



# Effects of cyclophosphamide administration on the in vitro fertilization of mice

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## Abstract

**Purpose:** To evaluate the oocyte fertilization ability and embryo growth after cyclophosphamide (CPA) treatment in mice.

**Methods:** Mice were treated with CPA at different doses (0–800 mg/kg body weight). The oocytes then were retrieved and evaluated for their in vitro fertilization efficiency.

**Results:** The average number of metaphase II (MII) oocytes significantly decreased by  $\geq 400$  mg/kg CPA administration. The fertilization rate also decreased in the group that was treated with  $\geq 400$  mg/kg CPA. However, after fertilization, the embryos demonstrated normal growth ability. Two weeks after CPA administration, the number of mice from which the oocytes could be retrieved markedly decreased, but the fertilization rate and development of morphological features in the embryos were similar to those of the controls. One month after CPA administration, the number of mice from which the oocytes could be retrieved, fertilization rate, and development of the morphological features in the embryos were similar to those of the controls.

**Conclusion:** The number of oocytes decreased as the CPA administration level increased; however, the oocytes' potential for fertilization and development to the blastocyst stage was not significantly affected. One month after CPA administration, the number of oocytes and the potential for development into blastocysts were recovered.

## KEYWORDS

chemotherapy, cyclophosphamide, dose, embryo development, ovarian function failure

## 1 | INTRODUCTION

Presently, although assisted reproductive technology offers several advances and benefits, it has been unable to resolve the issues arising in women who have lost ovarian function due to different reasons. Chemotherapy and radiotherapy can occasionally

induce gonadal disorders and permanent ovarian failure.<sup>1,2</sup> Chemotherapy has been reported to reduce fertility and cause ovarian dysfunction.<sup>3–5</sup> In addition, early menopause has been reported to occur, even if the ovarian function is maintained, in patients after the completion of cancer treatment.<sup>5–7</sup> However, even if the menstrual cycle recovers after chemotherapy in patients

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with cancer, pregnancy might not be possible due to decreased ovarian function.

Cyclophosphamide (CPA), an alkylating agent that has been used for a long time in chemotherapy, is often used in the treatment of blood diseases and autoimmune diseases and is known to act like a reproductive toxin, as demonstrated in several clinical reports and animal experiments.<sup>8-12</sup> Therefore, with CPA treatment, various fertility preservation strategies are applied, depending on the risk of gonadal dysfunction, the condition of the patient at the time of preservation of the fertile surames, and the presence or absence of a partner.<sup>13,14</sup> These strategies include in vitro fertilization (IVF), embryo cryopreservation, ovarian tissue cryopreservation, oocyte freezing, in vitro maturation, and hormonal treatment. Currently, the most effective methods are IVF and embryo or oocyte cryopreservation.

The administration of chemotherapy agents at the ovarian hyperstimulated stage could lead to toxicity to the ovaries. However, until date, investigations on the effects of ovarian stimulation being exposed to cytotoxic substances remain scarce. Recently, a study reported that CPA administration affected the ovarian reserve when ovarian stimulation was conducted prior to the treatment.<sup>15</sup> These results support that ovarian stimulation and IVF-embryo cryopreservation procedures should be performed prior to chemotherapy for the safety of fertility preservation.

Moreover, the vitrification method of unfertilized oocytes demonstrated improved results and a higher survival rate than those in the conventional slow-freezing methods. One study reviewed more than 936 cases of babies who were born by cryopreserved oocytes and found no apparent increase in the congenital abnormalities in them.<sup>16</sup> Young, unmarried patients who wish to preserve their unfertilized oocytes before or after chemotherapy administration can now cryopreserve their own oocytes for reproductive treatments in the future. The reports on the oocyte conditions during or after cancer drug administration are limited. Therefore, through the present study was investigated the effects of ovarian stimulation on ovarian function following CPA administration. In addition, the ability of oocyte fertilization after CPA administration was investigated in mice.

## 2 | MATERIALS AND METHODS

Mature ICR strain mice (female mice: aged 8 weeks; male mice: aged 9-12 weeks) were used in this study. All the experiments were approved by the ethics committee within St. Luke's Clinic, Oita, Japan. The experiments were conducted in two phases.

### 2.1 | Phase 1

To investigate the effects of ovarian stimulation immediately following CPA administration on future ovarian function, the mice were administered a single intra-peritoneal (i.p.) injection of CPA (Shionogi & Company, Ltd, Tokyo, Japan) dissolved in saline, at a dose of 0, 50, 100, 200, 400, or 800 mg/kg body weight (800 mg/kg was administered

over 2 days). An additional group of mice was injected with saline, serving as the controls. Immediately following CPA administration, the ovarian stimulation was started. The ovaries were stimulated though an i.p. injection of 5 IU pregnant mare's serum gonadotropin, followed by an administration of 5 IU human chorionic gonadotropin (hCG) after 48 hours. The mouse oocytes were collected from the oviducts at 14-16 hours after the hCG injection. The sperm were collected from the epididymis. Then, 2 hours after the sperm collection, the collected oocytes were inseminated in HFF99 medium (Fuso Pharmaceutical Industries, Osaka, Japan) that was supplemented with 0.3% bovine serum albumin (Sigma Chemical Company, St. Louis, MO, USA) and was cultured at 37°C under a 5% CO<sub>2</sub> atmosphere until blastocyst formation.

### 2.2 | Phase 2

To evaluate the oocyte fertilization efficiency and the influences of the residual drug in 2 weeks and 1 month after CPA administration regarding the IVF results, ovarian stimulation, oocyte retrieval, and IVF were attempted after 2 weeks and 1 month of administering a single i.p. injection of 400 mg/kg CPA in the experimental mice. The mice embryos then were checked morphologically, as previously described in the phase 1 protocol. The treatment protocol and the time frame of the experiment are shown in Figure 1.

### 2.3 | Statistical analysis

A chi-square test and *t* test were performed, with  $P < .05$  considered to be statistically significant.

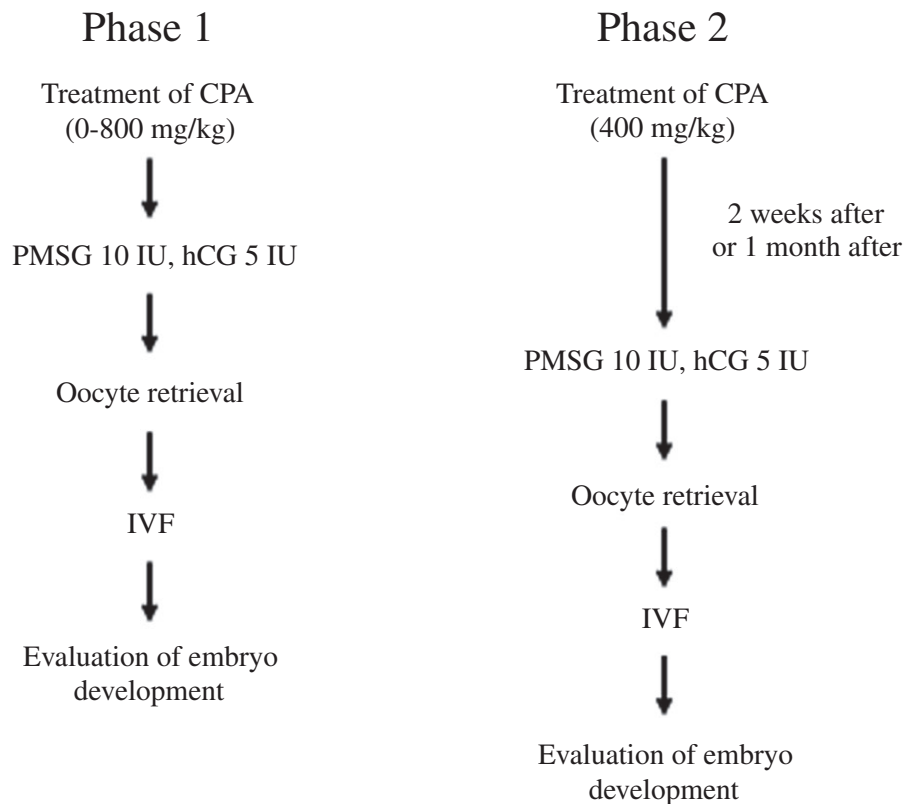
## 3 | RESULTS

### 3.1 | Phase 1

The number of mice in which the oocytes could be retrieved was compared among different CPA dose injections and found to be significantly decreased with a  $\geq 400$  mg/kg CPA dosage ( $P < .01$ ; Table 1). The average number of retrieved oocytes was dramatically decreased when the CPA dosage exceeded 400 mg/kg ( $P < .01$ ; Figure 1). The average number of oocytes that developed to metaphase II (MII) significantly decreased when 400 mg/kg and 800 mg/kg of CPA was administered ( $P < .01$ ; Figure 2). The fertilization rate and embryo development features are shown in Table 2. Although the fertilization rate decreased with the administration of 400 mg/kg of CPA, the morulation and blastocyst formation rates remained the same as the control group rate.

### 3.2 | Phase 2

After 2 weeks of CPA administration, IVF was performed. The number of mice from which the oocytes could be retrieved significantly decreased to 50%. However, after 1 month, the oocyte retrieval rate increased to 90% ( $P < .05$ ). The rate of MII oocytes per total cumulus oocytes complex was not significantly different



**FIGURE 1** Study design regarding the dose, which was 0, 50, 100, 200, or 400 mg/kg per day. The dose of 800 mg/kg was administered over 2 days (400 mg/kg per day for 2 days). The control group was injected with saline only. CPA, cyclophosphamide; hCG, human chorionic gonadotropin; IVF, in vitro fertilization; PMSG, pregnant mare's serum gonadotropin

**TABLE 1** Cyclophosphamide (CPA) concentration for mice oocyte retrieval

CPA (mg/kg)	No. of mice in which oocytes were retrieved <sup>a</sup> (%)	P
Control	22/27 (81.5)	
50	8/14 (64.3)	NS <sup>b</sup>
100	9/10 (90.0)	NS <sup>b</sup>
200	7/10 (70.0)	NS <sup>b</sup>
400	6/18 (33.3)	<.01**
800	2/10 (20.0)	<.01**

<sup>a</sup>Number of mice in which oocytes were retrieved/number of all mice (%).

<sup>b</sup>NS, no statistical significance using the chi-square test.

\*\*P < .01, according to the chi-square test.

from that of the control group (Table 3). In IVF, the fertilization rate and the morula and blastocyst formation rates were not different from those of the controls under a similar condition and time period (Table 4).

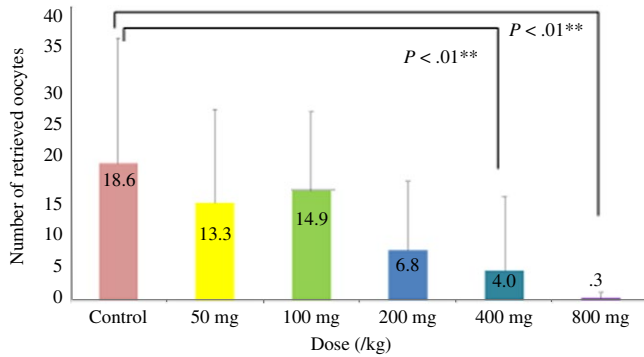
## 4 | DISCUSSION

The present animal study demonstrated that ovarian stimulation after CPA treatment affected the number of retrieved oocytes. Thus,

the production of oocytes was significantly decreased by a higher CPA dose. However, the recovered oocytes developed normally in IVF. Moreover, after 2 weeks of CPA treatment, the fertilization potential of the oocyte remained unchanged despite a significant decrease in the number of mice from which the oocytes could be retrieved. One month after CPA administration, the number of retrieved oocytes and the fertilization rate had recovered to the levels before CPA administration.

One reason for ovarian function failure was the depletion of the primordial follicle stockpile following treatment with the cytotoxic agent, CPA. The reactivity of the ovaries depends on the dose; the more the dose, the lower is the ovarian reserve.<sup>13</sup> A past animal and human study suggested the presence of the direct toxic effect of chemotherapy on follicles<sup>17</sup> and large apoptosis in the growing phase of CPA-administered mice follicles, but not in the resting follicles.<sup>18</sup> Moreover, injury to the entire ovary that resulted in blood vessel occlusion, focal cortical fibrosis, and the disappearance of the primordial follicle from fibrotic zones<sup>19</sup> also have been observed.

When patients who were treated with chemotherapy or surgery desired to maintain their fecundity after treatment, some time was required for the recovery of their ovarian function. Ovarian stimulation in IVF protocols usually altered the ovarian physiology, considering that the ovarian volume and blood flow dramatically increase on stimulation.<sup>20</sup> Therefore, it was hypothesized that overstimulation



**FIGURE 2** The metaphase II oocytes that were retrieved from one mouse. A comparison of the number of retrieved oocytes between the six treatment groups. \*\* $P < .01$ , according to the Student's  $t$  test. Values are the mean  $\pm$  standard deviation

prior to chemotherapy can reduce ovarian damage. It also has been hypothesized that the ovarian reserve might decline as more anti-cancer drugs increase follicular destruction. One study reported that ovarian stimulation before CPA administration did not adversely affect the ovarian reserve after treatment.<sup>15</sup> Another hypothesis suggested that increasing the blood flow before chemotherapy would prevent the annihilation of the blood vessels and fibrosis and reduce the decrease in the follicle number. These results support the safety of fertility preservation procedures by ovarian stimulation before chemotherapy. The present study was designed to evaluate the fertilization ability regarding CPA treatment immediately before ovarian stimulation and a safe time period from CPA administration.

This study's results clearly indicate a significant decrease in the number of oocytes that could be retrieved from the mice when a dose of  $\geq 400$  mg/kg CPA was administered. In addition, the number of retrieved MII oocytes was significantly decreased (Figure 1). However, when IVF procedures used the retrieved oocytes, the fertilization rate and the morula and blastocyst development rates were not significantly different from those of the controls. However, after 2 weeks of 400 mg/kg CPA administration, the number of retrieved oocytes decreased, while the fertilization, morula, and blastocyst rates remained similar to those of the controls. These doses are the usual treatment volume per cycle in humans. Therefore, these results suggest that, in mice experiments, CPA administration induces more severe follicular closure and oocyte damage to the

**TABLE 3** Oocyte retrieval and metaphase II (MII) oocyte ratio from mice variable

	2 weeks	1 month
No. of mice which retrieval oocytes (%)		
Control	9/12 (75)	9/10 (90)
CPA	6/12 (50)*	9/10 (90)
Recovery of MII oocyte <sup>a</sup> (%)		
Control	217/244 (88.9)	229/268 (85.4)
CPA (400 mg/kg)	195/240 (81.3)	313/327 (95.7)

CPA, cyclophosphamide.

<sup>a</sup>MII oocytes/cumulus oocyte complexes.

\* $P < .05$ , according to the chi-square test.

**TABLE 4** Fertilization and embryo development using the retrieved oocytes after various periods of administration

Period	Fertilization (%)	Morula (%)	Blastocysts (%)
60 h	5/5 (100.0)	4/5 (80.0)	4/5 (80)
Control	268/401 (66.8)	258/268 (96.3)	250/268 (93.3)
2 weeks	103/195 (52.8)	100/103 (97.1)	99/103 (96.1)
Control	106/217 (48.8)	90/106 (84.9)	87/106 (82.0)
1 month	162/313 (51.8)	156/162 (96.3)	146/162 (90.1)
Control	115/229 (50.2)	109/115 (94.5)	108/115 (93.8)

NS, no statistical significance, according to the chi-square test.

developing follicle than to the primordial follicle. The cumulative findings indicate that the effect of CPA toxicity tends to diminish after 1 month of CPA administration, as primordial follicles require a period of 1 month to mature into a follicle. Ovarian stimulation immediately following CPA administration does not affect the extent of damage to the follicle quality in IVF, nor did the ovarian reserve change after treatment.

The present study has some limitations. First, this study was performed only in mice. Future studies are required to investigate the effects of CPA administration in humans. Second, this study was a single-injection experiment only. Phase 2 used a higher CPA dose (400 mg/kg body weight) than usual for animal experiments (50-150 mg/kg) and human experiments (50 mg/kg per day for 7 days). Third, this study evaluated the effect of CPA treatment on

**TABLE 2** Fertilization and embryo development by using in vitro fertilization retrieval oocytes

CPA (mg/kg)	Fertilization (%)	Molura (%)	Blastocyst (%)
Control	310/493 (62.9)	300/310 (96.8)	292/310 (94.2)
50	107/176 (60.8)	84/107 (78.5)	82/107 (76.6)
100	123/146 (84.2)	120/123 (97.6)	119/123 (96.7)
200	49/61 (80.3)	49/49 (100)	47/49 (95.9)
400	35/71 (49.3)	33/35 (94.3)	32/35 (91.4)
800	2/2 (100)	1/2 (50.0)	1/2 (50.0)

CPA, cyclophosphamide.

The results in the table show no statistical significance using the chi-square test.

the oocyte fertilization ability and embryo growth in mice. Further studies are required to evaluate the effect of CPA on oocytes by evaluating the pregnancy rate, the number of fetuses, and the degree of growth by using embryos that are collected after CPA administration. Moreover, the effect of CPA on the mitochondrial function, DNA damage, and epigenetic change remain unknown.

In the future, ovarian histological evaluation of the follicular development status would enable the investigation of the influence of CPA administration on the ovary.

As the survival rate of young patients who are receiving cancer treatment is increasing, it has become important to improve the quality of life of cancer survivors. The preservation of fertility is an indispensable step across all treatment regimens in young patients with cancer; cancer treatment often induces a reduction in fertility. The most readily available method for fertility preservation is the cryopreservation of the embryo and ovarian tissues.<sup>21-25</sup>

One option is to perform natural-cycle IVF.<sup>26</sup> However, in order to preserve future fertility, the ovaries are stimulated with follicle-stimulating hormone or human menopausal gonadotropin to induce ovulation, after which multiple eggs are harvested, fertilized, and saved for future use. In most cases, chemotherapy is initiated shortly after fertility preservation. Therefore, it is important to attain more knowledge about oocyte efficiency in relation to chemotherapy.

In conclusion, the results of this study showed that ovarian stimulation immediately following CPA administration does not adversely affect the ovarian reserve despite a decrease in the number of oocytes after 2 weeks of CPA treatment, when the ovarian function recovers to the normal pre-CPA administration levels. These observations recommend conformance to safety in fertility preservation procedures (IVF-embryo cryopreservation or unfertilized oocyte cryopreservation) in patients with cancer who are undergoing ovarian stimulation.

## DISCLOSURES

**Conflict of interest:** The authors declare no conflict of interest. **Human Rights Statement and Informed Consent:** This article does not contain any study with human participants that have been performed by any of the authors. **Animal studies:** All the experiments in this research were approved by the ethics committee within St. Luke's clinic, Oita, Japan.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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