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Temsirolimus and anti-Müllerian hormone protect fertility and the ovarian reserve against chemotherapy-induced damage in a murine model

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Running title: Temsirolimus and AMH protect fertility

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Lara Houeis: Conceptualization, data analysis, investigation and writing of the original draft; Graziella van der Plancke: Data analysis and writing of the original draft; Luciana Cacciottola: Review and editing; Apolline Goudmaeker: Data curation; Candice Kluyskens: Data curation; Margherita Zipponi: Data curation; Jean Paul Dehoux: Data curation; Jacques Donnez: Review and editing; Marie-Madeleine Dolmans: Conceptualization, supervision, project administration, review and editing.

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Capsule: Our study shows that administration of temsirolimus and recombinant anti-Müllerian hormone concomitant with chemotherapy effectively protects fertility and the ovarian reserve in an in vivo murine model.

Keywords: Ovarian reserve, fertility, chemotherapy, temsirolimus, AMH, gonadoprotection.

Abstract

Objective: To investigate the effects of temsirolimus and recombinant anti-Müllerian hormone (AMH) on fertility and the ovarian reserve when associated with chemotherapy.

Design: Thirty-seven female mice were assigned to one of three treatment groups: (i) control group; (ii) chemotherapy group; and (iii) gonadoprotection group. Five weeks after treatment, the mice underwent ovarian stimulation and were mated with males to evaluate residual fertility. Two days after mating, the animals were euthanized for embryo collection, and ovaries were retrieved to assess the ovarian reserve and follicle damage.

Setting: Academic research center.

Subject: Thirty-seven Naval Medical Research Institute mice.

Exposure: The control group (n=10) was given a 100 µL injection of phosphate-buffered saline. The chemotherapy group (n=13) received a single 100 µL injection of chemotherapy (120 mg/kg cyclophosphamide and 12 mg/kg busulfan). The gonadoprotection group (n=14) was administered five injections of 5mg/kg temsirolimus over one week prior to chemotherapy, followed by a chemotherapy injection, then five injections of 5µg recombinant AMH over the next 24 hours.

Main outcome measures: Embryo count, follicle count and morphology, and immunostaining for apoptosis (cleaved caspase-3), follicle activation (phospho-Akt), and proliferation (Ki67).

Results: The control group showed the highest mean number of retrieved embryos (41.40 ± 14.74), closely followed by the gonadoprotection group (36.27 ± 17.22), while the chemotherapy group exhibited a significant reduction (20.63 ± 12.12) compared to both groups.

Regarding the ovarian reserve, the control group showed the highest total follicle count (897.4 ± 392.8), with no difference compared to the gonadoprotection group (714.4 ± 283.9), but there was a significant decline in the chemotherapy group (320.7 ± 145.5) compared to both groups. In terms of follicle apoptosis and activation, no significant difference was observed between groups. Follicle proliferation rates were significantly higher in the chemotherapy group ($59.38\% \pm 14.67\%$) than in the control group ($20.72\% \pm 10.52\%$), but gonadoprotective treatment did curtail follicle proliferation ($42.23\% \pm 18.37\%$) compared to the chemotherapy group.

Conclusion: Our findings suggest that temsirolimus and AMH show promise as gonadoprotective agents, potentially protecting the ovarian reserve against chemotherapy and thereby preserving fertility. This could offer patients who cannot benefit from currently available techniques brand new fertility preservation options.

Introduction

Chemotherapy-induced gonadotoxicity in children or women of reproductive age is a serious concern for cancer survivors. Indeed, while the 5-year survival rate for childhood cancer is currently over 85% (1), pregnancy rates among cancer survivors are approximately 40% lower than in the general population (2). Since the 1970s, there has been increasing evidence of the gonadotoxic effects of chemotherapy on the female reproductive system (3). Chemotherapeutic regimens can be classified into: (i) alkylating agents (like cyclophosphamide [Cy] and busulfan [Bu]); (ii) alkylating platinum-based complexes; (iii) taxanes; (iv) topoisomerase inhibitors; and (v) vinca alkaloids (4). Of those, alkylating agents are known to be the most gonadotoxic, and Cy the most notorious (3). This prodrug undergoes biotransformation in the liver by cytochrome P450 enzymes and is converted to its active form, phosphoramidate mustard, which alkylates DNA by covalent binding, leading to formation of DNA adducts and preventing DNA replication (5). The gonadotoxic dose of alkylating agents is determined by the Cy equivalent dose (CED) of 6,000-8,000 mg/m², a level at which toxicity is likely to occur (6).

Mechanisms by which chemotherapy depletes the ovarian reserve and ultimately impairs fertility in exposed women are well established and multifaceted (4). Chemotherapy triggers apoptosis in oocytes directly by initiating DNA double-strand breaks (DSBs) (7), while its indirect effects on the ovarian reserve happen through two distinct regulatory pathways. First, the cytotoxic impacts of chemotherapy target actively dividing cells, resulting in destruction of granulosa cells (GCs) in growing follicles (7,8). Apoptosis of growing follicles then leads to a drop in GC-derived paracrine growth factor secretion, such as anti-Müllerian hormone (AMH) (8). Since AMH is an inhibitor of primordial follicle recruitment, a downturn in its circulating levels causes excessive activation and depletion of these follicles (8). Second, chemotherapy triggers activation of quiescent follicles by upregulating the intrinsic regulatory pathway, namely PI3K signaling (7). Through these two mechanisms, chemotherapy triggers massive primordial follicle recruitment, also known as ‘burn-out’ (9). Moreover, the cytotoxicity of the treatment causes vascular damage and subsequent fibrosis in the ovarian stroma, making the local environment inhospitable for follicle development and survival (10).

This is why fertility preservation methods like oocyte cryopreservation or ovarian tissue cryopreservation are proposed to patients set to undergo gonadotoxic chemotherapy (11). However, some patients cannot benefit from either of these techniques, including those who have no time for ovarian stimulation, which rules out oocyte cryopreservation, those who live in places where the technique of ovarian tissue cryopreservation and transplantation is not

available, and those running the risk of malignant disease reintroduction (12–15). Our new approach therefore involves fertility protection, or gonadoprotection, against chemotherapy-induced damage. Considering the effects of alkylating agents on follicle overactivation and subsequent burn-out of the ovarian reserve, recent studies have sought to inhibit both activation pathways, through use of recombinant AMH (rAMH) and/or mTOR inhibitors, such as rapamycin and temsirolimus (Tem) (16–20). Indeed, Tem and other mTOR inhibitors act upon the PI3K pathway, while AMH, secreted by GCs, curbs primordial follicle activation by a mechanism that still remains elusive (7).

An approach applying dual suppression (AMH and mTOR inhibitor) tested in vitro on human ovarian tissue (17) and in vivo in a murine model (16) has demonstrated a protective effect of this therapy on the ovarian reserve. It was evaluated up to three weeks post-treatment in an in vivo mouse model. However, to our knowledge, no study has yet tested the impact of the dual inhibition approach on fertility or assessed ovarian markers like follicle count, activation and apoptosis rates at 5-8 weeks post-treatment. The goal of the current study was therefore to investigate the efficacy of concomitant in vivo administration of Tem, rAMH and alkylating agents to mice. Residual fertility was determined by the number of embryos retrieved, and depletion of the ovarian reserve and follicle-related chemotherapy-induced ovarian damage were evaluated 5 and 8 weeks post-treatment.

Material and methods

Study design and outcome measurement

To assess the efficacy of these novel gonadoprotective approaches, 37 female Naval Medical Research Institute (NMRI) mice were randomly assigned to one of three treatment groups: (i) control group (phosphate-buffered saline [PBS]) (n=10); (ii) chemotherapy group (Bu+Cy) (n=13); and (iii) gonadoprotection group (Bu+Cy+Tem+rAMH) (n=14). Five weeks after treatment, all the mice underwent controlled ovarian stimulation (COS) and were mated with fertile males to appraise residual fertility. If no vaginal plug was observed, a second round of COS was performed 3 weeks later, namely 8 weeks after treatment. After mating, the animals were euthanized for embryo collection and recovery of ovaries. Residual fertility was ascertained based on the total number of live day-2 embryos retrieved from pregnant mice. The ovarian reserve was determined by follicle counts after hematoxylin and eosin (H&E) staining,

while immunohistochemistry (IHC) was performed to investigate follicle apoptosis (cleaved caspase-3), and primordial follicle activation (p-Akt) and proliferation (Ki67).

Mice

Ethics approval

Animal welfare was fully respected and complied with all guidelines endorsed by the Committee on Animal Research of the Université Catholique de Louvain. All experiments were reviewed and approved by the Ethics Committee of the Université Catholique de Louvain under reference 2024/UCL/MD/18.

Mouse origin and housing conditions

Thirty-seven 8-week-old female NMRI mice and 37 8-week-old male NMRI mice (Charles River Laboratories, France) were used in this study. They were housed in groups of four per wire cage and maintained under standard laboratory conditions (12 hours light, 12 hours dark; 21°C; 40-60% relative humidity; food and deionized water ad libitum). They were acclimated for 7 days before the experiment.

Treatment protocols for experimental groups

(i) *Control group: vehicle*

Ten female NMRI mice were injected subcutaneously with 60 μ L PBS (10010-023, Gibco) and 40 μ L dimethyl sulfoxide (DMSO; WAK-DMSO-50, WAK-Chemie Medical).

(ii) *Chemotherapy group: Bu and Cy*

A total of 13 female NMRI mice were given a single intraperitoneal injection of 12 mg/kg Bu (728981, Pierre Fabre) and 120 mg/kg Cy (HA-65-01-826, Baxter), both resuspended in DMSO and diluted in PBS to a final volume of 100 μ L (21). Sixty percent of the final volume was PBS, while the rest was DMSO with Cy and Bu.

(iii) *Gonadoprotection group: Tem and AMH (Figure 1.)*

- Tem injection

Fourteen NMRI mice were given 5 subcutaneous injections of 100 μ L Tem (5 mg/kg) over the course of one week, administered on days -7, -5, -3, -1 and 0 relative to chemotherapy (16). Indeed, as mTOR inhibitor steady-state concentrations are reached within 5-7 days of dosing, this timing ensures maximal mTOR pathway inhibition during chemotherapy exposure (22,23).

Each injection contained 150 µg Tem (100 mg/mL, FR61494, Biosynth) diluted in 1.5 µL DMSO to achieve a dose of 5 mg/kg per mouse, based on average mouse weight of 30 grams. To ensure adequate dissolution of hydrophobic Tem, non-ionic surfactants with amphiphilic properties were added: 5 µL polysorbate 80 (Tween 80, 24A17, Fagron) and 5 µL polyethylene glycol 400 (PEG 400, 8074850050, Merck) (24). The final preparation volume was adjusted to 100 µL by adding 88.5 µL PBS.

- Chemotherapy injection

Mice in the gonadoprotection group were subjected to the same protocol as the chemotherapy group on day 0, looking to trigger chemotherapy-induced ovarian damage.

- AMH injection

The animals were given subcutaneous injections of 100 µL rAMH (5 µg/mouse) every 6 hours, totaling 5 injections over 24 hours, with hour 0 defined as the time of chemotherapy administration. This timing was chosen to obtain sufficient and stable systemic levels of AMH based on its half-life of 4-6 hours when administered subcutaneously (19,25). To obtain 5 µg rAMH in a 100 µL injection, 10 µL of a solution containing 1 mg rAMH powder (recombinant mouse Müllerian-inhibiting substance, DA05456k1g0, Cusabio Technology) dissolved in 1,000 µL sterile water and 1,000 µL glycerol (G-5516, Sigma) (to prevent crystallization) was added to 90 µL PBS.

Controlled ovarian stimulation protocol

Five weeks after treatment, the mice (n=37) underwent COS according to a previously published protocol (21), to synchronize their reproductive cycles and optimize ovulation. On days 1 and 2, they were given subcutaneous injections of 100 µL follicle-stimulating hormone (FSH) (U11587X, Menopur, Ferring) at a total dose of 3.75 IU to initiate development of multiple follicles. On day 3, the animals received a subcutaneous injection of 100 µL human chorionic gonadotropin (hCG; BA084037, Ovitrelle, Merck) at a dose of 5 IU to trigger final oocyte maturation and ovulation. The females were then paired with males (1:1) for mating. On day 4, the presence of vaginal plugs, a sign of sexual activity and likely pregnancy, was investigated. Animals without plugs underwent another round of COS three weeks later, while those with vaginal plugs were euthanized on day 5 for embryo counting and recovery of ovaries.

Embryo count for fertility assessment

Residual fertility was assessed by counting embryos after ovarian flushing. To this end, murine oviducts were dissected and placed in RPMI medium (2436400, Gibco) in a Petri dish. To retrieve embryos, a 34-gauge blunt microneedle was inserted into the infundibulum and RPMI medium was flushed through the oviducts. The embryos were then identified and classified under the microscope as either 2-cell or degenerated embryos. The total number of live embryos per mouse was counted to evaluate residual fertility after treatment.

Ovarian tissue preparation and processing

After euthanasia, mouse ovaries were collected, weighed and measured before being fixed in formalin. After 24 hours, they were transferred to ethanol and embedded in paraffin.

Histology

The whole ovary was cut into serial sections of 5 μm and every fifth section was stained with H&E (Sigma-Aldrich, Machelen, Belgium) to determine total follicle count. For histological evaluation, stained slides were scanned with the Panoramic P250 digital slide scanner (3DHISTECH, Hungary) and analyzed using CaseViewer v2.4 (3DHISTECH, Hungary). The follicle count was assessed blindly by 2 independent readers. Follicles in the selected sections were counted from one end of the ovary to the other. To ensure that no preantral follicles were counted twice, only those with a visible oocyte nucleus were included. Follicles were then classified as atretic if they exhibited signs of pyknosis, GC layer disorganization, oocyte fragmentation, and/or zona pellucida remnants, or viable when they did not show any of these features. (26–29).

Immunohistochemistry

Protocols for IHC have been previously described in detail (21,30). In brief, sections were deparaffinized with Histosafe (Yvsolab SA) and rehydrated in hydrogen peroxide for 30 minutes at room temperature. After demasking in citrate buffer (pH 6) for 75 minutes at 98°C, nonspecific binding sites were blocked by incubation with normal goat serum for 30 minutes at room temperature. The sections were then incubated overnight at 4°C with primary antibodies: rabbit monoclonal anti-mouse (anti-human) Ki67 antibody (M7240, 1:100 dilution; Dako, USA), rabbit polyclonal anti-mouse (anti-human) cleaved caspase-3 antibody (9661S, 1:100

dilution; Cell Signaling Technology, USA), or rabbit monoclonal anti-p-Akt antibody (4060P, 1:100 dilution; Cell Signaling Technology, USA). The slides were subsequently incubated for 60 minutes at room temperature with goat anti-rabbit secondary antibody (Envision anti-rabbit system horseradish peroxidase [HRP], Dako). Diaminobenzidine (Dako) was used as a chromogen and hematoxylin as a counterstain. Negative controls were incubated with universal negative controls (IS600, Dako).

Two non-consecutive sections at least 200 μm apart were examined per sample. Stained slides were then scanned with the Panoramic P250 digital slide scanner (3DHISTECH, Hungary) and analyzed using CaseViewer v2.4 (3DHISTECH, Hungary). Only nongrowing follicles (NGFs) (primordial follicles and intermediate follicles) were assessed for Ki67. NGFs containing at least one GC with Ki67 nuclear staining were classified as proliferative (31). For caspase-3 labeling, follicles at all stages were evaluated and classified as apoptotic when >50% of GCs and/or the oocyte stained positive for caspase-3 (32). Sections labeled with p-Akt were investigated using Fiji software. Each NGF present on a slide was individually isolated to exclude surrounding stromal tissue. Total area of each NGF was measured, followed by color deconvolution to isolate the DAB chromogen signal. This enabled quantification of DAB-stained area within each follicle. The percentage of positive-stained area was then calculated for each NGF, and the mean staining percentage was determined for each analyzed tissue section (30).

Statistical analysis

Statistical analysis was conducted using GraphPad Prism software, v8.0.1 (GraphPad Software Inc., CA). Normality and lognormality tests were performed to check for adherence to normal (Gaussian) distribution. One-way analysis of variance (ANOVA) was then done to assess any statistically significant differences between groups, with significance set at $p < 0.05$.

Results

Fertility assessment

There was a significantly higher proportion of mice with a plug in the control group where 10/10 stimulated mice presented with a plug, compared to the chemotherapy group where the figure was 6/13 animals ($p = 0.046$). In the gonadoprotection group, 12/14 mice exhibited a plug,

which is comparable to the control group ($p=0.239$), and is a higher number but not statistically different from the chemotherapy group ($p=0.420$).

The control group showed the highest mean number of retrieved embryos (41.40 ± 14.74), closely followed by the gonadoprotection group (36.27 ± 17.22), with comparable fertility outcomes. By contrast, the chemotherapy group yielded significantly fewer embryos (20.63 ± 12.12) than did both the control group ($p=0.0046$) and the gonadoprotection group ($p=0.0349$), as shown in Figure 2.

Ovarian reserve

The gonadoprotective effects of Tem and AMH on the ovarian reserve were determined based on total follicle and total NGF counts. The control group showed the highest total follicle count (897.4 ± 392.8), with no significant difference compared to the gonadoprotection group (714.4 ± 283.9). Conversely, the chemotherapy group exhibited a significant decline in total follicle count per ovary (320.7 ± 145.5) compared to both the control group ($p<0.0001$) and the gonadoprotection group ($p=0.0008$).

When conducted analysis normalized for ovarian volume, the mean $\pm$ SD of follicle density was $1,546 \pm 269.0$ foll/mm³ in the control group, 723 ± 342 foll/mm³ in the chemotherapy group, and $1,489 \pm 896$ foll/mm³ in the gonadoprotection group. Follicle density was significantly lower in the chemotherapy group than in the control ($p=0.0004$) and gonadoprotection ($p=0.0130$) groups. No difference was observed between the control group and the gonadoprotection group ($p=0.9724$).

In terms of viable follicle proportions out of total follicles, no significant differences were observed between the gonadoprotection group and the others. However, viable follicle proportions were significantly lower in the chemotherapy group ($89.54\% \pm 8.68\%$) than in the control group ($95.44\% \pm 4.36\%$) ($p=0.0303$). Follicle proportions are detailed in the supplementary material.

Follicle damage

As illustrated in Figure 4a, apoptosis rates were higher in the chemotherapy group ($3.96\% \pm 4.86\%$) than in the control group ($1.23\% \pm 1.51\%$) and, to a lesser extent, the gonadoprotection group ($3.32\% \pm 3.29\%$), although these differences were not statistically significant.

Concerning follicle activation rates, no significant differences were noted between the control group ($6.10\% \pm 5.28\%$), the chemotherapy group ($5.75\% \pm 3.52\%$) and the gonadoprotection group ($8.14\% \pm 6.21\%$), as shown in Figure 4b. Follicle proliferation rates were significantly higher in the chemotherapy group ($59.38\% \pm 14.67\%$) than in the control group ($20.72\% \pm 10.52\%$) ($p < 0.0001$). Gonadoprotective treatment did cause a reduction in follicle proliferation ($42.23\% \pm 18.37\%$) compared to the chemotherapy group ($p = 0.0083$), but the proliferation rate still remained significantly higher than in the control group ($p = 0.0020$) (Figure 4c).

Discussion

Alkylating agents are known to be the most gonadotoxic chemotherapeutic agents (6) and, as reviewed by Spears et al. (4), mechanisms by which chemotherapy depletes the ovarian reserve are well established and multifaceted. Chemotherapy triggers apoptosis in oocytes directly and targets actively dividing cells, resulting in destruction of GCs in growing follicles (7,8). This leads to a drop in AMH, an inhibitor of primordial follicle recruitment (8). Chemotherapy also activates quiescent follicles by upregulating the intrinsic regulatory pathway, namely PI3K signaling (7), inducing massive primordial follicle recruitment, known as the burn-out theory (9).

An interesting new approach involves fertility protection, also called gonadoprotection, against chemotherapy-induced damage. Recent experiments have sought to inhibit both activation pathways through use of rAMH and/or mTOR inhibitors (such as rapamycin and Tem) (12–14,16, 26). In studies evaluating the effects of mTOR inhibitor-based gonadoprotection, analyses were performed for up to 8 weeks following its administration (20). In studies involving AMH, some assessed the effects at 8 weeks post-treatment (18), while others did so every 7 weeks (across 5 stimulation cycles), starting from 5 weeks after administration (19). However, the only study to investigate combined use of both gonadoprotective strategies (AMH and mTOR inhibitor) in vivo only evaluated the ovarian reserve up to 21 days post-treatment, but did not look at fertility outcomes (16).

We assessed the efficacy of concomitant in vivo administration of Tem and rAMH with alkylating agents after a longer period, namely 5-8 weeks post-treatment. Residual fertility was evaluated by the number of embryos retrieved, as was depletion of the ovarian reserve and

follicle-related chemotherapy-induced ovarian damage. In our study, we demonstrated that the number of viable embryos following chemotherapy combined with gonadoprotection (Tem + rAMH) was comparable to that observed in the untreated control group, and significantly higher than in the chemotherapy-only group. Furthermore, in terms of the ovarian reserve, we observed a protective effect of the combined treatment, as the total follicle count per ovary in the gonadoprotection group was similar to that of controls and significantly higher than in the chemotherapy group. These findings are consistent with previous studies using either mTOR inhibitors alone (20) or AMH alone (18,19), which showed preservation of both the ovarian reserve and fertility following chemotherapy, reinforcing the rigor and validity of previous studies. By demonstrating that the combination of Tem and AMH also confers fertility protection, our results expand upon those published by Kashi et al. (16).

Treatment choice

It is nevertheless important to identify and discuss the protective impact of each molecule on the ovarian reserve. Upregulation of the PI3K-mTOR pathway by chemotherapy is crucial to primordial follicle activation (34), mTOR being a serine/threonine kinase that regulates protein synthesis, cell growth and proliferation. In mice, activation of mTOR in the oocyte acts as a catalyst for the entire follicle pool, resulting in premature ovarian failure (9). Inhibiting the mTOR signaling pathway, on the other hand, blocks primordial-to-primary follicle transition. Rapamycin and its analogs like Tem are inhibitors of the mTOR pathway and studies have proved that their administration along with Cy decreases follicle loss induced by chemotherapy (20,35). One drawback of mTOR inhibitors is their broad systemic activity in all cells (36), triggering side effects like mucositis, rashes, hyperglycemia and hyperlipidemia (37). However, it was recently demonstrated that when administered at the same time as Cy, they do not have any adverse effects on the cancer being treated, as they also have antitumoral attributes (33).

There are different mTOR inhibitors, all of which have been previously used for gonadoprotection (16,17,20,33,35). Rapamycin is a lipophilic drug with low bioavailability but a long half-life (around 60 hours). Rapamycin analogs have superior 'drug-like' effects with increased efficacy thanks to better solubility and bioavailability, leading to more consistent and effective inhibition of the mTOR pathway in vivo (38). Everolimus is a chemically modified version of rapamycin, taken orally and has a shorter half-life than rapamycin (around 30 hours) (39). Finally, Tem, a prodrug of rapamycin, shows better solubility in water, making it suitable for intravenous and intraperitoneal injections, and has a half-life comparable to rapamycin (39).

We chose Tem because its solubility is higher than rapamycin and its half-life longer than everolimus. Moreover, as everolimus is designed for oral administration while Tem is designed for injections, we felt that injections would allow more precise dosing than including the drug in animal food, as gavage is frowned upon by our ethics committees.

Several studies have also shown AMH to be protective of the ovarian reserve against chemotherapy-induced damage (18,19). Indeed, AMH, which is produced by early growing follicles, has been reported to inhibit chemotherapy-induced primordial follicle activation too (40,41), although the downstream pathway is so far uncharted. It is, however, possible that this suppression of activation is also regulated by the PI3K pathway (18). Unlike mTOR inhibitors, AMH has a selective influence on the ovary (42).

There are numerous studies that have demonstrated both the beneficial effects of mTOR inhibitors in protecting ovarian tissue from chemotherapy-induced damage (20,33,35), and also the protective role of AMH (18,19). Two recent studies (16,17) investigated the effects of each compound individually as well as in combination. They found not only individual protective effects of mTOR inhibitors and AMH, but actually a synergistic effect of both agents together, yielding enhanced outcomes. We decided to build upon the work of these two studies, which underscore the added value of combining an mTOR inhibitor with AMH, to further scrutinize the potential of this combined approach to preserving fertility. Moreover, use of Tem, as opposed to other mTOR inhibitors, was also based on the paper by Kashi et al (16), as we wanted to confirm their results and assess fertility by embryo counting with the same protocol to avoid treatment-related discrepancies between our results and theirs.

Treatment timing

In their study, Tanaka et al administered daily rapamycin injections (5 mg/kg for 4 weeks) and cyclic chemotherapy (120 mg/kg Cy once a week for 3 weeks) to mimic the clinical setting, where patients require multiple injections. They were able to confirm the protective effect of rapamycin on the ovary, with lower rates of apoptosis encountered in growing follicles after chemotherapy (33). We opted for 'one cycle' of treatment, with 5 Tem injections in the week prior to chemotherapy, followed by 5 AMH injections during the 24 hours post-chemotherapy.

This timing was selected because mTOR inhibitor steady-state concentrations are reached within 5-7 days of administration, ensuring maximal mTOR pathway inhibition during chemotherapy exposure (22,23), as well as sufficient and stable systemic levels of AMH based on its half-life of 4-6 hours when given subcutaneously (19,25). This protocol was also chosen based on previously published studies to allow comparison and discussion (16).

Fertility outcomes

In the literature, litter size and embryo rates have been explored with administration of mTOR inhibitors or AMH alone, but not both at the same time (18,20). However, both treatments did appear to have a positive impact on fertility rates (18,20). To our knowledge, our study is the first to evaluate fertility potential by analyzing the number of embryos obtained after gonadoprotection by concomitant administration of Tem and rAMH, demonstrating that these treatments safeguard not only the ovarian reserve, but also subsequent fertility. With gonadoprotection, embryo rates were comparable to the control group and significantly higher than in the chemotherapy group. It should be noted that COS was performed between 5 and 8 weeks after treatment to avoid the deleterious effects demonstrated by Meirow et al when stimulation was performed sooner (43).

Apoptosis

Chemotherapy-induced apoptosis in primordial follicles due to DNA DSBs is contentious, with some studies showing the presence of apoptotic primordial follicles (44–46) and others not (9,47,48). In our study, we did not detect any difference in apoptosis rates between groups either in primordial or growing follicles. These discrepancies may be explained by different timing of analysis. Indeed, apoptosis after chemotherapy happens within a timeframe of 24 hours post-exposure, as shown by Tanaka et al (33). By conducting our analysis 5 to 8 weeks after chemotherapy, we were looking to exclude later damage. Knowing that the murine follicle cycle lasts 3 weeks, growing follicles analyzed 5-8 weeks post-chemotherapy were at the primordial stage when exposure to chemotherapy occurred. As we found no difference in growing follicle apoptosis rates 5 weeks after chemotherapy exposure, it means that surviving primordial follicles were not harmed by chemotherapy.

Proliferation

We also found that administration of Tem with rAMH partially inhibited chemotherapy-induced proliferation, confirming the study by Kashi et al. (16) who observed that Tem could curb proliferation triggered by chemotherapy. However, it is very important to point out that our analysis was conducted on primordial follicles 5 to 8 weeks after treatment, while Kashi's study analyzed early growing follicles 24 hours after treatment.

Limitations

In our study, we did not see any difference between groups in terms of follicle activation rates assessed by p-Akt immunostaining. Some studies show an increase in activation when analyzed 24 hours post-chemotherapy, and a decrease when gonadoprotection is added (whether Tem alone, AMH alone, or both together) (16). At our chosen timepoint (5 to 8 weeks post-chemotherapy), there was no evidence of changes in activation rates according to treatment. It is important to note that assessing activation using only p-Akt is not optimal compared to other downstream activation markers in case of treatment with mTOR1 inhibitors. However, at 5 weeks post-treatment, we did not expect to observe significant changes in other activation markers either, as follicle activation occurs within the first 24 hours of chemotherapy. As our aim was to evaluate embryo rates, we could thereby not retrieve ovaries at 24 hours post-treatment.

Our study is the first to demonstrate that Tem and rAMH administered concomitantly are able to effectively protect both the ovarian reserve and murine fertility against alkylating agent-induced damage. It is nevertheless essential to identify the best gonadoprotection protocol and timing, and investigate its effectiveness in the longer term, to determine whether the protective effect observed at 5-8 weeks persists. Evaluating the effects of Tem + chemotherapy and AMH + chemotherapy separately (rather than only in combination) would have provided additional insights and strengthened our findings. However, our decision to focus on the concomitant use of both compounds was guided by previously published data demonstrating their added benefit when used together, particularly because they act on distinct pathways involved in follicle activation.

Conclusion

In conclusion, concomitant administration of Tem and rAMH during chemotherapy effectively protects the ovarian reserve, and hence fertility outcomes, but further research is required to study the longer-term efficacy of this treatment and its use on human tissue.

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References

1. Siegel RL, Giaquinto AN, Jemal A. Cancer statistics, 2024. *CA Cancer J Clin* 2024;74:12–49.
2. van Dorp W, Haupt R, Anderson RA, Mulder RL, van den Heuvel-Eibrink MM, van Dulmen-den Broeder E, et al. Reproductive Function and Outcomes in Female Survivors of Childhood, Adolescent, and Young Adult Cancer: A Review. *J Clin Oncol* 2018;36:2169–80.
3. Miller JJ, Williams GF, Leissring JC. Multiple late complications of therapy with cyclophosphamide, including ovarian destruction. *Am J Med* 1971;50:530–5.
4. Spears N, Lopes F, Stefansdottir A, Rossi V, De Felici M, Anderson RA, et al. Ovarian damage from chemotherapy and current approaches to its protection. *Human Reproduction Update* 2019;25:673–93.
5. Plowchalk DR, Mattison DR. Phosphoramidate mustard is responsible for the ovarian toxicity of cyclophosphamide. *Toxicol Appl Pharmacol* 1991;107:472–81.
6. van Dorp W, Mulder RL, Kremer LCM, Hudson MM, van den Heuvel-Eibrink MM, van den Berg MH, et al. Recommendations for Premature Ovarian Insufficiency Surveillance for Female Survivors of Childhood, Adolescent, and Young Adult Cancer: A Report From the International Late Effects of Childhood Cancer Guideline Harmonization Group in Collaboration With the PanCareSurFup Consortium. *JCO* 2016;34:3440–50.
7. Kashi O, Meirow D. Overactivation or Apoptosis: Which Mechanisms Affect Chemotherapy-Induced Ovarian Reserve Depletion? *Int J Mol Sci* 2023;24:16291.
8. Meirow D, Biederman H, Anderson RA, Wallace WHB. Toxicity of chemotherapy and radiation on female reproduction. *Clin Obstet Gynecol* 2010;53:727–39.
9. Kalich-Philosoph L, Roness H, Carmely A, Fishel-Bartal M, Ligumsky H, Paglin S, et al. Cyclophosphamide Triggers Follicle Activation and “Burnout”; AS101 Prevents Follicle Loss and Preserves Fertility. *Science Translational Medicine* 2013;5:185ra62-185ra62.
10. Meirow D, Dor J, Kaufman B, Shrim A, Rabinovici J, Schiff E, et al. Cortical fibrosis and blood-vessels damage in human ovaries exposed to chemotherapy. Potential mechanisms of ovarian injury. *Human Reproduction* 2007;22:1626–33.
11. Donnez J, Dolmans M-M. Fertility Preservation in Women. *N Engl J Med* 2017;377:1657–65.
12. Meirow D, Hardan I, Dor J, Fridman E, Elizur S, Ra’anani H, et al. Searching for evidence of disease and malignant cell contamination in ovarian tissue stored from hematologic cancer patients. *Hum Reprod* 2008;23:1007–13.
13. Dolmans M-M, Marinescu C, Saussoy P, Van Langendonck A, Amorim C, Donnez J. Reimplantation of cryopreserved ovarian tissue from patients with acute lymphoblastic leukemia is potentially unsafe. *Blood* 2010;116:2908–14.

14. Greve T, Clasen-Linde E, Andersen MT, Andersen MK, Sørensen SD, Rosendahl M, et al. Cryopreserved ovarian cortex from patients with leukemia in complete remission contains no apparent viable malignant cells. *Blood* 2012;120:4311–6.
15. Dolmans M-M. Safety of ovarian autotransplantation. *Blood* 2012;120:4275–6.
16. Kashi O, Roness H, Spector I, Derech-Haim S, Meirow D. Dual suppression of follicle activation pathways completely prevents the cyclophosphamide-induced loss of ovarian reserve. *Hum Reprod* 2023;38:1086–98.
17. Rosario R, Stewart HL, Spears N, Telfer EE, Anderson RA. Anti-Mullerian hormone attenuates both cyclophosphamide-induced damage and PI3K signalling activation, while rapamycin attenuates only PI3K signalling activation, in human ovarian cortex *in vitro*. *Human Reproduction* 2023;dead255.
18. Sonigo C, Beau I, Grynberg M, Binart N. AMH prevents primordial ovarian follicle loss and fertility alteration in cyclophosphamide-treated mice. *The FASEB Journal* 2019;33:1278–87.
19. Roness H, Spector I, Leichtmann-Bardoogo Y, Savino AM, Derech-Haim S, Meirow D. Pharmacological administration of recombinant human AMH rescues ovarian reserve and preserves fertility in a mouse model of chemotherapy, without interfering with anti-tumoural effects. *J Assist Reprod Genet* 2019;36:1793–803.
20. Goldman KN, Chenette D, Arju R, Duncan FE, Keefe DL, Grifo JA, et al. mTORC1/2 inhibition preserves ovarian function and fertility during genotoxic chemotherapy. *Proc Natl Acad Sci U S A* 2017;114:3186–91.
21. Houeis L, van der Plancke G, Wen JY, Dupuy L, Kara E, Cacciottola L, Maurel MC, Donnez J, Dolmans MM. Chemotherapy-induced diminished murine ovarian reserve model and impact of low-dose chemotherapy on fertility. *F S Sci*. 2025 May;6:177-185..
22. MacDonald A, Scarola J, Burke JT, Zimmerman JJ. Clinical pharmacokinetics and therapeutic drug monitoring of sirolimus. *Clin Ther* 2000;22 Suppl B:B101-121.
23. Kirchner GI, Meier-Wiedenbach I, Manns MP. Clinical Pharmacokinetics of Everolimus. *Clin Pharmacokinet* 2004;43:83–95.
24. Chaudhari SP, Dugar RP. Application of surfactants in solid dispersion technology for improving solubility of poorly water soluble drugs. *Journal of Drug Delivery Science and Technology* 2017;41:68–77.
25. Kano M, Sosulski AE, Zhang L, Saatcioglu HD, Wang D, Nagykerly N, et al. AMH/MIS as a contraceptive that protects the ovarian reserve during chemotherapy. *Proc Natl Acad Sci U S A* 2017;114:E1688–97.
26. Monniaux D, Caraty A, Clement F, Dalbies-Tran R, Dupont J, Fabre S, et al. Développement folliculaire ovarien et ovulation chez les mammifères. *INRAE Productions Animales* 2009;22:59–76.
27. Roig I, Brieno-Enriquez MA, Caldes MG. Meiosis in a bottle: new approaches to overcome Mammalian meiocyte study limitations. *Genes (Basel)* 2011;2:152–68.

28. Cheon Y-P. Regulation and 3 dimensional culture of tertiary follicle growth. *Clin Exp Reprod Med* 2012;39:95–106.
29. Liu X, Craig ZR. Environmentally relevant exposure to dibutyl phthalate disrupts DNA damage repair gene expression in the mouse ovary†. *Biol Reprod* 2019;101:854–67.
30. Hossay C, Tramacere F, Cacciottola L, Camboni A, Squifflet J-L, Donnez J, et al. Follicle outcomes in human ovarian tissue: effect of freezing, culture, and grafting. *Fertil Steril* 2023;119:135–45.
31. Luyckx V, Scalercio S, Jadoul P, Amorim CA, Soares M, Donnez J, et al. Evaluation of cryopreserved ovarian tissue from prepubertal patients after long-term xenografting and exogenous stimulation. *Fertil Steril* 2013;100:1350–7.
32. Amorim CA, Donnez J, Dehoux J-P, Scalercio SR, Squifflet J, Dolmans M-M. Long-term follow-up of vitrified and autografted baboon (*Papio anubis*) ovarian tissue. *Hum Reprod* 2019;34:323–34.
33. Tanaka Y, Amano T, Nakamura A, Yoshino F, Takebayashi A, Takahashi A, et al. Rapamycin prevents cyclophosphamide-induced ovarian follicular loss and potentially inhibits tumour proliferation in a breast cancer xenograft mouse model. *Human Reproduction* 2024;deae085.
34. Adhikari D, Zheng W, Shen Y, Gorre N, Härmäläinen T, Cooney AJ, et al. Tsc/mTORC1 signaling in oocytes governs the quiescence and activation of primordial follicles. *Hum Mol Genet* 2010;19:397–410.
35. Tanaka Y, Kimura F, Zheng L, Kaku S, Takebayashi A, Kasahara K, et al. Protective effect of a mechanistic target of rapamycin inhibitor on an in vivo model of cisplatin-induced ovarian gonadotoxicity. *Exp Anim* 2018;67:493–500.
36. Manning BD, Cantley LC. AKT/PKB signaling: navigating downstream. *Cell* 2007;129:1261–74.
37. Soefje SA, Karnad A, Brenner AJ. Common toxicities of mammalian target of rapamycin inhibitors. *Target Oncol* 2011;6:125–9.
38. Ali ES, Mitra K, Akter S, Ramproshad S, Mondal B, Khan IN, et al. Recent advances and limitations of mTOR inhibitors in the treatment of cancer. *Cancer Cell International* 2022;22:284.
39. Danesi R, Boni JP, Ravaud A. Oral and intravenously administered mTOR inhibitors for metastatic renal cell carcinoma: pharmacokinetic considerations and clinical implications. *Cancer Treat Rev* 2013;39:784–92.
40. Durlinger ALL, Gruijters MJG, Kramer P, Karels B, Ingraham HA, Nachtigal MW, et al. Anti-Müllerian hormone inhibits initiation of primordial follicle growth in the mouse ovary. *Endocrinology* 2002;143:1076–84.
41. Carlsson IB, Scott JE, Visser JA, Ritvos O, Themmen APN, Hovatta O. Anti-Müllerian hormone inhibits initiation of growth of human primordial ovarian follicles in vitro. *Hum Reprod* 2006;21:2223–7.

42. Bedenk J, Vrtačnik-Bokal E, Virant-Klun I. The role of anti-Müllerian hormone (AMH) in ovarian disease and infertility. *J Assist Reprod Genet* 2020;37:89–100.
43. Meirow D, Epstein M, Lewis H, Nugent D, Gosden RG. Administration of cyclophosphamide at different stages of follicular maturation in mice: effects on reproductive performance and fetal malformations. *Hum Reprod* 2001;16:632–7.
44. Luan Y, Edmonds ME, Woodruff TK, Kim S-Y. Inhibitors of apoptosis protect the ovarian reserve from cyclophosphamide. *J Endocrinol* 2019;240:243–56.
45. Oktem O, Oktay K. Quantitative assessment of the impact of chemotherapy on ovarian follicle reserve and stromal function. *Cancer* 2007;110:2222–9.
46. Titus S, Szymanska KJ, Musul B, Turan V, Taylan E, Garcia-Milian R, et al. Individual-oocyte transcriptomic analysis shows that genotoxic chemotherapy depletes human primordial follicle reserve in vivo by triggering proapoptotic pathways without growth activation. *Sci Rep* 2021;11:407.
47. Zhou J, Chen Y, Zhang P, Lou H. Ovarian preservation in adenocarcinoma of the uterine cervix. *J Ovarian Res* 2017;10:48.
48. Shai D, Aviel-Ronen S, Spector I, Raanani H, Shapira M, Gat I, et al. Ovaries of patients recently treated with alkylating agent chemotherapy indicate the presence of acute follicle activation, elucidating its role among other proposed mechanisms of follicle loss. *Fertility and Sterility* 2021;115:1239–49.

Legends to the figures

Figure 1. Experimental design

The control group (n=10) was given a 100 μ L intraperitoneal injection of PBS. The chemotherapy group (n=13) received a single 100 μ L intraperitoneal injection of chemotherapy (120 mg/kg Cy + 12 mg/kg Bu). The gonadoprotection group (n=14) was given five injections of 5 mg/kg Tem over the course of one week prior to chemotherapy, followed by a chemotherapy injection, then five injections of 5 μ g rAMH over the next 24 hours. All mice were stimulated five weeks later with 3.75 IU Menopur, then mated with males with proven fertility. If a vaginal plug was observed, the mice were euthanized for embryo and ovary recovery. If no plug was present, the mice underwent another round of COS and mating two weeks later, namely 8 weeks after chemotherapy. Created in BioRender. Dolmans, M. (2025)

Figure 2.

- a) Embryo count: The control group yielded the highest mean number of retrieved embryos (41.40 ± 14.74), closely followed by the gonadoprotection group (36.27 ± 17.22), with comparable fertility outcomes. By contrast, the chemotherapy group exhibited significantly fewer embryos (20.63 ± 12.12) than both the control group ($p=0.0046$) and the gonadoprotection group ($p=0.0349$).
- b) Picture of day-2 embryos (10x, Axio Imager 2, ZEISS, Germany).

Figure 3.

- a) Follicle count: The control group showed the highest total follicle count (897.4 ± 392.8), with no significant difference compared to the gonadoprotection group (714.4 ± 283.9). Conversely, the chemotherapy group demonstrated a significant decline in total follicle count per ovary (320.7 ± 145.5) compared to both the control group ($p<0.0001$) and the gonadoprotection group ($p=0.0008$).
- b) Control group: representative histology section of the ovary (16x, Pannoramic P250 digital slide scanner (3DHISTECH, Hungary)).
- c) Chemotherapy group: representative histology section of the ovary (16x, Pannoramic P250 digital slide scanner (3DHISTECH, Hungary)).

- d) Gonadoprotection group: representative histology section of the ovary (16x, Pannoramic P250 digital slide scanner (3DHISTECH, Hungary)).

Figure 4.

- a. Apoptosis rates: Apoptosis rates were higher in the chemotherapy group ($3.96\% \pm 4.86\%$) than in the control group ($1.23\% \pm 1.51\%$) and, to a lesser extent, the gonadoprotection group ($3.32\% \pm 3.29\%$), although these differences were not statistically significant.
- b. Activation rates: No significant differences were observed between the control group ($6.10\% \pm 5.28\%$), the chemotherapy group ($5.75\% \pm 3.52\%$) and the gonadoprotection group ($8.14\% \pm 6.21\%$).
- c. Proliferation rates: NGF proliferation rates were significantly higher in the chemotherapy group ($59.38\% \pm 14.67\%$) than in the control group ($20.72\% \pm 10.52\%$) ($p < 0.0001$). Gonadoprotective treatment caused a reduction in follicle proliferation ($42.23\% \pm 18.37\%$) compared to the chemotherapy group ($p = 0.0083$), but the proliferation rate still remained significantly higher than in the control group ($p = 0.0020$).







