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Effects of mTOR inhibitor administration for attenuation of cyclophosphamide-induced ovarian damage in a mouse model

마우스에서 cyclophosphamide 투여로 인한 난소 손상을 완화하기 위한 mTOR 억제제 투여의 효과

2023 년 8 월

서울대학교 대학원

의학과 산부인과학전공

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Abstract

Effects of mTOR inhibitor administration for attenuation of cyclophosphamide-induced ovarian damage in a mouse model

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AIM: To investigate if pre-treatment with everolimus or rapamycin could prevent cyclophosphamide (Cp)-induced ovarian follicle damage in mice.

METHODS: A total of 120 BDF-1 female mice were randomly divided into four groups (30 mice per group) according to specific treatment. In the Control Group, normal saline (0.1 mL) was injected on Day 1 (D1), D3, D5, and D13. In the Cp Group, normal saline (0.1 mL) was injected one D1, D3, and D5 and Cp (75 mg/kg) was injected on D13. In the Everolimus + Cp Group, everolimus (0.75 mg/kg) was injected on D1, D3, and D5 and Cp was injected on D13. In the Rapamycin + Cp Group, rapamycin (4.0 mg/kg) was injected on D1, D3, and D5 and Cp was injected on D13. On D20, all mice were euthanized to recover ovaries. Ovarian samples were processed for ovarian histology. In addition, protein expression levels of

BCL-xL (anti-apoptotic marker), Caspase 3 (apoptotic marker), and mTOR were assessed quantitatively by Western blot analysis.

RESULTS: The number of primordial follicles in the Cp Group was significantly lower than that in the Control Group (median: 8 vs. 22, p = 0.001). The Everolimus + Cp Group (11.5 vs. 22, p = 0.001) and the Rapamycin + Cp Group (8.5 vs. 22, p = 0.003) also showed significantly lower numbers of primordial follicles than the Control Group.

In all four groups, there was no significant difference in the proportion of G1 primordial or G1 secondary follicles. Compared with the Control Group, the Cp Group and the Everolimus + Cp Group showed significantly lower proportions of G1 primary follicles. However, the Rapamycin + Cp group (64.1% ± 3.3%) showed a proportion of G1 primary follicles similar to that in the Control Group (62.5% ± 3.2%). The Cp Group, Everolimus + Cp Group, and Rapamycin + Cp Group showed significantly lower proportions of G1 antral follicles than the Control Group. However, intra-ovarian protein expression levels of BCL-XL, Caspase 3, and mTOR were not significantly different among the four groups.

CONCLUSION: Pretreatment with rapamycin did preserve the proportion of G1 primary follicles. However, pretreatment with everolimus or rapamycin did not preserve the number of primordial follicles.

Keywords: Fertility Preservation, Chemotherapy, Ovary, Everolimus, Rapamycin

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Chapter 1. Introduction

1.1. Study background

The total number of ovarian follicles is determined at birth. Depletion of this pool can lead to reproductive senescence. Recent studies have provided insights into intracellular signaling mechanisms essential for primordial follicle activation from a dormant state [1,2]. During normal follicular development, the ovary is in a state of equilibrium. Activation of primordial follicles is regulated by the phosphatidylinositol-3-kinase (PI3K) / Ak strain transforming (AKT) / mammalian target of rapamycin (mTOR) signaling pathway. In addition, suppressive factors produced by growing follicles ensure that the vast majority of primordial follicles are maintained in a dormant state.

mTOR is a conserved serine/threonine kinase that regulates cell growth and proliferation. The rapamycin-sensitive mTOR-Complex 1 (mTORC1) positively regulates cell growth and proliferation by promoting the biosynthesis of proteins, lipids, and organelles and by limiting catabolic processes such as autophagy. Suppression of mTORC1 activity in oocytes appears to be a prerequisite for maintaining dormancy of primordial follicles based on extensive studies using mice [3,4]. The essential and coordinated roles of AKT and mTORC1 signaling pathways in regulating primordial follicle dormancy and preserving the length of female reproductive life have been demonstrated [5]. Therefore, any agents that can cause aberrant activation of the PI3K/AKT/mTOR signaling pathway can induce follicular depletion and primary ovarian insufficiency (POI).

Alkylating chemotherapeutic agents, particularly cyclophosphamide (Cp), are gonadotoxic that can lead to POI [6–9]. Treatment with Cp can disturb the balance, activate follicles by up-regulating the PI3K/AKT/mTOR signaling pathway, and cause growing follicles to undergo apoptosis, thereby reducing the secretion of inhibitory factors [10]. As a consequence, more primordial follicles are recruited. They develop and die, eventually leading to loss of ovarian function and POI [11,12].

To minimize ovarian toxicity of anticancer drugs, various drugs (i.e., fertoprotective agents) have been researched. However, they have not shown clear effects yet. Table 1 summarizes previous studies investigating effects of fertoprotective agents.

Table 1. Previous studies investigating the effect of fertoprotective agents

Mechanism of action	Fertoprotective agents	Studies
Prevention of follicle activation	Ossirene (AS101)	Kalich-Philosoph 2013
	AMH	Roness 2015
	Melatonin and ghrelin	Jang 2017
	Everolimus	Goldman 2017
	Rapamycin	Chen 2022
Anti-apoptosis	Imatinib	Gonfloni 2009, Morgan 2013, Kim 2013
		Chun 2014, Kim 2022
	S1P	Morita 2000, Jurisicova 2006, Hancke 2007, Li 2014
Vascular effects	G-CSF	Skaznik-Wikiel 2013
		Akdemir 2014
Transport block	Bortezomib	Roti Roti 2014
Upregulation of MDR1	MDR1	Salih 2011
Nano-encapsulation	As2O3-loaded Nanobins	Ahn 2013
Scavenger of free radicals	Amifostine	Barekati 2012
Antioxidants	Resveratrol	Atli 2017

In theory, inhibiting the PI3K–AKT–mTOR signaling pathway could induce follicle dormancy, thereby reducing apoptosis, enhancing autophagy, and improving follicle survival in cyclophosphamide–treated patients.

Rapamycin is a macrolide produced by bacteria Streptomyces hygroscopicus. It can induce autophagy in mammalian cells as well as in Saccharomyces cerevisiae and Drosophila melanogaster [13,14]. It can act as a universal inhibitor of mTORC1 and a cell–type–dependent inhibitor of mTORC2 [15]. Rapamycin is already used to prevent transplant rejection. Some studies have shown that rapamycin can increase the phosphorylation of glycogen synthase in skeletal muscles [16]. These findings represent a novel treatment for glycogen storage disease that involves glycogen accumulation in muscle.

Everolimus is a rapamycin–derived macrolide antibiotic with immunosuppressive properties. It has a high affinity for intracellular receptor FKBP12 to form a complex that can act as an mTOR inhibitor. Everolimus is used to treat patients with renal cell carcinoma. It has been studied as a targeted therapy in various types of malignancies, including pancreatic cancer, breast cancer, and ovarian cancer [17, 18, 19].

These mTOR inhibitors are thought to impair tumor angiogenesis and cause impairment of the G1/S transition. Recently, they have been studied for treating/preventing several age–associated conditions, including neurodegenerative diseases [20].

So far, few animal studies have assessed protective effects of those mTOR inhibitors in alkylating chemotherapeutic agent–induced ovarian damage. To the best of our knowledge, only a few studies have evaluated the effectiveness of everolimus or rapamycin among mTOR inhibitors [21, 22]. In these studies,

everolimus or rapamycin could maintain ovarian follicles in their primordial state, maintain normal serum AMH levels, and preserve normal fertility. Although these studies showed that treatment with everolimus or rapamycin could protect ovaries from anti-cancer drug toxicity, further experiments are needed to assess the extent of their protective effects. In addition, no studies have shown the degree of protection compared to other agents.

1.2. Purpose of research

The purpose of this research was to investigate whether pre-treatment with everolimus or rapamycin could protect against ovarian follicle damage in Cp-induced mice. This is the first study to compare effects of these two mTOR inhibitor agents.

Chapter 2. Materials and methods

2.1. Study animals

This study was approved by the Institutional Animal Care and Use Committee (IACUC) of Seoul National University Bundang Hospital (IACUC number BA-2108-325-081-01). All animal care and use during the experiment followed institutional guidelines established by the IACUC.

Six-week-old BDF-1 female mice (Orient Bio., Seongnam, Republic of Korea) were housed under a 12-hour light/dark cycle and temperature-controlled (22℃) conditions. They were provided food and water ad libitum [23,24]. We observed weight changes, appearance changes, and behavior changes two to three times a day. Euthanasia was considered when body weight decreased by more than 20%, or when there was no response or movement to a stimulus. All mice were killed by cervical dislocation.

2.2. Experimental design

After one week of adaptation, a total of 120 BDF-1 female mice were randomly divided into four groups (30 mice per group) according to a specific treatment.

For the Cp group, 75 mg/kg of Cp monohydrate (Cat. no. 29875, Sigma-Aldrich, St. Louis, MO, USA) was used.

Everolimus (LC Laboratories, New Boston Street Woburn, MA, USA) was used at a dose of 0.75 mg/kg.

Rapamycin (LC Laboratories, New Boston Street Woburn, MA, USA) was used at a dose of 4.0 mg/kg.

The experimental design is illustrated in Table 2:

In the Control Group, normal saline (0.1 mL) was injected on D1, D3, D5, and D13.

In the Cp Group, normal saline (0.1 mL) was injected on D1, D3, and D5 and Cp was injected on D13.

In the Everolimus + Cp Group, everolimus was injected on D1, D3, and D5 and Cp was injected on D13.

In the Rapamycin + Cp Group, rapamycin was injected on D1, D3, and D5 and Cp was injected on D13.

On D20, all mice were euthanized and ovaries were recovered. Part of the ovary was processed for ovarian histology. The remaining ovaries were used to assess protein expression levels of BCL-xL (anti-apoptotic marker), Caspase 3 (apoptotic marker), and mTOR quantitatively by western blot

analysis. Regarding the internal control, α-tubulin was used.

Table 2. Experimental design

Time schedule	Control	Cp	Everolimus + Cp	Rapamycin + Cp
D1	normal saline	normal saline	Everolimus	Rapamycin
D3	normal saline	normal saline	Everolimus	Rapamycin
D5	normal saline	normal saline	Everolimus	Rapamycin
D13	normal saline	Cp	Cp	Cp
D20	Ovariectomy	Ovariectomy	Ovariectomy	Ovariectomy

Cp, cyclophosphamide.

2.3. Histological analysis and follicle counting

Histological analysis was performed as mentioned in a previous study from our team [25]. Ovarian tissue was fixed with 4% buffered paraformaldehyde for one day followed by paraffin embedding. Paraffin-embedded tissue was serially sectioned at 4-㎛ in thickness. Slides were stained with hematoxylin and eosin (Merck, Darmstadt, Germany) and analyzed for follicle counting. Every section was examined under a light microscope at least twice by a single experienced inspector (B.Y. Choi). The average number of follicles was used as results. Only follicles with a visible nucleus in the oocyte were counted.

Each follicle type was classified into the following categories [26]:

1) primordial, a single layer of flattened pre-granulosa cells;

2) primary, a single layer of granulosa cells, 1 or more being cuboidal cells;

3) secondary, 2 or more layers of cuboidal granulosa cells, with the antrum absent; and

4) antral, multiple layers of cuboidal granulosa cells, with the antrum present.

Each follicle was evaluated for its integrity according to the following criteria as mentioned in a previous study [27]:

1) G1 (good quality) follicle, intact spherical follicle and oocyte

2) G2 (fair quality) follicle, granulosa cells pulled away from the edge of follicles, but with an intact oocyte

3) G3 (poor quality) follicle, disruption and/or loss of granulosa-theca cells, with pyknotic nuclei and/or a missing oocyte.

Representative histological images of ovarian follicles are shown in Figure 1.

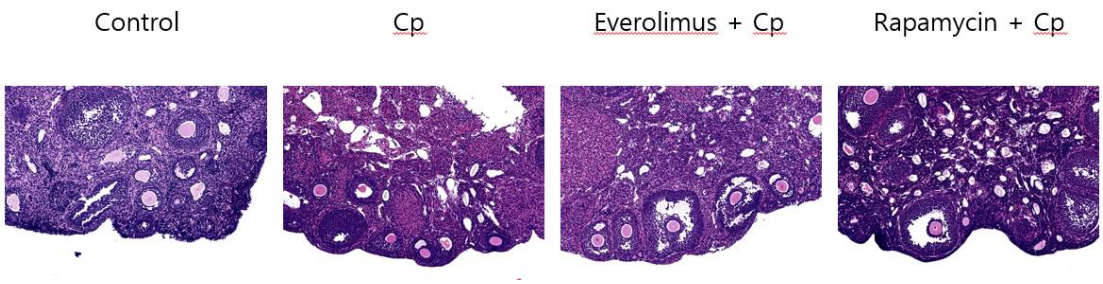

Figure 1. Representative histologic images of mouse ovarian follicles according to each treatment group (Mayer' s H&E, × 100). Control, 0.1 mL of normal saline; Cp, cyclophosphamide 75 mg/kg; Everolimus 0.75 mg/kg; Rapamycin 4.0 mg/kg.

2.4. Western blotting

Western analysis was performed by collecting samples from five mice and repeating the measurement six times for each marker. Ovaries were washed with PBS and lysed with cell lysis buffer (20 mM Tris-HCl at a pH of 8.0, 137 mM NaCl, 1% Nonidet P-40, and 10% glycerol) supplemented with protease inhibitors (0.5 mM PMSF, 0.025 mM N-CBZ-L-phenylalanine chloromethyl ketone, 0.025 mM N-p-tosyl-lysine chloromethyl ketone, and 0.025 mM L-1-tosylamide-2-phenyl-ethylchloromethyl ketone) on ice for 20 min. After centrifugation at 10,000 x g for 15 min at 4℃, supernatants were harvested and protein concentrations were determined by BCA protein assay kit (Thermo Scientific Pierce, Rockford, IL, USA). Each sample at 20 ㎍/㎕ was then resolved and transferred to a PVDF membrane (Millipore, Billerica, MA, USA). Membranes were blocked and incubated overnight at 4℃ with appropriate primary antibodies: BCL-xL (1:100, sc-271121; Santa Cruz Biotechnology), cleaved caspase-3 (1:500, 5a1e; Cell Signaling Technology, Danvers, MA, USA), and mTOR (1:500, orb99435; Biorbyt Ltd, Cambridge, United Kingdom). BCL-xL is an anti-apoptotic marker. Caspase-3 is a well-known marker of late apoptosis.

After washing with Tris Buffered Saline with Tween (TBST) for 15 min three times, membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies at room temperature for 1 hr. After washing with TBST, membranes were developed using an ECL Prime Western Blotting detection reagent (GE Healthcare). Representative bands from Western blot analysis are presented in Figure 2.

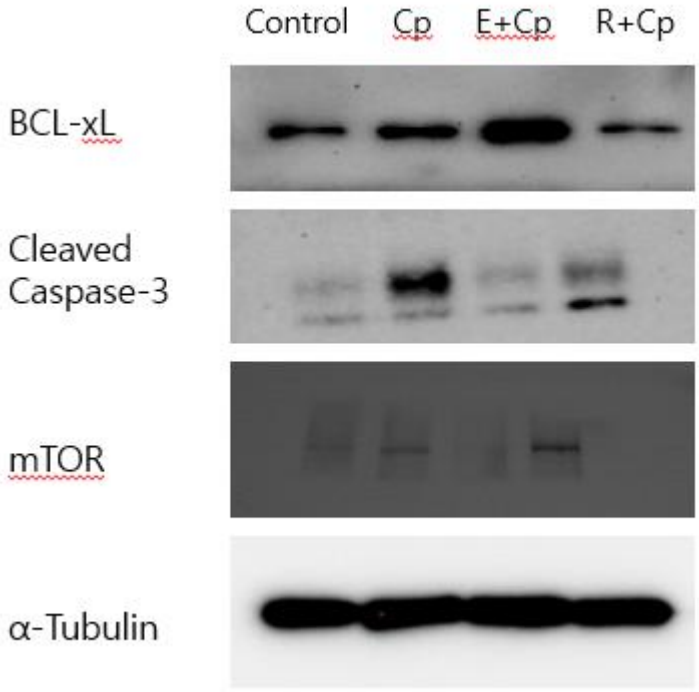

Figure 2. The expression levels of three proteins within the whole ovaries as determined by western blot. Control, 0.1 mL of normal saline; Cp, cyclophosphamide 75 mg/kg; E, everolimus 0.75 mg/kg; R, rapamycin 4.0 mg/kg.

2.5. Statistical analysis

All statistical analyses were performed using R software version 2.14.2 (http://www.rproject.org). Ovarian follicle counts were compared using a one-way analysis of variance (ANOVA) followed by the Tukey multiple-comparison test. All samples were tested for the normality of data distribution before ANOVA. Protein levels were compared using the Kruskal-Wallis test followed by the Mann-Whitney U-test. A p-value < 0.05 indicated statistical significance.

Chapter 3. Results

3.1. Histological analysis of follicles

Detailed ovarian follicle counts in four groups are presented in Table 3. The median number of primordial follicles was significantly lower in the Cp Group than in the Control Group (8 vs. 22, p = 0.001). The Everolimus + Cp Group (11.5 vs. 22, p = 0.001) and Rapamycin + Cp Group (8.5 vs. 22, p = 0.003) also showed significantly lower numbers of primordial follicles than the Control Group. There were no significant differences in the number of primordial follicles among Cp Group, Everolimus + Cp Group, and Rapamycin + Cp Group. Among the four groups, numbers of primary, secondary, and antral follicles were similar.

Table 3. Ovarian subtype follicle counts

Group	Control	Cp	Everolimus + Cp	Rapamycin + Cp	*p*− value
Primordial	22 [13−42]^{a}	8 [4.5−15.5]^{b}	11.5 [3.7−14.7]^{b}	8.5 [4.7−21.0]^{b}	0.002
Primary	19 [16−28]	23 [14−27]	22 [18−26.2]	17 [15−24.7]	0.607
Secondary	40 [31−45]	35.5 [27.7−44.2]	36 [30.7−42.0]	38.5 [32.2−42.0]	0.649
Antral	13 [9−16]	14 [12.7−17.5]	14.5 [12.7−19]	12.5 [8.5−15.5]	0.119

Kruskal−Wallis test (a,b; different superscripts mean a statistical significance within the same row).

Values are expressed as median [IQR].

Cp, cyclophosphamide.

Proportions of G1 follicles in the four groups are presented in Figure 3. There was no significant difference in the proportion of G1 primordial or G1 secondary follicles among the four groups,

The Cp Group and the Everolimus + Cp Group had significantly lower proportions of G1 primary follicles than the Control Group. However, the proportion of G1 primary follicles in the Rapamycin + Cp group was well preserved at a level similar to that in the Control Group.

Proportions of G1 antral follicles were significantly lower in the Cp Group, Everolimus + Cp Group, and the Rapamycin + Cp Group than in the Control Group.

To summarize findings of the ovarian subtype follicle counts and proportions of G1 follicles, pretreatment with everolimus or rapamycin did not preserve the number of primordial follicles. On the other hand, pretreatment with rapamycin did preserve the number of G1 primary follicles.

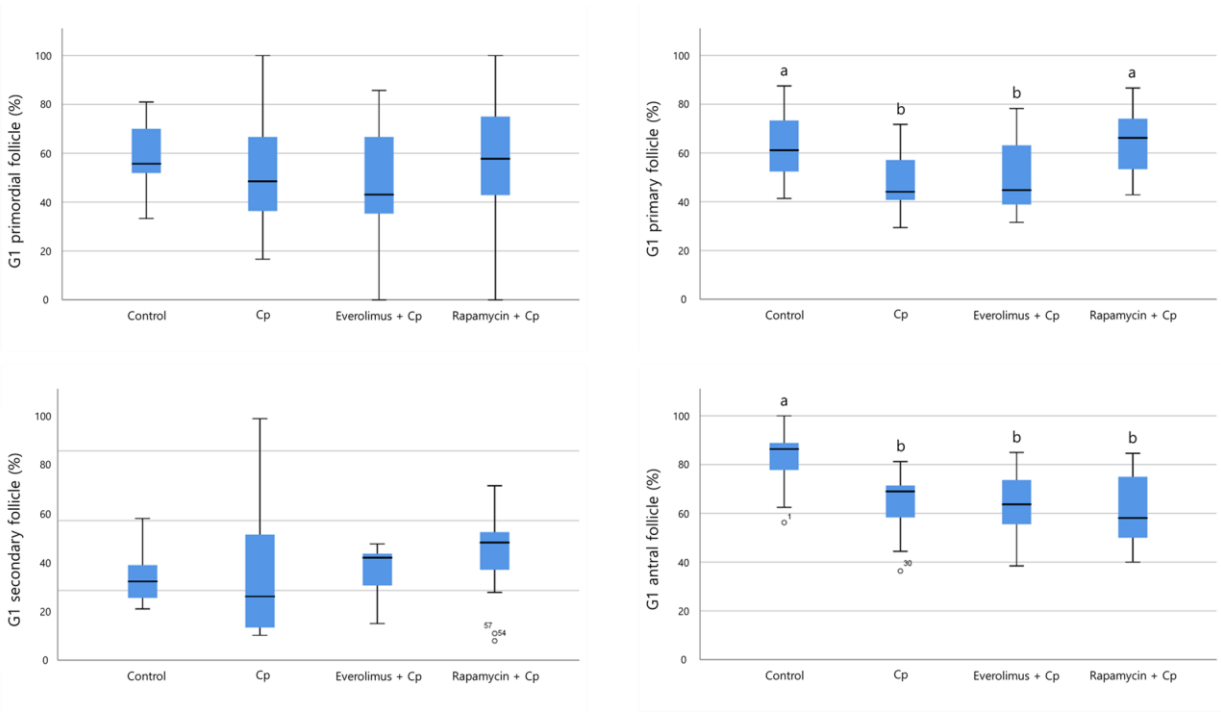

Figure 3. The proportion of good-quality (G1) subtype follicles in the four groups.

a,b; different superscripts mean a statistical significance

Cp, cyclophosphamide

3.2. Western blot analysis to assess apoptosis and mTOR expression

Expression levels of BCL-xL, cleaved Caspase 3, and mTOR in the four groups are depicted in Figure 4. Expression levels of these three proteins (BCL-xL, cleaved Caspase 3, and mTOR) were not significantly different among the four groups.

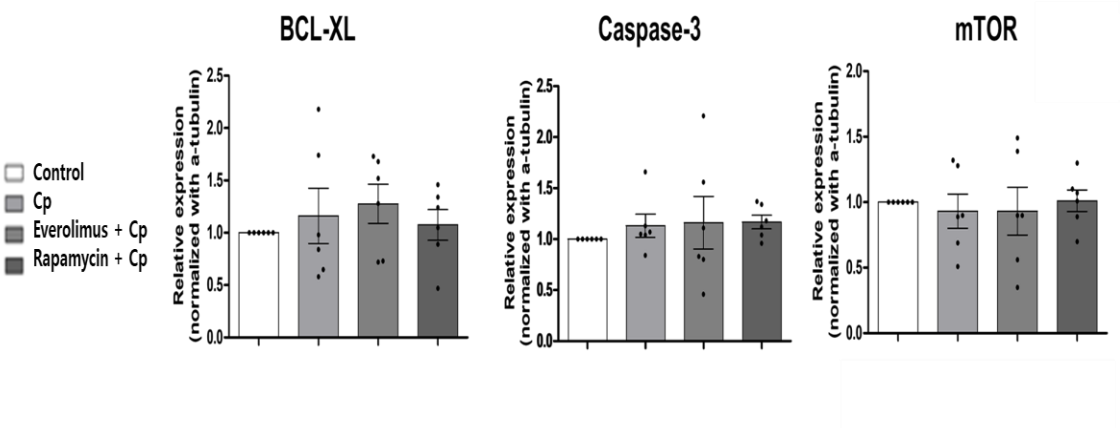

Figure 4. Western blot analysis to assess apoptosis and mTOR expression

Chapter 4. Discussion

Fertoprotective effects of mTOR inhibitors

Rapamycin pretreatment before Cp administration was effective in preserving the proportion of G1 primary follicles. However, according to research results so far, it is difficult to conclude that there is a sufficient fertoprotective effect of rapamycin in attenuating Cp-induced follicular damage.

As a result of this study, premedication with everolimus or rapamycin prior to Cp administration was not effective in preserving the number of primordial follicles or the proportion of G1 primordial follicles.

Mechanism of chemotherapy-induced ovarian damage

The most recent theory of chemotherapy-induced ovotoxic effects is called a “burn-out theory”. Chemotherapy agents such as Cp can trigger an initiation of dormant follicle growth and speed up depletion of the follicle pool. While the "burn-out theory" has been widely accepted and supported by a growing body of evidence, it is important to note that mechanisms behind ovarian follicle pool depletion and its relationship with infertility and early menopause are not fully understood yet.

In the present study, Cp treatment decreased the number of primordial follicles without affecting primary, secondary, or antral follicle count, consistent with our previous study [28]. Based on our studies, it can be inferred that Cp could specifically target primordial follicles by inducing apoptotic cell death rather than depleting the primordial follicle reserve

through activation or destruction of growing follicles.

Follicle activation is not the sole mechanism underlying chemotherapy-induced ovarian damage. Chemotherapy can also induce apoptosis or oxidative stress. It is crucial to acknowledge that complete understanding of the mechanisms responsible for chemotherapy-induced ovarian damage remains elusive. Additional research is necessary to elucidate why certain women experience more pronounced impacts on their ovarian function than others.

Dosage regimen of Cp

The specific dosage of Cp used to establish a mouse model of ovarian dysfunction can vary depending on the specific study and research objectives. However, a commonly used dosage range for Cp-induced ovarian dysfunction in mice is typically between 75 and 150 mg/kg. Within this range, 75 mg/kg is considered as a conventional dose and 150 mg/kg is considered as a sterilizing dose [21].

In our previous study [28], administration of Cp at 50 mg/kg did not result in a reduction in the number of primordial follicles. However, a single dose of Cp at 75 mg/kg significantly reduced the number of primordial follicles. Therefore, we selected 75 mg/kg, the lowest dose capable of achieving the desired effect, and applied it to this experiment.

Dosage regimen of everolimus

Certican® is indicated for the prophylaxis of organ rejection in adult patients who have undergone renal, cardiac, or hepatic transplantation. Certican® tablets contain 0.25 mg, 0.5 mg, 0.75 mg, or 1.0 mg everolimus. The dosage of

everolimus for mice can vary depending on the specific study and the objective of the experiment.

In a previous study [21], mice were treated with 75 mg/kg Cp intraperitoneally on a weekly basis for 3 weeks. Throughout this treatment, everolimus was co-administered at a dose of 2.5 mg/kg daily via oral gavage. In that study, mice treated with a combination of Cp and everolimus exhibited twice the number of primordial follicles compared to those treated with Cp alone. Everolimus demonstrated an ability to preserve varian reserve, serum anti-Mullerian hormone (AMH) levels, and fertility.

In ovariectomized mice, intraperitoneal injections of 1 mg/kg/day everolimus for a duration of 4 weeks effectively prevented observed bone loss [29]. In a study evaluating the role of everolimus in preventing the progression from ductal carcinoma in situ to invasive ductal carcinoma, mice were orally administered everolimus at a dosage of 0.75 mg/kg/day [30].

In the current study, everolimus was administered at a dose of 0.75 mg/kg. Everolimus was used to treat mice on days 1, 3, and 5, while Cp was injected on day 13. We attempted to achieve a protective effect using the minimum dose. The dose and duration of administration might be too short.

Dosage regimen of rapamycin

In a previous study [31], 5 mg/kg rapamycin daily intraperitoneal injection for 19 days effectively prevented global follicular activation and preserved the ovarian reserve of PTEN (phosphatase and tensin homolog deleted on chromosome ten)-mutant mice.

In another study [22], mice received intraperitoneal injections of different

doses (5, 10, or 20 mg/kg) of rapamycin daily for 15 or 30 days. The 5 mg/kg rapamycin exposure demonstrated notable effects on folliculogenesis without obvious side effects.

In studies regarding rapamycin-mediated mouse lifespan extension, the dose was in the range of 2 – 4 mg/kg [32, 33].

In the present study, a dosage of 4 mg/kg of rapamycin was chosen and injected on days 1, 3, and 5, while Cp was injected on day 13. It should be noted that due to small doses administered over a short duration, the anticipated protective effect might not have been achieved.

Western blot analysis

Ovarian protein levels of BCL-xL (anti-apoptotic), Caspase 3 (apoptotic), and mTOR were similar in all four groups.

Similar expression of BCL-XL between Control Group and Cp Group was consistent with our previous study [28]. Taken together, BCL-xL might be not involved in the apoptotic mechanism induced by Cp.

Caspase-3 is a major downstream effector enzyme in the late apoptosis process. It has been reported that Cp can induce increased levels of caspase-3 [28, 34]. However, in the present study, protein expression level of Caspase 3 was not increased in the Cp group. It is essential to evaluate and consider various experimental factors that could have contributed to the discrepancy between our results and previous reports. These considerations may include differences in Cp concentration, treatment duration, and techniques used for protein analysis. In addition, apoptosis is a tightly regulated process characterized by distinct temporal stages. Caspase-3

activation occurs during the late phase of apoptosis, leading to cleavage of numerous cellular substrates. It was plausible that our study captured an earlier time point or a different phase of apoptosis, where caspase-3 activation had not yet occurred or had already subsided. Further investigation using time-course experiments or examining additional markers of apoptosis at different time points might shed light on the dynamic nature of caspase-3 activation.

If mTOR inhibitors everolimus or rapamycin premedication prevented Cp-induced follicular damage, ovarian mTOR levels were expected to increase. However, the protein expression level of mTOR was not changed after treatment, consistent with a previous study [35]. In this study, ovaries of Cp-treated mice showed increased phosphorylation of mTOR without affecting total mTOR level.

Limitations

This study has several limitations that should be acknowledged. Firstly, we utilized a single fixed dose of everolimus and rapamycin with limited exposure period. It is worth considering co-administration of mTOR inhibitors during administration of anticancer drugs rather than solely as a pre-treatment measure. Further investigations are warranted to assess effects of different durations and doses of everolimus and rapamycin. Secondly, the number of markers available for evaluating the underlying mechanism of drug effects was limited. It would have been beneficial to assess phosphorylation of various proteins associated with the PI3K/AKT/mTOR signaling pathway as this could have strengthened the data and provided a more comprehensive understanding

of our findings.

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국문 초록

목적: 본 연구에서는 시클로포스파미드를 투여하기 전 mTOR 억제제인 에베로리무스 또는 라파마이신 처리가 난소 난포 손상을 보호할 수 있는지 평가하고자 하였다.

방법: 총 120마리의 BDF-1 마우스를 무작위로 4개 그룹으로 나누었다 (각 그룹 30마리). 대조군은 생리식염수 0.1 mL를 D1/D3/D5/D13에 주사하였다. 시클로포스파미드 군은 생리식염수 0.1 mL를 D1/D3/D5에, 그리고 시클로포스파미드 75 mg/kg를 D13에 투여하였다. 에베로리무스 + 시클로포스파미드 군은 에베로리무스 0.75 mg/kg를 D1/D3/D5에, 그리고 시클로포스파미드를 D13에 투여하였다. 라파마이신 + 시클로포스파미드 군은 라파마이신 4.0 mg/kg을 D1/D3/D5에, 그리고 시클로포스파미드를 D13에 투여하였다. 모든 마우스는 D20에 안락사하여 난소를 적출하였고, 난소 난포수를 확인하기 위하여 H&E 염색을 시행하였다. 일부 난소는 BCL-XL (항세포사멸 표지자), Caspase 3 (세포사멸 표지자) 및 mTOR 단백질 발현을 보고자 웨스턴 (western blot) 정량적 분석을 시행하였다.

결과: 시클로포스파미드 군은 대조군에 비하여 원시난포수가 유의하게 낮았다 (8 대 22, p = 0.001). 에베로리무스 + 시클로포스파미드 군 (11.5 vs. 22, p=0.001), 라파마이신 + 시클로포스파미드 군에서도 (8.5 vs. 22, p=0.003) 대조군에 비하여 원시난포수가 유의하게 낮았다. 원시난포수는 시클로포스파미드 군, 에베로리무스 + 시클로포스파미드 군, 라파마이신 + 시클로포스파미드 군에서 차이가 없었다. 일차난포수, 이차난포수 및 동난포수는 네 군에서 차이가 없었다.

원시난포의 G1 비율과 이차난포의 G1 비율은 네 군에서 차이가 없었다.

일차난포의 G1 비율은 시클로포스파미드 군 및 에베로리무스 + 시클로포스파미드 군에서 대조군에 비해 감소하였으나 라파마이신 + 시클로포스파미드 군에서는

대조군과 비슷한 수치를 보여 일차난포의 G1 비율이 잘 유지되었다.

동난포의 G1 비율은 시클로포스파미드 군, 에베로리무스 + 시클로포스파미드 군 및 라파마이신 + 시클로포스파미드 군 모두 대조군에 비해 감소하였다.

난소내 BCL-XL, Caspase 3 및 mTOR 단백질 발현 수준은 네 군에서 차이가 없었다.

결론: 에베로리무스와 라파마이신 전처치는 원시난포수 보존에 효과를 보이지는 않았지만 라파마이신 전처치는 일차난포의 G1 비율 보존에 효과적이었다.

주요어: 가임력 보존, 항암치료, 난소, 에베로리무스, 라파마이신

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