醫學博士學位論文

Effect of imatinib co-administration on *in vitro* oocyte acquisition and subsequent embryo development in cyclophosphamide-treated mice

사이클로포스파마이드 처치 마우스에서 이마티닙 병용 투여가 난자 회수 및 배아 발달에 미치는 영향

2014년 2월

서울대학교 대학원
의학과 산부인과전공
천 은 경

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지도교수 지 병 철

이 논문을 의학박사 학위논문으로 제출함

2013년 12월

서울대학교 대학원
의학과 산부인과학 전공
천 은 경

천은경의 의학박사 학위논문을 인준함

2014년 1월

위 원 장 _____(인)
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위 원 _____(인)
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논문 제목: **Effect of imatinib co-administration on in vitro oocyte acquisition and subsequent embryo development in cyclophosphamide-treated mice**

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ABSTRACT

Objective: To investigate the effect of imatinib co-administration on *in vitro* oocyte acquisition and subsequent embryo development in cyclophosphamide (Cp)-treated mice.

Design: Experimental study.

Materials and Methods: Female 4–5-week-old BDF1 mice (n=112) were injected with 5 IU eCG followed by 5 IU hCG 48 hours later and then oocytes were retrieved 14 hours later. They were grouped according to the dose of Cp and/or imatinib which were injected 24 hours prior to eCG injection (group 1: saline 0.1 mg/kg; group 2, 3, 4: Cp 25, 50, 75 mg/kg, group 5: imatinib 7.5mg/kg, group 6, 7, 8: Cp 25, 50, 75 with imatinib 7.5 mg/kg). Each number of mature, dead and total oocytes was counted. After in vitro fertilization, fertilization rate and percentage of blastocyst formation were assessed. Blastocyst cell numbers and percentage of apoptotic cells were assessed by TUNEL method. Meiotic spindle integrity within mature oocytes was also assessed

Results: In the 25 and 50 mg/kg Cp group, imatinib co-administration significantly enhanced the percentage of mature oocytes (+16.4%, $p<0.001$ and +10.4%, $p<0.02$, respectively) and significantly decreased the percentage of dead oocytes (-25.9%, $p<0.001$ and -15.3%, $p<0.001$, respectively). Imatinib co-administration significantly enhanced the fertilization rate in the 50 mg/kg Cp group (+12.2%, $p<0.05$). The percentage of blastocysts formation and their cell number as well as

the percentage of apoptotic cells was similar whether imatinib was co-administered or not. Spindle integrity within oocyte was significantly affected by the Cp treatment and was rescued by the imatinib co-administration.

Conclusions: Imatinib co-administration prior to ovarian stimulation improved oocyte maturity and in vitro fertilization rate in Cp-treated mice. Spindle integrity within oocyte was well-conserved by the imatinib co-administration.

Keywords: cyclophosphamide, imatinib, in vitro fertilization, blastocyst

Student Number: 2003-30634

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INTRODUCTION

Alkylating chemotherapeutic agents, especially cyclophosphamide (Cp), are harmful to gonadal function [1,2]. Its administration causes ovarian follicle depletion and eventually ovarian failure [3-6].

For preserving fertility of patients who needs chemotherapy, in vitro production of embryo via in vitro fertilization (IVF) is a well-established option [7]. Alternatively, oocyte or ovarian tissue could be preserved, although the latter approach requires further research. Chemotherapy must be postponed until mature oocytes can be obtained in cancer patients who wish to cryopreserve their oocytes or embryos for future fertility. Cryopreservation of ovarian tissue can usually be performed without delaying chemotherapy, as it does not require ovarian stimulation but patients should undergo surgery to excise her ovary.

Usually we do not perform ovarian stimulation promptly after chemotherapy, but patients with chemotherapy in their medical history always have concern about the effect of these kinds of treatments on their future reproductive health.

It has been reported that administration chemotherapeutic agents before ovarian stimulation can affect the integrity of resultant oocytes or embryos. An earlier report in a murine model indicated that Cp, if administered *in vivo* at one day before

ovarian stimulation, reduces the oocyte number and impairs fertilization and subsequent embryo development [8]. A recent report suggested that the oocyte number can be reduced by a single injection of Cp but embryo development after IVF is dependent on the treatment dose [9].

After single injection of Cp, even oocytes retrieved 6 weeks later showed significantly reduced fertilization and embryo formation rate [10]. *In vitro* Cp treatment also inhibited dissolution of the cumulus and reduced fertilization and early cleavage rates in a dose-dependent manner [11]. Indeed, significantly decreased oocyte yield (6.5 vs. 14.1) and number of embryos available for cryopreservation (3.9 vs. 6.8) were observed in 23 female breast cancer patients who underwent ovarian stimulation immediately after chemotherapy compared to 144 patients who underwent ovarian stimulation before chemotherapy [12].

Although incidence of aneuploid oocytes was similar between *in vivo* treatment of Cp and non-treated control [13], a recent report indicated that aneuploid embryos are significantly increased in the Cp treatment group [10]. In the offspring of mice treated with Cp 1–4 weeks before mating, the malformation rate was increased at least 10-fold [14].

Imatinib is a selective tyrosine kinase inhibitor that has been successfully used to treat chronic myeloid leukemia (CML) and gastrointestinal stromal tumors [15]. This agent was recently reported to counteract cisplatin-induced oocyte damage in

cultured mouse ovaries [16]. Here, we investigated the effect of co-administration of imatinib on the quantity and quality of *in vitro* produced oocytes and subsequent embryo developmental outcomes in Cp-treated mice. Intra-oocyte spindle integrity was also examined.

MATERIALS AND METHODS

Animals

Four- to five-week-old female BDF1 mice (Orient Co., Seoul, Korea) were maintained under a 12-hours light:12-hours dark cycle at 23°C and fed ad libitum. Animal care and use were in accordance with the institutional guidelines established by the Animal Care and Use Committee of Seoul National University Bundang Hospital.

Collection of mature oocyte and in vitro fertilization

After one week of adaptation, mice were treated with ip injection of 5 IU eCG (Sigma-Aldrich, SL, MO) followed by ip injection of 5 IU hCG (Sigma-Aldrich) 48 hours later. Mice were sacrificed by cervical dislocation 13–14 hours later, and the oviducts were collected. The oviducts were dissected and placed in a Petri dish containing modified mouse tubal fluid (mMTF) medium supplemented with 0.4% (w/v) bovine serum albumin (BSA; Sigma-Aldrich). Cumulus-oocyte complexes were released by tearing the ampulla of the oviducts. The cumulus cells were removed enzymatically using 85 IU/mL hyaluronidase (Sigma-Aldrich) and by mechanical dissociation using a glass pipette. Oocytes were classified as mature, immature, dead and fragmented and the numbers were recorded. Only morphologically normal mature oocytes, as judged by the presence of a first polar body, were used for IVF..

The epididymal spermatozoa were retrieved from the cauda epididymis of 8- to 10-week-old BDF-1 mice, and the sperm suspensions were pre-incubated for 1.5 hours in capacitation medium (mMTF supplemented with 0.8% BSA). The oocytes were then inseminated at a final dilution of 2 million cells/mL and incubated at 37°C in humidified 5% CO_2 in air. Inseminated oocytes were washed by pipetting 6 hours later and then placed in embryo maintenance medium (Global medium supplemented with 10% human serum albumin; Life Global, Guilford, CT). Fertilization was assessed by the formation of 2-cell on day 1 after insemination. The cleaved embryos were transferred to new embryo maintenance medium, and blastocyst development was recorded on day 5 after insemination.

Blastocyst cell counts and TUNEL staining

DNA integrity was measured by the TUNEL method. The blastocysts were fixed with 4% paraformaldehyde for 1 hour at RT. After washing with phosphate-buffered saline (PBS), the blastocysts were permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate (Sigma-Aldrich). A commercial apoptosis detection kit was used (In Situ Cell Death Detection Kit; Roche Diagnostics GmbH, Mannheim, Germany). The remaining procedures were performed as directed by the kit instructions. The blastocysts were air-dried on a silane-coated slide (DAKO, Glostrup, Denmark). Counterstaining was performed using a mounting medium with 4,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA). The blastomeres with fragmented DNA had green stained nuclei, whereas the nuclei of the other

blastomeres were stained blue and visualized with a fluorescence microscope (Carl Zeiss, Axio Imager A1, Germany) with a Hamamatsu digital camera imaging system (Figure 1). Blastomeres with >50% of the area stained green were considered positive. The total blastomere count and the percentage of positive blastomeres were determined in each blastocyst.

Meiotic spindle integrity

Spindle integrity was assessed using previously described methods [17,18]. The mature oocytes were washed three times with 1% BSA in PBS for 5 min and then then fixed with 4% paraformaldehyde for 1 hour at RT. After washing twice by 1% BSA in PBS, permeabilization was performed with 0.25% Triton X-100 in PBS at RT. After washing twice by 1% BSA in PBS, blocking was performed with 3% BSA in PBS for 1 hour at RT, and then washed twice by 1% BSA in PBS. A primary antibody for α-tubulin (Cell Signaling, Danver, MA) diluted in 1% BSA (1:100) was added and incubated overnight at 4°C. After washing three times with 1% BSA in PBS, a secondary antibody (Alexa flour® 488 goat anti-rabbit IgG; Invitrogen, Calsbad, CA, diluted in 1% BSA [1:100]) was added for 1 hour at RT in the dark. After washing three times by 1% BSA in PBS, the oocytes were air-dried and on a silane-coated slide (DAKO). The slide was counterstained with DAPI and examined using confocal microscope (Carl Zeiss, LSM 710) (Figure 2). A typical barrel-shaped microtubule structure between both poles with centrally aligned chromosomes (metaphase II) was considered normal.

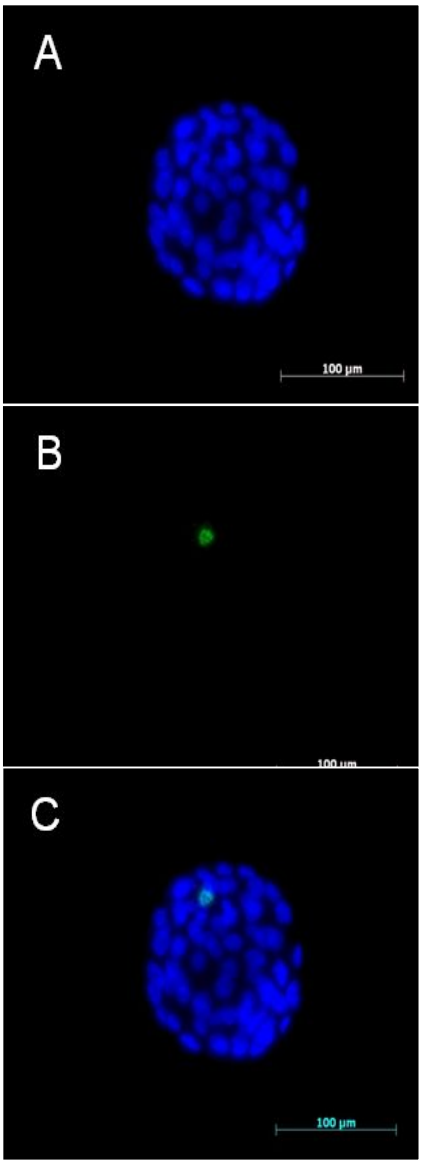


Figure 1. Microphotographs showing fluorescent TUNEL staining of blastocysts derived from mature mouse oocytes to determine DNA integrity. Nuclei are stained blue (A: DAPI) and apoptotic blastomeres appear green (B: TUNEL). Merged capture (C) (400x). A blastomere with less than 50% of area stained with green regarded as negative.

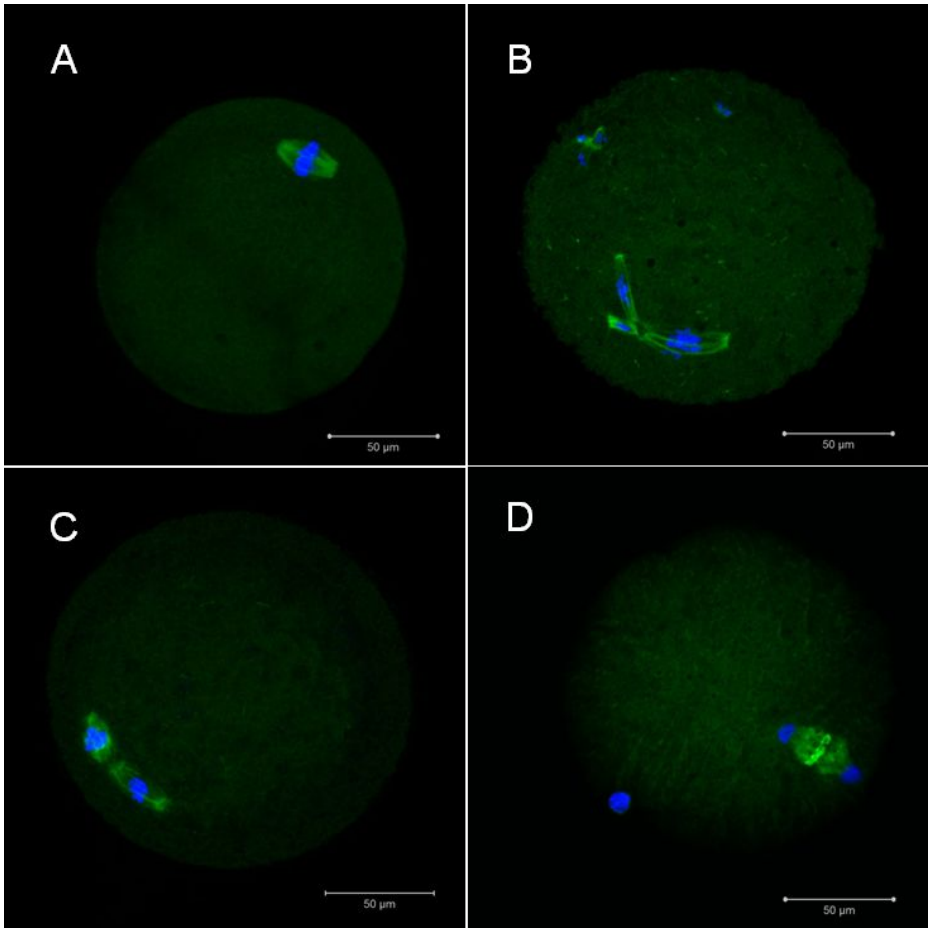


Figure 2. Representative confocal microphotographs showing the meiotic spindle organization and chromosome alignment in mature mouse oocytes: (A) normal metaphase II, typical barrel-shaped microtubule structure between both poles with centrally aligned chromosomes (metaphase II) was considered normal. (B and C) abnormal metaphase II, and (D) normal anaphase II, showing parthenogenesis (400x).

Experimental design

Experimental design is depicted in Figure 3. In the untreated control group, 0.1 mL of normal saline only was injected ip once. In group 2, 3 and 4, Cp (cyclophosphamide monohydrate; Sigma-Aldrich; Cat# 29875) 25, 50 or 75 mg/kg body weight was injected ip once into mice at 24 hours prior to eCG administration. In group 5, imatinib (Sigma-Aldrich) 7.5 mg/kg body weight was injected ip once to mice at 24 hours prior to eCG administration. An imatinib stock solution (5 mg/mL) was obtained by dissolving imatinib powder in PBS[19]. In group 6, 7, and 8, CP (25, 50 or 75 mg/kg body weight) in combination with imatinib (7.5 mg/kg body weight) was injected ip once into mice at 24 hours prior to eCG administration. All final ip injection volumes were adjusted as 0.1 mL mixed with normal saline.

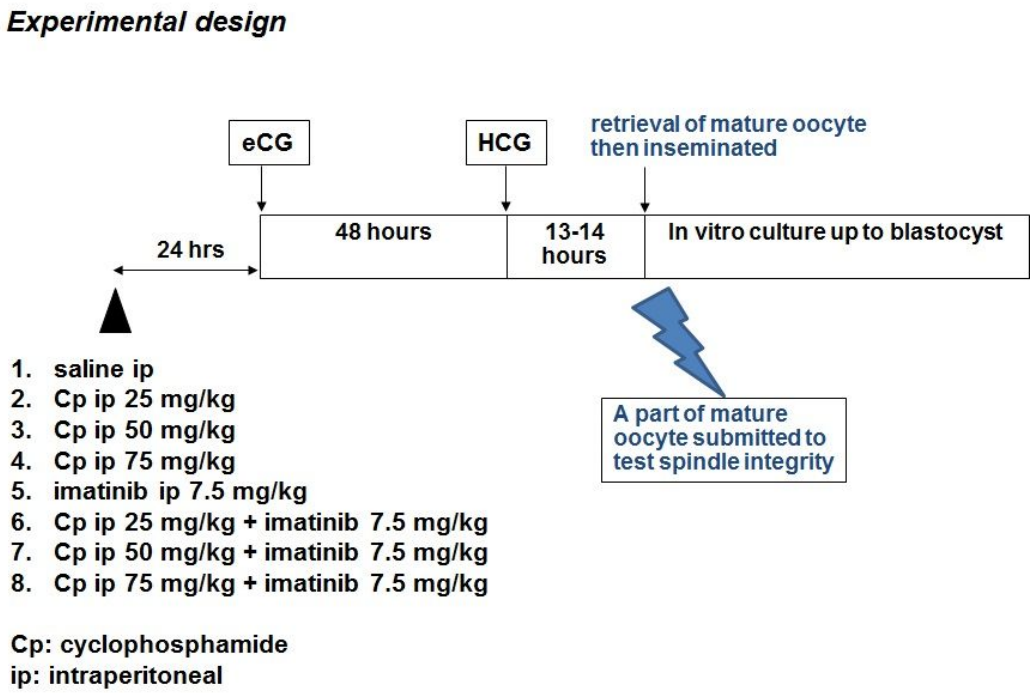


Figure 3. Experimental design Female 4–5-week-old BDF1 mice (n=112) were injected with different dose of Cp and/or imatinib. Twenty-four hours later, they were hyperstimulated with 5 IU eCG followed by 5 IU hCG 48 hours later and then oocytes were retrieved 14 hours later.

Statistical Analysis

Data were analyzed with SPSS (v.17, SPSS Inc., Chicago, IL). From 12-15 replicates, each median number of total, mature and in vitro fertilized oocytes, and the resultant blastocysts was counted in the 8 experimental groups and compared using the Wilcoxon rank sum test. Blastocysts cell numbers were compared using the Student's t-test. The proportions were compared using the Chi-square test. A p-value of <0.05 was considered as significant.

RESULTS

Oocyte yield and maturity and IVF outcomes

The median numbers (±SEM) of total, mature, fertilized oocyte and the resultant blastocysts are listed in Table 1. Overall, all treatment groups (except the imatinib only group) showed decreased numbers of mature and fertilized oocytes or blastocysts relative to untreated control. Moreover, co-administration of imatinib did not enhance the number of total, mature and fertilized oocytes or the resultant blastocysts when compared with each corresponding Cp-only group. Insignificant benefits were observed (not significant) in obtaining more mature 0ocytes (+1 and +2), fertilized oocyte (+2 and +1) or blastocysts (+2 and +1) after co-administration of imatinib in the 25 and 50 mg/kg Cp-treated group. In the 75 mg/kg Cp-treated group, the median number of mature and fertilized oocytes or blastocyst was markedly decreased than untreated controls whether imatinib co-administrated or not.

The data were pooled and the percentages of mature and dead oocytes are presented in Figure 4. All treatment groups showed a significantly lower percentage of mature oocytes and significantly higher percentage of dead oocytes compared with untreated controls. However, when compared with the corresponding Cp-only group, the percentage of mature oocyte was significantly enhanced (+16.4%, $p<0.001$ and

+10.4%, $p<0.02$) and the percentage of dead oocytes was significantly decreased (-25.9%, $p<0.001$ and -15.3%, $p<0.001$) after co-administration of imatinib; this phenomenon was observed only in the 25 and 50 mg/kg Cp-treated group.

Fertilization rate (FR) (per mature oocyte) and percentage of blastocysts (per fertilized oocyte) are depicted in Figure 5. In the imatinib-only group, the FR and the percentage of blastocysts were similar to those of untreated controls. After *in vivo* treatment of Cp only, the FR of the 25 and 50 mg/kg Cp-treated groups was significantly lower, and after co-administration of imatinib, the FR of the 25 and 75 mg/kg Cp-treated groups was significantly lower compared to the untreated controls. This led to a significantly enhanced FR in the 50 mg/kg Cp group: 64.8% without imatinib and 77% with imatinib (+12.2%, $p < 0.05$).

After *in vivo* treatment of Cp only, the percentage of blastocysts in the 25 and 75 mg/kg Cp-treated groups was significantly lower than in the untreated controls, and after co-administration of imatinib, the percentage of blastocysts in the 50 and 75 mg/kg Cp-treated groups was significantly lower than in the untreated controls. This resulted in an enhanced blastocyst percentage in the 25 mg/kg Cp-treated group: 62.9% without imatinib and 66.2% with imatinib, although this difference was not significant (+3.3%, $p > 0.05$).

As summarized above, co-administration of imatinib significantly enhanced the percentage of mature oocyte and significantly decreased the percentage of dead oocytes in the 25 and 50 mg/kg Cp-treated group. Co-administration of imatinib

significantly enhanced the FR only in the 50 mg/kg Cp-treated group, but did not enhance the percentage of blastocysts in any of the three Cp-treated groups.

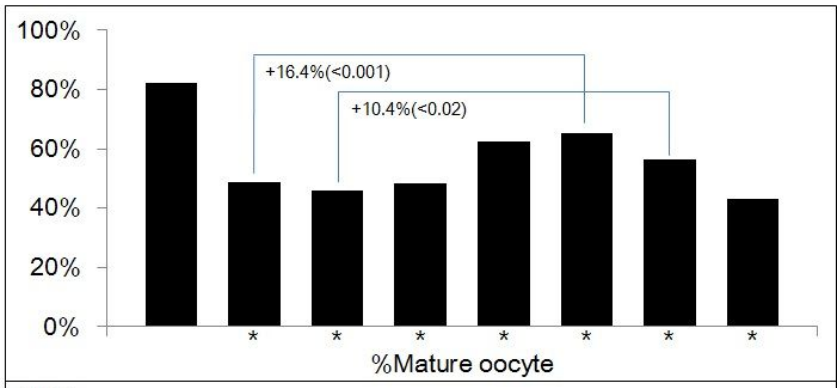
Table 1. Median numbers of total, mature, and fertilized oocytes and blastocysts

Group	1	2	3	4	5	6	7	8
Cyclophosphamide	-	25	50	75	-	25	50	75
Imatinib	-2	-	-	-	7.5	7.5	7.5	7.5
Mice	12	15	15	15	12	14	14	15
Replicates	12	15	15	15	12	14	14	15
Total oocyte	23	23	13	12	26	21	19	14
p-value [a]	-	NS	<0.05	<0.05	NS	NS	NS	<0.001
Δmedian	-	-	-	-	-	-2	+6	+2
p-value [b]	-	-	-	-	-	NS	NS	NS
Mature oocyte	19	10	7	6	14	11	9	5
p-value [a]	-	<0.001	<0.001	<0.001	NS	<0.05	<0.01	<0.001
Δmedian	-	-	-	-	-	+1	+2	-1
p-value [b]	-	-	-	-	-	NS	NS	NS
Fertilized oocyte	17	6	6	5	11	8	7	2
p-value [a]	-	<0.001	<0.001	<0.001	NS	<0.01	<0.01	<0.001
Δmedian	-	-	-	-	-	+2	+1	-3
p-value [b]	-	-	-	-	-	NS	NS	NS
Blastocyst	13	3	4	2	10	5	5	1
p-value [a]	-	<0.001	<0.001	<0.001	NS	<0.01	<0.001	<0.001
Δmedian	-	-	-	-	-	+2	+1	-1
p-value [b]	-	-	-	-	-	NS	NS	NS

Doses indicate mg/kg body weight.

[a]: when compared to untreated controls (group 1) by the Wilcoxon test.

[b]: when compared to imatinib control (i.e. between group 2&6, between group 3&7, and between group 4&8) by the Wilcoxon test.



Group	1	2	3	4	5	6	7	8
Cyclophosphamide	-	25	50	75	-	25	50	75
Imatinib	-	-	-	-	7.5	7.5	7.5	7.5
Mice	12	15	15	15	12	14	14	15
Total oocytes	323	363	271	263	292	299	285	207

Figure 4. Percentages of mature (upper panel) and dead oocytes (lower panel) in the eight experimental groups. Doses indicate mg/kg body weight. All treatment groups showed a significantly lower percentage of mature oocytes and significantly higher percentage of dead oocytes compared with untreated controls (group 1) (asterisks). In the 25 and 50 mg/kg Cp-treated group, significantly enhanced percentage of mature oocyte (+16.4%, $p<0.001$ and +10.4%, $p<0.02$) and significantly decreased percentage of dead oocytes (-25.9%, $p<0.001$ and -15.3%, $p<0.001$) with imatinib co-administration were observed, compared with the corresponding Cp-only group.

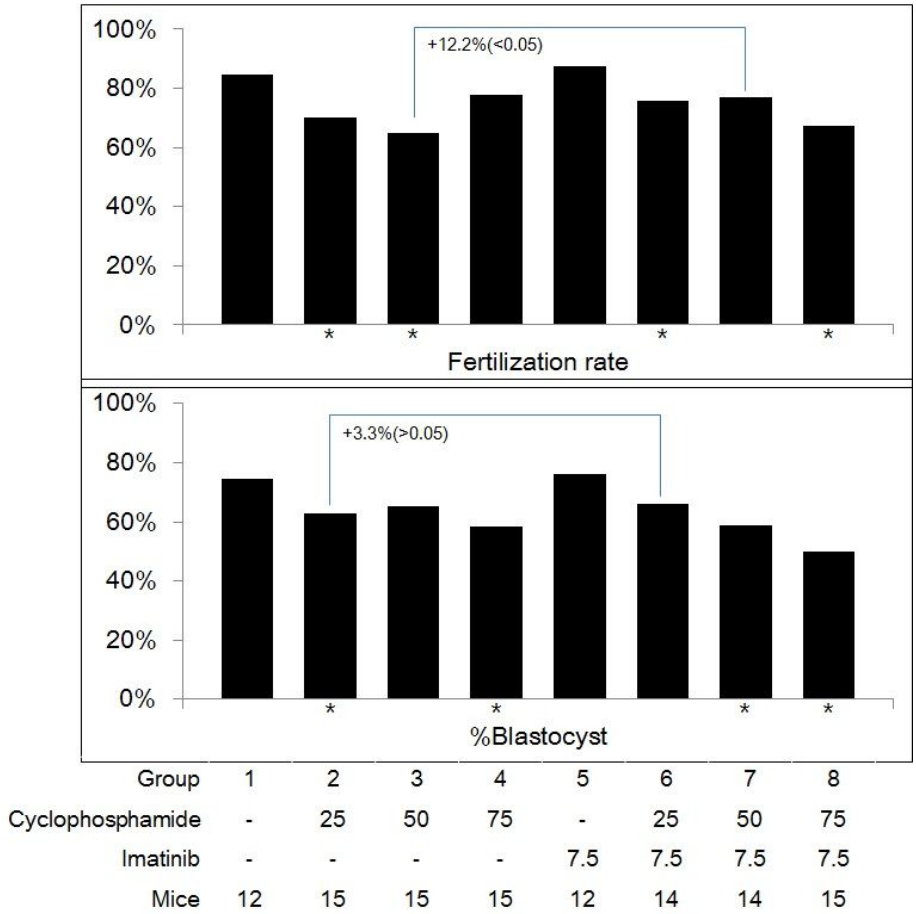


Figure 5. Fertilization rate (upper panel) and blastocyst formation rate (lower panel) in the eight experimental groups. Doses indicate mg/kg body weight. Asterisks indicate a significant difference compared to untreated controls (group 1). Imatinib only didn't affect FR and the percentage of blastocysts. A significantly enhanced FR was observed by imatinib co-administration in the 50 mg/kg Cp group (64.8 v.s. 77% , +12.2%, $p < 0.05$). An enhanced percentage of blastocyst was observed without statistical significance (62.9% v.s. 66.2% , +3.3%, $p > 0.05$) in the 25 mg/kg Cp-treated group.

Cell counts, apoptosis, and spindle integrity

In the 25 and 50 mg/kg Cp-treated groups, the cell number and the percentage of apoptotic cells was similar irrespective of whether imatinib was co-administered (Table 2). In the 75 mg/kg Cp-treated group, the cell number was significantly lower ($p<0.05$) and the percentage of apoptotic cells was significantly higher ($p<0.001$) when imatinib was co-administered.

Intra-oocyte spindle integrity was significantly affected by the Cp treatment in a dose-dependent manner (Table 3). However, this harmful effect disappeared when imatinib was co-administered. The percentage of oocytes with normal metaphase II spindles was higher after co-administration of imatinib in all of three Cp-treated groups (79.4% vs. 97.1% [$p = 0.054$], 78.8% vs. 100% [$p < 0.05$], and 67.7% vs. 93.8% [$p < 0.05$], respectively).

Table 2. **Cell numbers and percentage of apoptotic cells of blastocysts in eight experimental groups**

Cyclophosphamide	-	25	50	75	-	25	50	75
Imatinib	-	-	-	-	7.5	7.5	7.5	7.5
Blastocysts examined	77	62	53	57	70	73	67	28
Mean cell number	57.8	75.9	60.5	66.2	73.4	75.3	60.2	57.5
(SD)	(23.7)	(31.8)	(26.3)	(24.5)	(27.4)	(25.9)	(28.3)	(20.9)
p-value [a]	-	<0.001	NS	NS	<0.01	<0.001	NS	NS
p-value [b]	-	-	-	-	-	NS	NS	<0.05
Total cell number	4,451	4,704	3,206	3,773	5,163	5,695	4,036	1,611
Cells with positive signal	148	107	122	100	219	99	149	73
Percent apoptotic cells	3.33%	2.27%	3.81%	2.65%	4.24%	1.74%	3.69%	4.53%
p-value [c]	-	<0.01	NS	NS	<0.05	<0.001	NS	<0.05
p-value [d]	-	-	-	-	-	NS	NS	<0.001

Doses indicate mg/kg body weight.

[a]: when compared to untreated controls (group 1) by the Student's t test.

[b]: when compared to imatinib control (i.e. between group 2&6, between group 3&7, and between group 4&8) by the Student's t-test.

[c]: when compared to untreated controls (group 1) by the Chi-square test.

[d]: when compared to imatinib control (i.e. between group 2&6, between group 3&7, and between group 4&8) by the Chi-square test.

Table 3. Percentage of mature oocytes with normal spindle integrity in eight experimental groups

Group	1	2	3	4	5	6	7	8
Cyclophosphamide	-	25	50	75	-	25	50	75
Imatinib	-	-	-	-	7.5	7.5	7.5	7.5
Oocytes examined	32	34	33	31	32	34	30	32
Normal metaphase II spindle	32	27	26	21	32	33	30	30
Abnormal metaphase II spindle	0	2	5	8	0	0	0	2
Normal anaphase II spindle	0	5	2	2	0	1	0	0
Percentage of oocytes with normal metaphase II spindle	100%	79.4%	78.8%	67.7%	100%	97.1%	100%	93.8%
p-value [a]	-	<0.01	<0.01	<0.005	NS	NS	NS	NS
p-value [b]	-	-	-	-	-	0.054	<0.05	<0.05

Doses indicate mg/kg body weight.

[a]: when compared to untreated controls (group 1) by the Chi-square test.

[b]: when compared to imatinib control (i.e. between group 2&6, between group 3&7, and between group 4&8) by the Chi-square test.

DISCUSSION

Our study demonstrated that co-administration of imatinib has a benefit of enhancing oocyte maturity and decreasing the percentage of dead oocytes in relatively low dose Cp-treated mice. Acquisition of mature oocytes is an important first step in fertility preservation. In this context, most clinicians attempt to obtain as many mature and healthy oocytes as possible. It is well-known that the number of oocytes obtained in vitro is reduced if patients receive chemotherapy. This was demonstrated in several mouse studies [8-10] and one human report [12]. In the latter study, a significantly lower oocyte yield (6.5 vs. 14.1) and fewer embryos available for cryopreservation (3.9 vs. 6.8) were observed in 23 female breast cancer patients who underwent ovarian stimulation immediately after chemotherapy than in those who did not [12].

We also observed a significantly lower mature oocyte yield (10/7 vs. 19), 2-cell embryo yield (6/6 vs. 17) and blastocyst yield (3/4 vs. 13) in 25/50 mg/kg Cp-treated mice relative to untreated controls. After co-administration of imatinib, still fewer mature oocytes (11/9), 2-cell embryos (8/7) and blastocysts (5/5) than the untreated controls were obtained. However under the tested Cp dosages, insignificant benefits were observed with the co-administration of imatinib, as +1/+2 more mature 00cytes, +2/+1 more 2-cell embryos and +2/+1 more blastocysts could be obtained, respectively.

Interestingly, after co-administration of imatinib, the percentage of mature oocytes was significantly increased (+16.4%/+10.4%) and the percentage of dead oocytes was significantly decreased (-25.9%/-15.3%) in 25/50 mg/kg Cp-treated mice. This finding indicates that oocyte maturity could be enhanced by the co-administration of imatinib although absolute number of total or mature oocytes was decreased. In addition, we observed that oocyte spindle integrity was rescued by imatinib co-administration.

Although a similar incidence of aneuploid oocytes after in vivo Cp treatment was reported previously [13], we observed that Cp treatment induced spindle abnormalities within oocytes more frequently. Alkylating chemotherapeutic agents work on non-proliferative cells by interfering with cellular DNA function[6]. Cp also damages oocytes and surrounding granulosa cells in a dose-dependent manner[7]. Our findings clearly indicate that Cp treatment could induce meiotic errors or parthenogenesis (evident by emerging anaphase) in oocytes and co-administration of imatinib could conserve the spindle integrity and prevent parthenogenesis. This finding is important because *in vitro*-produced oocytes in the emergency IVF setting will undergo cryopreservation, which can further induce oocyte spindle damage.

A recent report indicates that co-injection of imatinib significantly attenuates the toxic effect of cisplatin on the ovarian follicular reserve and partially rescues female mice from cisplatin-induced infertility[16]. The investigators explained that imatinib

inhibits c-Abl-TAp63 pathway, which can induce oocyte death following chemotherapy. In a mouse model, the TA isoform of p63 is expressed in the oocytes of primordial follicles[20] and this has been reported to be essential in the process of p53-independent DNA damage–induced oocyte death [21].

Gonfloni et al. observed an accumulation of p63 and c-Abl protein levels following cisplatin exposure, which eventually leads to cell death of in vitro cultured ovaries[16]. However, inhibition of c-Abl, by treatment with c-Abl kinase inhibitor imatinib, could prevent p63 accumulation, thus counteracting the depletion of ovarian follicles induced by cisplatin. This indicates that imatinib co-treatment can preserve oocytes from p63-dependent death.

Inspired by this observation, we explored whether imatinib co-injection has a protective effect against short-term Cp treatment on the quantity and quality of *in vitro*-produced oocytes, their fertilization potential, and subsequent embryo development up to blastocyst stage. Although we did not observe any advantage regarding blastocyst formation, co-administration of imatinib has a benefit of enhancing oocyte maturity and decreasing the percentage of dead oocytes in Cp-treated mice. Our observations are in line with the findings by Gonfloni et al., in which oocyte deaths within cultured ovaries could be prevented by imatinib co-treatment[16].

In the present study, no benefit of imatinib co-administration was observed in the

high dose Cp-treated group (i.e. 75 mg/kg). The oocyte yield, FR and the percentage and cell number of blastocysts were markedly decreased and blastocyst apoptosis was significantly increased. Nonetheless, spindle integrity was well-conserved via co-administration of imatinib in this higher Cp dose. This finding suggests that imatinib co-treatment can prevent spindle abnormalities in oocyte but has a negative impact on apoptosis of the resultant blastocyst in this high Cp dose. However, such a high dose of Cp is not currently used in cancer patients. The usual dose of Cp for breast cancer is 600 mg/m^2. If a woman is 161.5 cm in height and 54.7kg in weight, 942 mg of Cp is needed (body surface area 1.57). This means a dose of 17.2 mg/kg would be used.

The currently available strategies for preserving female fertility are promising for women undergoing chemotherapy. However, such strategies do not totally guarantee female fertility preservation. Thus, alternative methods should be developed that offer more protection and preservation of ovarian follicles against chemotherapy. We herein suggest that imatinib could prevent chemotherapeutic agent-induced oocyte death and preserve oocyte spindle integrity.

Recently, a protective effect of amifostine against ovarian damage induced by chemotherapy has been reported[22]. Amifostine is a prodrug that is dephosphorylated in the tissues by alkaline phosphatase to a free thiol active metabolite, which behaves as a potent scavenger of free radicals induced by chemotherapy protocols and ionizing radiation[23]. Barekati et al. reported the cytoprotective effects of

amifostine (250 mg/kg) against the adverse effects of Cp (75 mg/kg) on preantral follicle survival (harvested 24 hours after treatment) of pubertal mice [22]. Three weeks after treatment with amifostine and Cp, mice were superovulated and fertilized in vitro. The fertilization potency was significantly improved compared to mice treated with Cp only (88% vs. 39%).

Although the offspring of women exposed to cancer treatment do not have a greater risk of chromosomal or congenital abnormalities[24,25], the safety of IVF in cancer patients who have already undergone chemotherapy is questionable. Therefore, the genetic risk of embryos fertilized from oocytes recovered after Cp treatment should be evaluated. In a mouse model, it has been reported that aneuploidy of *in vitro* produced embryos was significantly increased in the Cp treatment group (29.2% vs. 8.7%)[10]. In regard to the adverse effects of Cp on chromosomal integrity, pre-implantation genetic screening is needed before transfer of embryos produced from women treated with chemotherapeutic agents.

In the present study, we used a single chemotherapeutic agent (Cp) and a single dose of imatinib (7.5 mg/kg body weight, as like in experiment performed by Gonfloni et al.)[16] with a fixed exposure time (24 hours). Currently, most patients receive a combination of chemotherapeutic agents including platinum-based chemotherapy; therefore, further research is needed to examine the possible protective effects of imatinib against various chemotherapeutic agents. In addition, possible protective effects of various doses of imatinib against a single

chemotherapeutic agent should be evaluated. Finally, to confirm its long-term safety, production of normal pups should be assessed in this model.

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국문 초록

목적: 항암제인 사이클로포스파마이드 (cyclophosphamide, Cp) 처치 마우스에서 이마티닙 병용 투여(imatinib co-administration)가 난자 회수 및 배아 발달에 미치는 영향에 관하여 연구하고자 하였다.

대상 및 방법: 태생 4-5주령 암컷 마우스(총 112마리)를 8개의 group으로 나누어 group 1은 saline 0.1 mg/kg을, group 2, 3, 4는 Cp 25, 50, 75 mg/kg을, group 5는 이마티닙 7.5 mg/kg을, group 6, 7, 8은 Cp 25, 50, 75 mg/kg와 이마티닙 7.5 mg/kg을 각각 주사하였다. 24시간 후 각각의 마우스에 eCG 5 IU을 주사하고 48시간이 경과 후 hCG 5 IU을 주사하고 다시 14시간 후 난자를 채취하였다. 각각의 group에서 회수된 총 난자 개수, 성숙/사멸 난자 개수를 측정하였다. 체외수정을 시도하였으며, 이에 따른 수정률, 배반포형성률을 측정하였다. 배반포세포수와 TUNEL을 이용하여 아포프토시스 세포 분율을 측정하였다. 회수한 성숙난자에서 방추사 건전성을 확인하였다.

결과: 이마티닙을 함께 투여한 group 6, 7에서 Cp만 투여했던 group 2, 3에 비하여 통계적으로 유의한 성숙난자 백분율의 증가(+16.4%, $p<0.001$, +10.4%, $p<0.02$)와 사멸난자 백분율의 감소를 보였다(-

25.9%, $p<0.001$, −15.3%, $p<0.001$). 수정률은 이마티닙을 함께 투여했던 group 7에서 group 3와 비교하여 통계적으로 유의한 증가를 보였다 (+12.2%, $p<0.05$). 정상 방추사 형태를 보이는 난자의 분율은 Cp 처치로 대조군에 비하여 유의하게 감소하였으나 이마티닙 병용 처치군에서는 대조군과 비슷하였다.

결론: Cp를 투여 받은 마우스에서 과배란유도를 시행할 때 난자성숙도와 수정률, 배반포형성률은 유의하게 감소하나 이마티닙의 병용 투여로 난자성숙도와 수정률을 어느 정도 향상시킬 수 있었으며 난자내의 방추사 건전성에도 유익한 결과를 보였다

주요어: **사이클로포스파마이드, 이마티닙, 체외수정, 배반포**

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