2015). Accordingly, it is possible that adopting vitrification would enable more hospitals to perform ovarian cryopreservation, which would benefit cancer patients who want to preserve their fertility.

The present study had 3 limitations. First, rate of live pups was low. In general, rate of live pups in in-vitro fertilization and embryo transfer (IVF-ET) is around 50%. There is a report about live pups rate of IVF-ET with ovarian transplantation and the rate is also almost 50% (Migishima et al., 2003). They did partial resection of recipient ovary followed by transplantation. In contrast, we performed total resection of recipient ovary and transplantation. The difference in the procedure of transplantation might cause different live pups rate. Second, mouse ovaries were used. Sensitivity to low-temperature storage differs among mammalian species, therefore the possibility of differences between humans and mice cannot be excluded (Isachenko *et al.*, 2009). However, protection of follicles from external damage such as oxidative damage is crucial for high rate of live pups in ovarian preservation. This study has focused on methods to protect follicles and indeed several conditions have shown better implantation and live pups rates. On the other hand, mouse model has used in this study and menstrual cycle and follicular recruitment are different in human and mouse. However, our study is

performed for preventing accumulation of damage in oocytes, therefore we suspect that it should be applicable in clinical work. Ovarian preservation before ovarian tissue cryopreservation is expected increase as demand grows clinically. However, it requires considerable time for the patients who already have cryopreserved their ovaries to conduct autotransplantation and then to obtain fertility results. Ideal results cannot be expected from a suboptimal technique which might cost the future fertility of many patients. Therefore, before applying the method clinically, it is essential that each laboratory conduct fundamental research and perform thorough validation of its technique. At present, rodents are some of the best experimental models available for research on fertility. We suspect that even if ovarian preservation was already conducted in the clinic, accumulation of data derived from animal experiment is prove useful for further optimization of ovarian preservation. Therefore we believe our study will help for human ovarian preservation. Of course, further optimization is necessary in these techniques for human ovarian preservation. Thus, further investigations are important in mammals with ovaries of approximately the same weight as human ovaries. Finally, we did not investigate fertility when ovaries were frozen, thawed, and transplanted after storage. Because our aim was to determine the specific effects of storage before cryopreservation, we assessed fertility when ovaries were stored without freezing. However, this means that the effects of freezing/thawing the ovaries were not taken into consideration, so investigation of the combined influence of storage and cryopreservation is needed.

In conclusion, this study revealed that histological changes of mouse ovaries were minimal after storage at 4°C for up to 24 hours, but that fertility declined as storage was prolonged. This suggests that it is preferable to cryopreserve ovaries immediately after removal or minimize the storage time during transport. To improve the preservation of fertility in women undergoing treatment for cancer, it is important for more medical centres to develop the capability for performing ovarian tissue cryopreservation.

Authors' roles

The authors were responsible for the following roles in the study. K.K.: study conception and design, study execution, data interpretation, manuscript writing; N.O.: study execution, data interpretation, revision of the article; M.N.: study execution; T.H.: study conception and design, critical discussion; K.S.: critical discussion, revision of the article; A.O.: critical discussion, revision of the article; Y.S.: study conception and design, study execution, critical discussion; N.S.: study conception and design, data interpretation, critical discussion, revision and final approval of the article.

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Conflict of interest

The authors have no conflicts of interest to declare.

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Figure Legends

Figure 1

Outline of the experimental protocol. In histology assay of each condition was used three ovaries respectively. In fertility assay of each condition was used two mice per experiment respectively. This experiment was repeated at least 3 times.

Figure 2

Grade and stage of follicles

A: Morphological classification of follicles

The horizontal axis indicates the stage of follicular development and the vertical axis indicates the grade (G1, G2, and G3 correspond to good, average, and poor, respectively). Follicles were graded as follows.

Primary follicles: G1, the egg is spherical and the granulosa cells show a regular arrangement; G2, the egg is spherical, but the granulosa cells are partly detached; and G3, the egg has a condensed or deformed nucleus or shows vacuolation.

Secondary follicle/vesicular follicle: G1, intact spherical follicle with uniformly arranged granulosa cells/theca cells, small gaps between the granulosa and theca cells, and a spherical egg; G2, intact theca cells, but damaged granulosa cells, and a spherical egg; and G3, damage or loss of granulosa cells and theca cells, and nuclear condensation or loss of the egg.

B: Percentage of G1 primary and secondary follicles per ovary

Figure 3

Findings on electron microscopy

A: Morphological classification of primordial follicles by electron microscopy

G1: The nucleus of the egg is round and the nuclear membrane is regular.

G2: The nuclear membrane is irregular, or there is a gap between the cytoplasm of the egg and the granulosa cells. Neither mitochondrial ballooning nor aggregation of chromatin is seen.

G3: The nuclear membrane of the egg is lost, or mitochondrial ballooning and chromatin aggregates are seen.

B: Characteristic electron microscopic findings

In the control group, the percentage of GI and G2 primordial follicles was 80.0% (a) and 20.0% (b), respectively. After storage for 4 hours or 8 hours, all of the primordial follicles were G2. The nuclear membrane of the egg was irregular, and there was a gap between the cytoplasm of the egg and the granulosa cells (d, e, g, h). After storage for 24 hours,

all of the primordial follicles were G3 (j, k). Panels c, f, i, and l show images of mitochondria. Mitochondrial ballooning was only seen in the 24-hour storage group (l).

Figure 4

HE staining of ovarian tissue after storage for 24 hours at different temperatures. (A,B) Fresh ovary (Control). Storage at 4°C (C,D), 8°C (E,F), RT (G,H) and 37°C (I,J) respectively. Upper (x40). Bottom (x200).

Figure 5

HE staining of ovarian tissues after storage at 4°C for different times. (A,B) Fresh ovary (Control). Storage for 4 hours (C,D), 8 hours (E,F) and 24 hours (G,H). Upper (x40). Bottom (x200).

Figure 6

Apoptosis and cell proliferation. (A) Investigation of apoptosis by TUNEL staining (×40). (B) Investigation of cell proliferation by Ki-67 staining (×40).

Figure 7

Fertility of eggs obtained from ovaries stored at 4°C. (A) Number of MII oocytes. (B) Fertilization rate. (C) Blastocyst development rate. (D) Implantation rate. (E) Rate of live pups. (mean ± SEM).

Supplementary Figure 1

(A) Total number of primary follicles, secondary follicles, and vesicular follicles per ovary. (B) Number of primordial follicles per ovary. Follicles were classified as below (Lundy *et al.*, 1999). Primordial follicles: a flat layer of pre-granulosa cells. Primary follicles: a layer of granulosa cells that are at least cuboidal. Secondary follicles: at least 2 layers of cuboidal granulosa cells without a follicular antrum. Vesicular follicles: multiple layers of cuboidal granulosa cells with a follicular antrum.

figure1

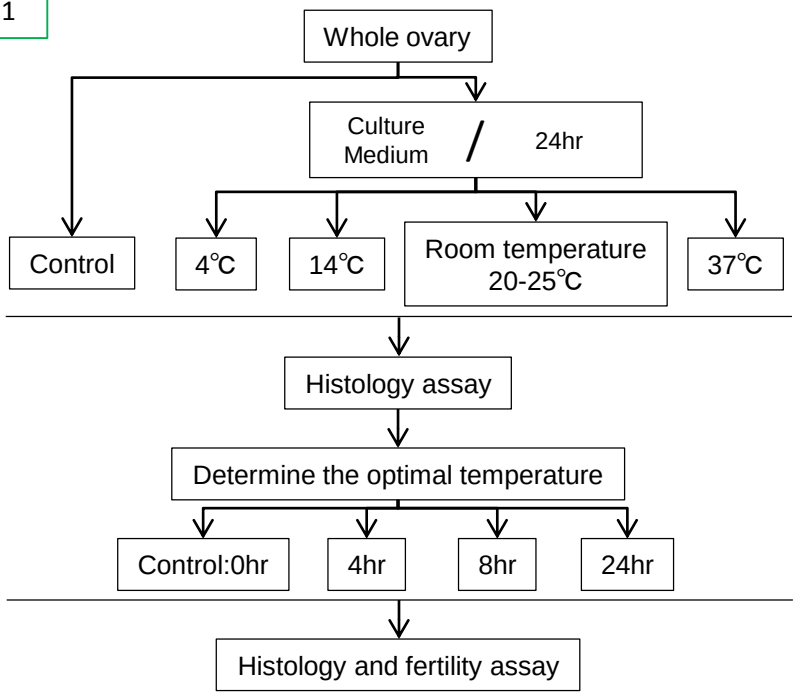
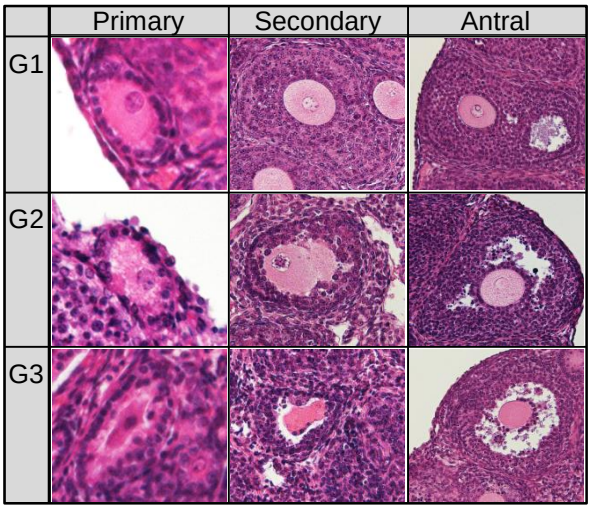

Whole ovary
Culture Medium
24hr
Control
4℃
14℃
Room temperature 20-25℃
37℃
Histology assay
Determine the optimal temperature
Control:0hr
4hr
8hr
24hr
Histology and fertility assay

Figure2

A


Primary
Secondary
Antral
G1
G2
G3

B

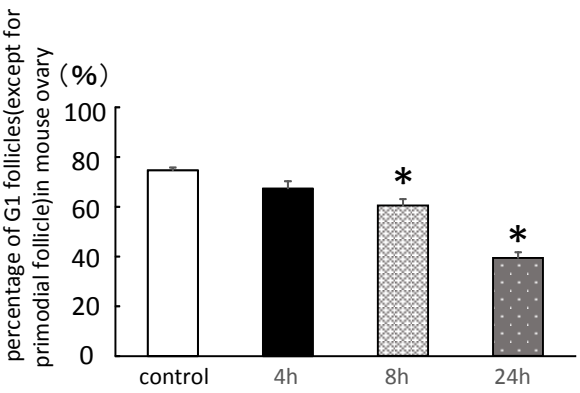
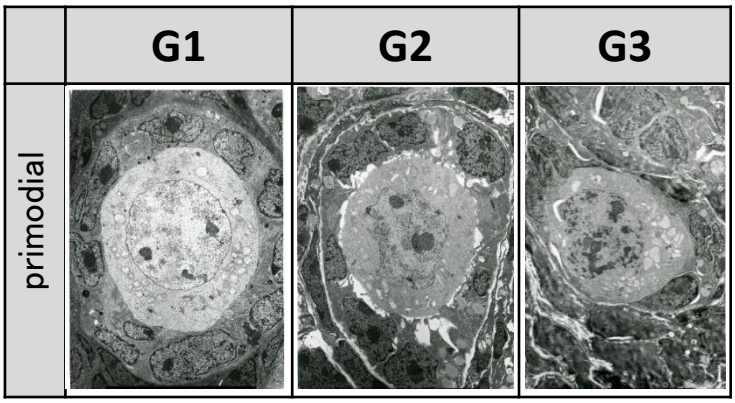

percentage of G1 follicles(except for primodial follicle)in mouse ovary
（%）
100
80
60
40
20
0
*
*
control
4h
8h
24h

Figure3 A



B

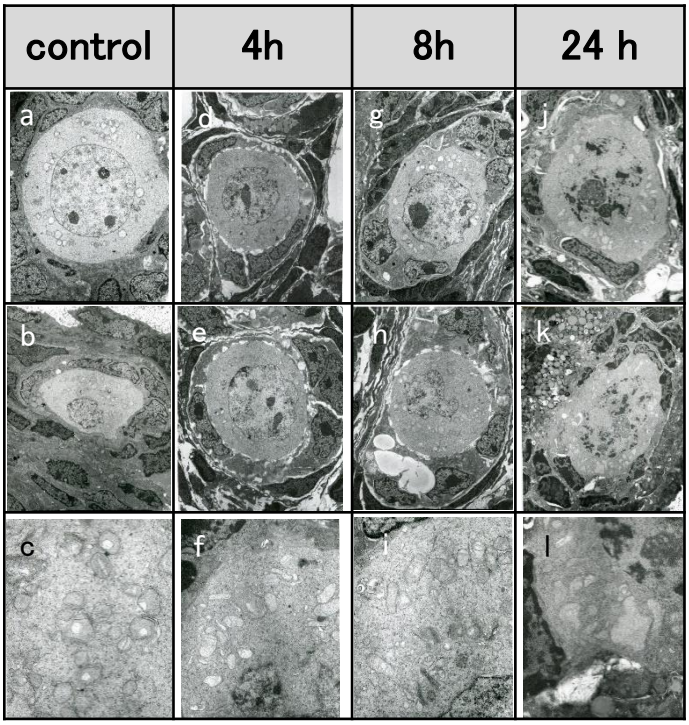
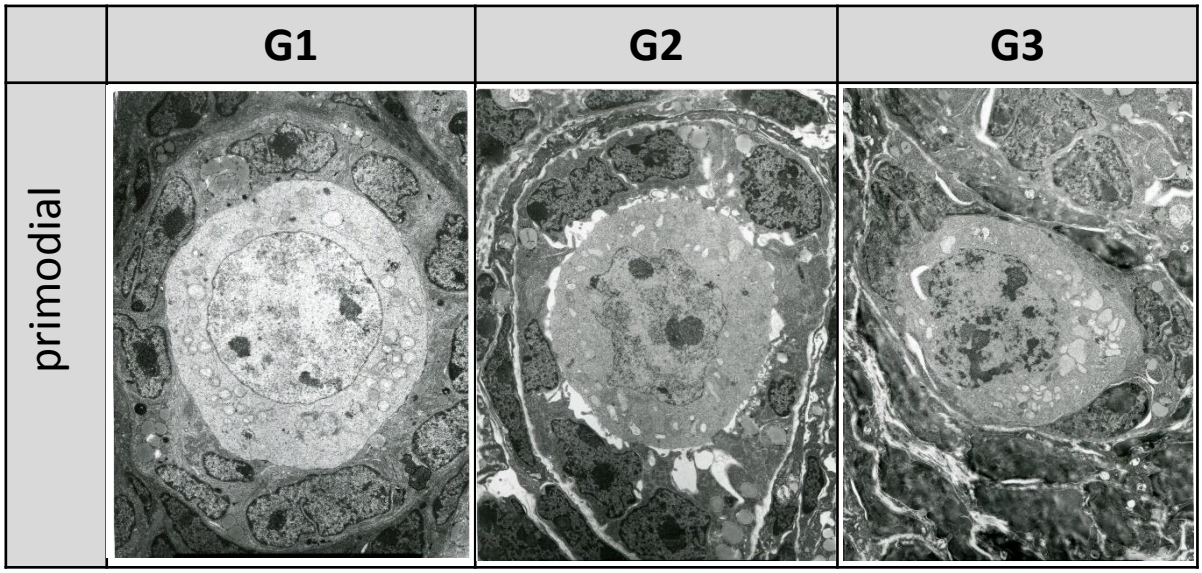


Figure3

A



B

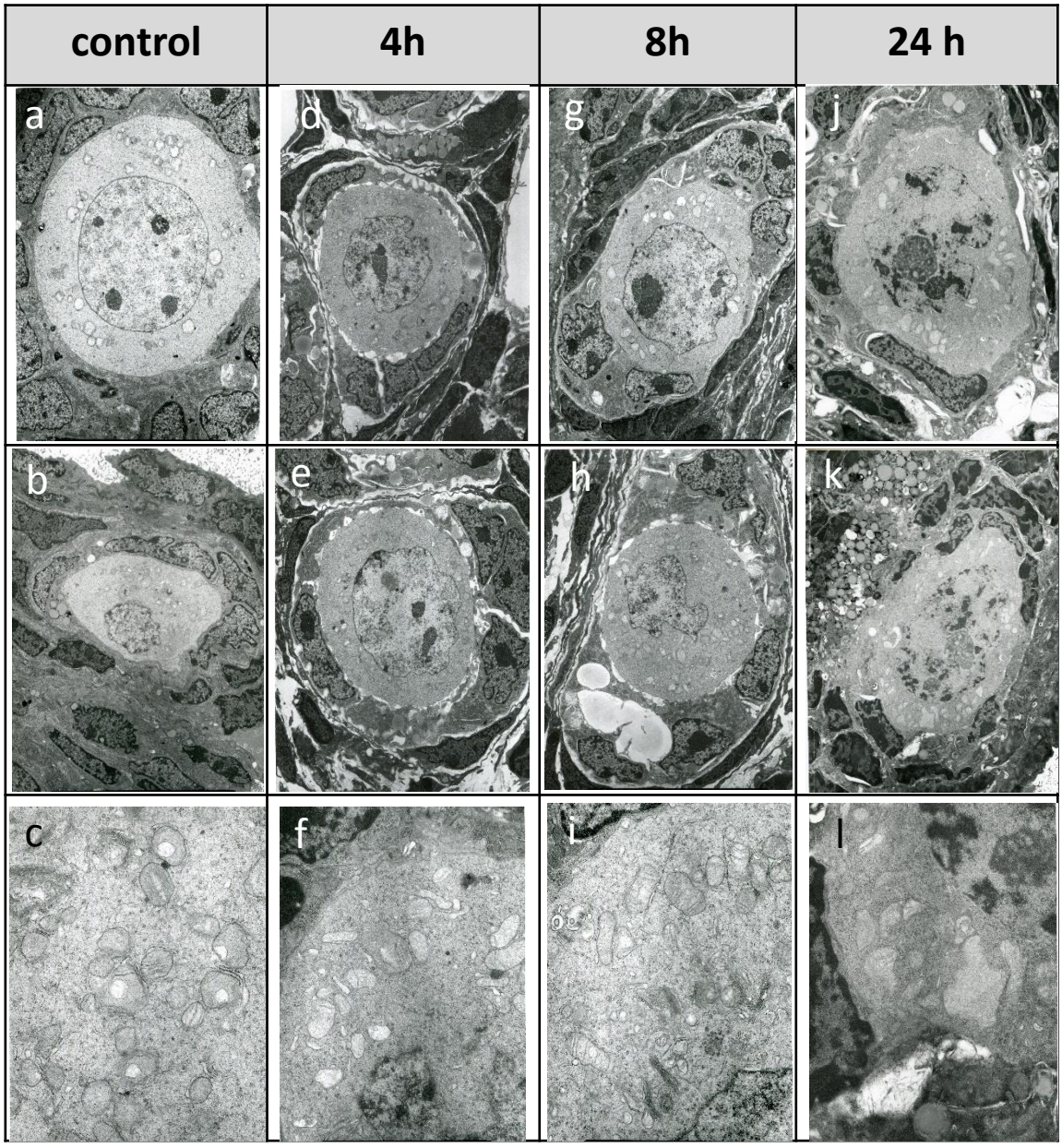


Figure4

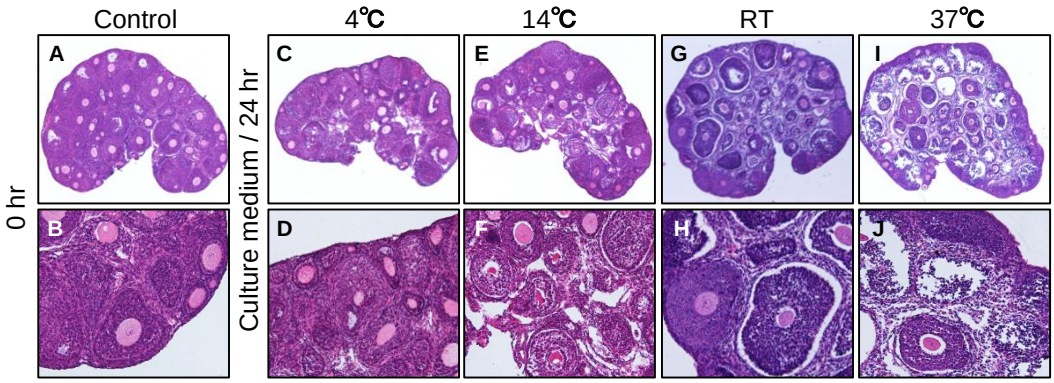


Figure5

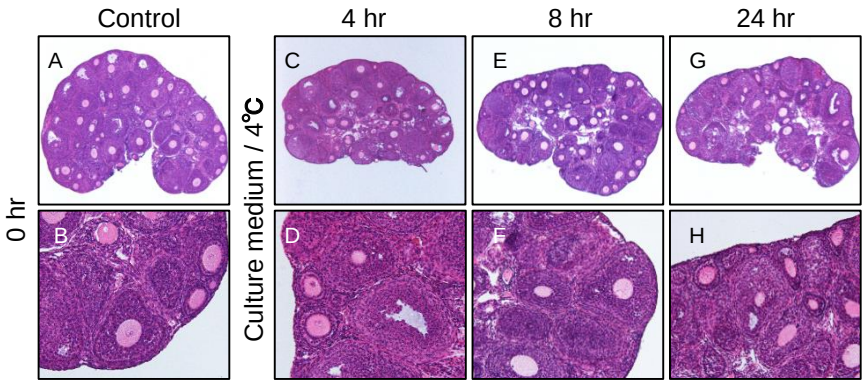


Figure6

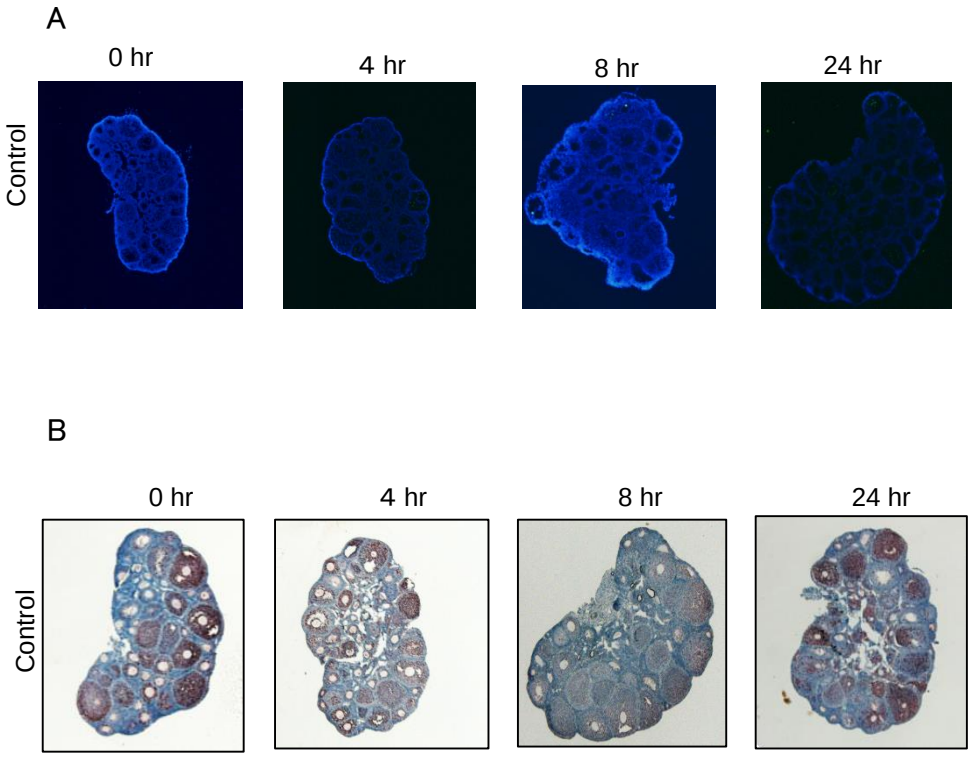
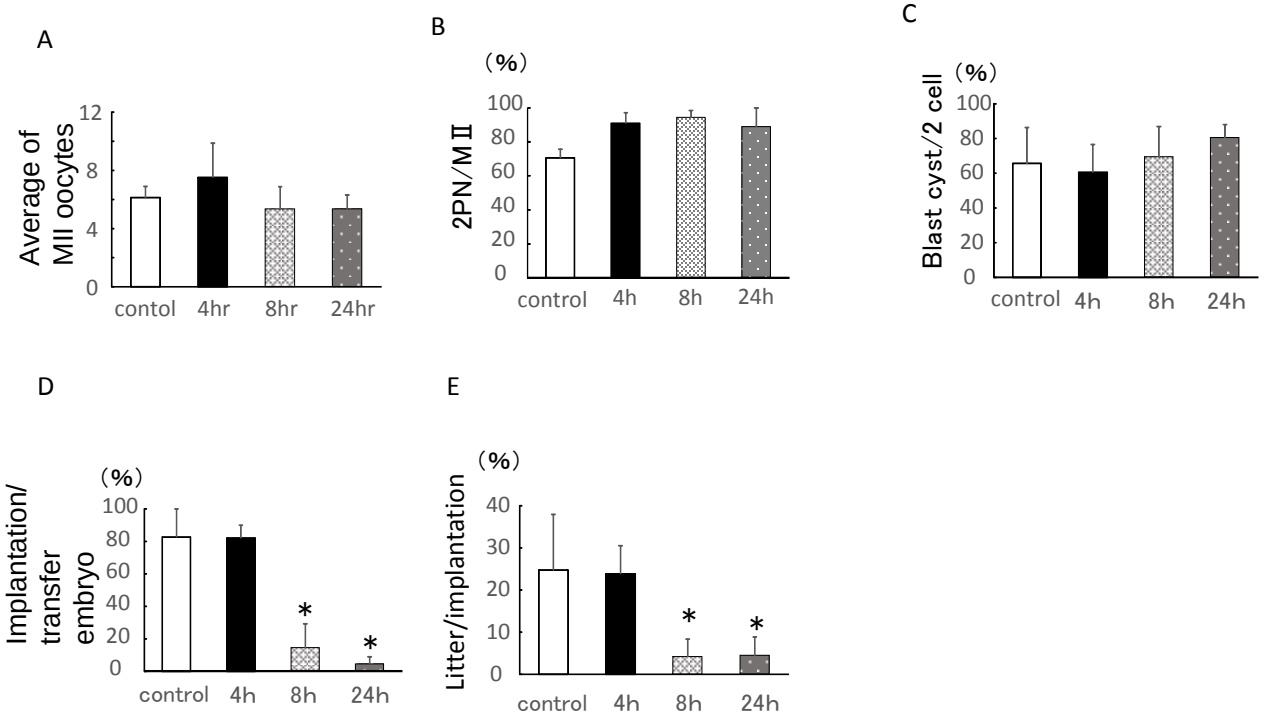
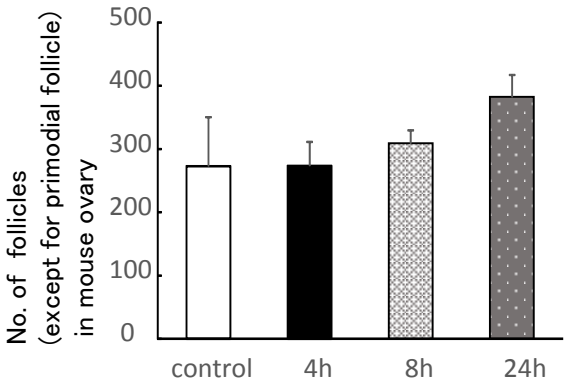


Figure7


A
Average of MII oocytes
12
8
4
0
contol
4hr
8hr
24hr
B
(%)
2PN/MⅡ
100
80
60
40
20
0
control
4h
8h
24h
C
(%)
Blast cyst/2 cell
100
80
60
40
20
0
control
4h
8h
24h
D
(%)
Implantation/ transfer embryo
100
80
60
40
20
0
*
*
control
4h
8h
24h
E
(%)
Litter/implantation
40
30
20
10
0
*
*
control
4h
8h
24h

Supplementary figure1

A



B