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Ovarian cryopreservation with rapamycin improves fertility restoration in a murine orthotopic transplantation model

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Currently, the only fertility preservation option of prepubertal patients is ovarian tissue cryopreservation followed by autotransplantation (OTCTP). Once in remission and patients desire to conceive, autotransplantation of frozen/thawed tissue is performed. A major issue of this technique is follicular loss directly after transplantation, mainly due to follicle activation. Our previous research showed that adding rapamycin to the freezing medium counteracted follicle proliferation and activation induced by OTCTP in heterotopic autotransplantation of ovaries in mice. Our current study aimed to test the potential of this approach to improve fertility restoration in mice. Forty 4-week-old female C57BL/6 mice underwent unilateral oophorectomy followed by slow-freezing of ovaries with or without rapamycin. After chemically disabling the remaining ovary, orthotopic autotransplantation was performed. After recovery, estrous cycle analysis was conducted using daily vaginal smears. The mice were mated with males for 4 months, and pregnancy outcomes were recorded. After mating, half the females were super-ovulated for oocyte quantification and ovarian analysis, while the others had their ovaries collected for analysis of remaining primordial follicles using immunohistochemistry. Female mice whose ovaries were cryopreserved with rapamycin prior to chemically disabling the remaining ovary and orthotopic autotransplantation, gave birth to more pups (102 rapamycin, 48 control). The live birth rate was also higher ($P = 0.0025$) when ovaries were cryopreserved in rapamycin compared to control medium. Additionally, more mice in the rapamycin group gave birth (13 rapamycin, 8 control) with a higher average litter size ($P = 0.0837$). More mice had primordial follicles left at the end of the experiment in the rapamycin group ($P = 0.0397$). Superovulation showed a similar number of oocytes collected ($P = 0.4462$). While rapamycin did not influence cyst formation after autotransplantation, mice that developed ovarian cysts gave birth to fewer pups per dam ($P = 0.0119$) with a lower live birth rate compared to mice without ovarian cysts ($P = 0.0032$). The use of rapamycin improved fertility restoration in mice. Using rapamycin during OTCTP in humans could potentially resolve the massive follicular loss directly after grafting, and thus eventually lead to better opportunities for women to become pregnant.

Keywords Ovarian cryopreservation, Follicle activation, Fertility preservation, Rapamycin, Ovarian transplantation, Animal models

Abbreviations

DDX4	DEAD-box helicase 4
DMSO	dimethylsulfoxide
FBS	Fetal bovine serum
hCG	human chorionic gonadotrophin
IHC	Immunohistochemistry
IP	Intraperitoneally
mTOR	Mammalian target of rapamycin
OTCTP	Ovarian tissue cryopreservation followed by autotransplantation
PI3K	Phosphatidylinositol-3-kinase

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PTEN	Phosphatase and tensin homolog
RT	Room temperature
SF	Slow-frozen
SFct	Ovaries slow-frozen in control medium
SFrapa	Ovaries slow-frozen in medium supplemented with rapamycin
VCD	4-vinylcyclohexene diepoxide

Advancements in cancer treatments have significantly raised the likelihood of survival over recent decades. Consequently, the long-term quality of life after remission has gained heightened significance. For many young cancer survivors who aspire to start families, a critical concern is their ability to conceive biological children. Unfortunately, specific chemotherapy and radiotherapy treatments can lead to ovarian failure and, consequently, infertility[1–3]. Currently, the only option available to preserve the fertility of prepubertal girls and young women requiring urgent oncological care is the cryopreservation of cortical ovarian tissue followed by autotransplantation (OTCTP)[4]. One of the major advantages of this technique is the ability to restore natural fertility. To date, over 200 live births have taken place after the use of OTCTP[5,6]. However, this technique has certain limitations, including the loss of follicles directly after transplantation[7,8]. This rapid depletion of the follicle reserve is possibly due to apoptosis, delayed neovascularization, and/or immense follicle recruitment, known as follicular "burn-out", and can drastically decrease graft longevity[9–11]. Under physiological conditions, the primordial follicle reserve is preserved through a balance between activation signals, provided by, e.g., granulosa cells, and inhibition signals provided by growing follicles. Unfortunately, many growing follicles do not survive the OTCTP process, resulting in a reduction of the inhibition signals and an imbalance towards follicle activation. This leads to excessive follicle recruitment and a decrease in the primordial follicle reserve[9,12,13]. Two of the pathways involved in follicle activation are the phosphatidylinositol-3-kinase (PI3K)/phosphatase and tensin homolog (PTEN)/AKT and the mammalian target of rapamycin (mTOR) pathways. Various inhibitors of these pathways have been investigated to limit follicle "burn out" and protect the follicle reserve[13–19]. One of these inhibitors is rapamycin, a specific mTOR inhibitor already used as an immunosuppressant in clinical settings after organ transplantation[20,21]. Rapamycin has shown promising results in protecting the primordial follicle pool in several experimental settings, e.g., in cultured cisplatin-treated fresh rat ovaries[17]. Furthermore, the injection of rapamycin has been shown to effectively preserve the primordial follicle pool in various models, including rats and SCID mice transplanted with ovarian tissue[18,19]. These findings, obtained with fresh or vitrified ovaries, highlight the potential of rapamycin in protecting the primordial follicle reserve. However, further investigation, particularly focusing on slow-frozen (SF) ovaries, the current gold-standard cryopreservation method, is essential before clinical application[22]. In vitro, we previously found that adding rapamycin to the freezing medium could counteract slow-freezing-induced follicle activation in mouse ovaries[23]. Following these promising in vitro results, we aimed to confirm in vivo that cryopreservation- and/or transplantation-induced primordial follicle activation could be inhibited by freezing ovaries with rapamycin using a heterotopic ovarian transplantation mouse model. Indeed, we found that the addition of rapamycin to the freezing medium can counteract follicle proliferation and activation induced by the slow-freezing and transplantation process three weeks after autotransplanting ovaries under the kidney capsule of mice[24]. While the heterotopic transplantation experiment demonstrated that rapamycin can maintain primordial follicles in a quiescent state, our ultimate goal is to improve the restoration of fertility. Therefore, we aimed to investigate whether adding rapamycin to the slow-freezing medium can enhance fertility restoration in mice through an orthotopic autotransplantation after chemically-induced ovarian failure.

Methods

Experimental design (Fig. 1)

A total of 40 four-week-old female C57BL/6 mice were obtained from Charles River Laboratories (France) and maintained at the accredited Mouse Facility of the University of Liège (Belgium). The mice were housed at

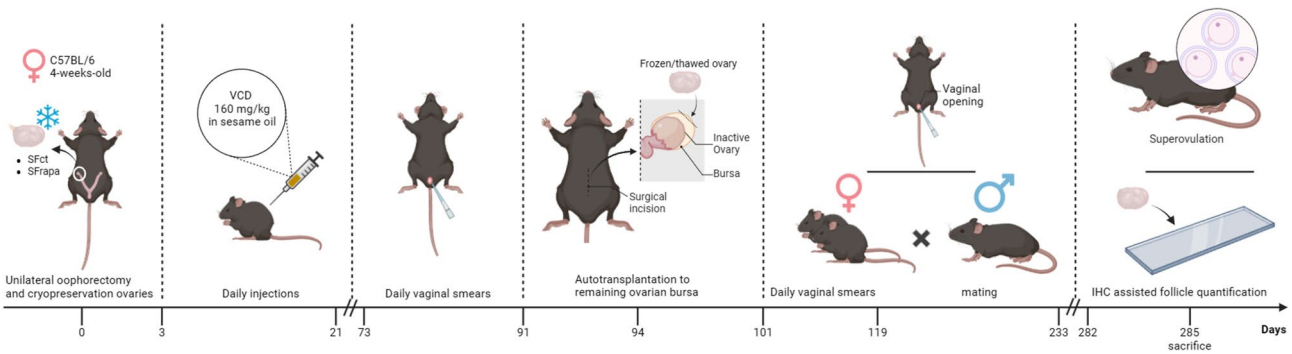

Fig. 1. Schematic of the experimental design used in the murine model to investigate the effects of adding rapamycin to the freezing medium on fertility restoration. VCD = 4-vinylcyclohexene diepoxide, IHC = immunohistochemistry, SFct = ovaries slow-frozen in control medium, SFrapa = ovaries slow-frozen in medium supplemented with rapamycin.

± 21 °C in a 12 h light/dark cycle with a maximum of five mice per cage, with food and water provided ad libitum. All mice underwent unilateral oophorectomy and the ovaries were slow-frozen with or without rapamycin in the freezing medium. The mice were randomly assigned to the groups. To induce failure of the remaining ovary, the mice were intraperitoneally (IP) injected daily with 160 mg/kg (100 µl) 4-vinylcyclohexene diepoxide (VCD; Merck, Darmstadt, Germany) diluted in sesame oil (Biofood) for a total of 19 days[25]. Once the failure of the remaining ovaries was verified using vaginal cytology, the cryopreserved ovaries were thawed and orthotopically autotransplanted to the remaining ovarian bursa[26,27]. Afterward, the recovery of estrous cyclicity was monitored using vaginal cytology. Fertility restoration was analyzed by mating the females with males, and recording details on deliveries and pup numbers. After 4 months of mating, 20 females were sacrificed by cervical dislocation and their ovaries were collected and fixed in 4% formaldehyde overnight. The fixed ovaries were embedded in paraffin, sectioned at 5 µm using a microtome, and mounted on slides for immunohistochemistry. Simultaneously, 16 females underwent superovulation, followed by their sacrifice by cervical dislocation, and subsequent collection and counting of oocytes. As manipulation of ovaries can lead to cyst formation, the ovaries from super-ovulated mice were analyzed for the presence of cysts[28,29].

Oophorectomy, slow-freezing, and thawing procedure

Using gas anesthesia (Isoflurane, Dechra, Northwich, UK), unilateral oophorectomy was performed on all mice, and the ovaries were placed in a slow-freezing transport medium consisting of Leibovitz L-15 medium (Lonza, Verviers, Belgium) supplemented with 10% Fetal Bovine Serum (FBS; Thermo Fisher Scientific, Gibco, Waltham, MA, USA). The adjacent fat tissue and oviduct were removed from the ovaries using a scalpel under a binocular microscope, after which the ovaries were cryopreserved as described before[27]. Briefly, whole ovaries were placed in a slow-freezing medium consisting of Leibovitz L-15 medium supplemented with 10% FBS, 10% dimethylsulfoxide (DMSO; Merck, Darmstadt, Germany), and 0.1 M sucrose. After a 30-minute equilibration at 4 °C, ovaries were placed in cryovial tubes (Simport, Montreal, QC, Canada) containing the slow-freezing medium and thereafter cooled down in a programmable freezing machine (CL-8800i System; CryoLogic, Mulgrave, Victoria, Australia) as previously described and stored in liquid nitrogen[30]. For the SFrapa group, rapamycin (1 µM, InvivoGen, Toulouse, France) was added to the transport and slow-freezing media. The 1 µM concentration of rapamycin was selected based on our previous in vitro experiments and supportive data from the literature[31]. These prior studies indicated that 1 µM effectively reduced follicle activation without causing detectable toxicity, and therefore no additional dose-response tests were performed for the present study. The thawing procedure involved incubating the cryovials at room temperature (RT) for 2 min followed by a 2-minute incubation in a 37 °C water bath. Cryoprotectants and/or rapamycin were removed by washing the ovaries in Leibovitz L-15 medium 3 times for 5 min at 37 °C.

Vaginal cytology

To validate ovarian failure, the status of the estrous cycle was examined once a day (between 8:30 and 10:30) for a total of 3 times 5 consecutive days by collecting smears via vaginal lavage. The estrous cycle stage was determined by examining the cell population in the smears using a hematoxylin-eosin staining[26]. Briefly, the murine estrous cycle consists of four phases: Proestrus, when follicles start to grow and the smears contain mostly nucleated epithelial cells; Estrus, the fertile phase when cornified epithelial cells are present; In the absence of conception, the metestrus phase begins, characterized by the presence of cornified epithelial cells and leukocytes in the smears; Diestrus, when the females are no longer receptive to males and smears contain a high amount of leukocytes[32]. Mice were considered to have ovarian failure when they remained in the metestrus or diestrus phase for the final five days of vaginal cytology.

Vaginal cytology was also performed daily for 3 weeks after transplantation to analyze the recovery of the estrous cycle.

Autotransplantation

After confirming ovarian failure, orthotopic transplantation of cryopreserved ovaries to the remaining ovarian bursa was performed based on a protocol by *Behringer*[33]. Briefly, the remaining non-functional ovary and uterus were surgically exposed under gas anesthesia. A small incision in the bursa was made using spring scissors on the site opposing the oviduct. The slow-frozen/thawed whole ovary was then inserted through the incision into the bursal sac next to the remaining ovary. The ovaries were then placed back into the peritoneal cavity followed by the suturing of the peritoneum and closing of the skin with surgical wound clips.

Fertility assessment

To analyze whether rapamycin increased fertility restoration, all female mice were mated after the transplantation with 8-week-old C57BL/6 males (1 male per 2 females) for a total of 4 months. Vaginal plugs were checked every morning and the females were weighed 3 times a week to determine pregnancies. The number of females giving birth, litter size, litter number, and live birth rate were recorded. When calculating the mean number of deliveries per mouse, only mice with a minimum of one delivery were included. To ensure that the absence of conception was not due to male subfertility, males used for mating with the control females were exchanged with the males used for mating with females in the rapamycin group every 4 weeks.

Superovulation

At the end of the experiment, mice were super-ovulated ($n = 8$ per group). They were injected IP with 5 IU (100 µl) of pregnant mare serum gonadotrophin (Folligon, MSD, Kenilworth, NJ, USA). Forty-nine hours later, 7.5 IU (100 µl) of human chorionic gonadotrophin (hCG, Merck, Darmstadt, Germany) was administered similarly. The mice were sacrificed fifteen hours after hCG administration and the ovary/oviduct complex was

collected and placed in M16 medium (Merck, Darmstadt, Germany). The ampulla of the oviducts was cut with spring scissors to release the cumulus-oocyte complexes, and oocytes were quantified.

Immunohistochemistry

To perform follicle quantification and analyze whether primordial follicles remained in the ovaries at the end of the experiment, ovarian sections were labeled with DEAD-box helicase 4 (DDX4; Abcam ab41519, Cambridge, UK) ($n = 8–10$ sections per mouse per group). DDX4 is a helicase involved in gamete generation and is localized in germ cells. It can therefore be used as a biomarker for easy follicle identification[34]. In short, ovarian sections were deparaffinized and rehydrated, followed by antigen retrieval using an autoclave (11 min, 126 °C, 1.3 Bar) in citrate buffer (Dako, Glostrup, Denmark). After cooling for 20 min, endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxide for 20 min at RT. Non-specific binding sites were blocked using Animal-Free Blocking Solution (Cell Signaling, Danvers, MA, USA) for 20 min at RT. The DDX4 primary antibody, diluted 1/600 in REAL antibody diluent (Dako, Glostrup, Denmark), was incubated for 1 h at RT. Next, the sections were incubated with the secondary antibody linked with horseradish peroxidase (ENVISION/HRP ready to use, Dako, Glostrup, Denmark) for 30 min at RT. Afterward, the revelation was performed using DAB+ (Dako, Glostrup, Denmark) followed by hematoxylin counterstaining, and sections were mounted using Entellan new mounting medium (Sigma-Aldrich, St. Louis, MO, USA). Stained sections were scanned using the NanoZoomer 2.0 HT digital slide scanner (Hamamatsu Photonics K.K., Hamamatsu, Japan).

Follicle quantification

Scanned DDX4 labeled sections were analyzed using the NDP.view2 software (Hamamatsu Photonics K.K., Hamamatsu, Japan). Follicles were classified into primordial, primary, and secondary or more growing according to morphological mouse follicle classification, to evaluate the presence (or not) and number of primordial follicles at the end of the experiment[35]. Every tenth section was analyzed, with a total of eight sections per ovary, to get a well-established representation of the whole ovaries. Total follicle density was defined as the number of primordial follicles per mm^2 after manually outlining the ovarian surface of each section, taking into account only mice with at least one remaining primordial follicle. Every section was individually analyzed, only using sections with a minimum of one primordial follicle, with the results being expressed as the mean of each section per ovary.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 9 (GraphPad, San Diego, CA, USA). A two-way ANOVA with šídák's multiple comparisons test was used to compare the days the mice were in each phase of the estrous cycle. A Chi-square test was used to calculate the correlation between the absolute mice numbers of two groups, as well as between percentages of two groups. The Mann-Whitney test was used for comparison between the median of two experimental groups. For all tests, $P \leq 0.05$ was considered statistically significant.

Results

Rapamycin does not alter recovery of estrous cyclicity after ovarian transplantation

Vaginal cytology analysis was performed from the 10th week after the first VCD injection until and including the 13th week to analyze ovarian function. An absence of estrous cyclicity was found in all of the mice at the end of the 13th week, indicating that all mice had ovarian failure (data not shown). Subsequently, the frozen/thawed ovaries were autotransplanted into the remaining bursa of the non-functional ovary, followed by another round of vaginal cytology analysis starting one week after transplantation. No difference was found in the number of mice that recovered their estrous cyclicity after transplantation between the two experimental groups (SFct: 13 vs. SFrapa: 14) (Fig. 2A). Furthermore, the number of days the mice were in each of the 4 phases of the estrous cycle was calculated, and we found no significant differences between the group autotransplanted with ovaries slow-frozen with rapamycin and the control group (Fig. 2B).

Rapamycin in the freezing medium improved the recovery of fertility after autotransplantation

To investigate whether the addition of rapamycin to the freezing medium could improve fertility recovery in mice with ovarian failure, an ovarian orthotopic autotransplantation model was used. After transplantation, the female mice were mated with 8-week-old males for 4 months. The number of females giving birth, litter size, litter number, and live birth rate were recorded, and the results are summarized in Table 1. During this period, 8 out of 20 control mice gave birth, compared to 13 out of 20 mice whose ovaries were slow-frozen with rapamycin. Furthermore, twice as many pups were born from females with ovaries slow-frozen with rapamycin compared to control mice. Specifically, a total of 48 pups were born from females in the control group, while 102 pups were born from females in the rapamycin group. The live birth rate showed that the addition of rapamycin to the freezing medium resulted in a significantly higher percentage of pups born alive compared to the control ($^{**}P = 0.0025$). The live birth rate for pups born from control mice was 41.7%, compared to 67.7% for pups born from mice in the rapamycin group. Additionally, no difference in the number of litters per female was found between the two experimental groups (SFct: 2.38 ± 0.46; SFrapa: 2.31 ± 0.31), considering only mice with at least one litter. Although not statistically significant, a higher average litter size was observed in mice whose ovaries were slow-frozen with rapamycin (3.43 ± 0.37) compared to mice in the control group (2.53 ± 0.35, $P = 0.0837$). Since not all mice regained their estrous cyclicity post-transplantation, and not all were able to deliver offspring, we explored potential correlations between these factors. A chi-square test revealed a significant association between the restoration of estrous cyclicity and the capacity to produce offspring following transplantation ($^{**}P = 0.0011$, results contain pooled data from the control and rapamycin groups, see figure S1).

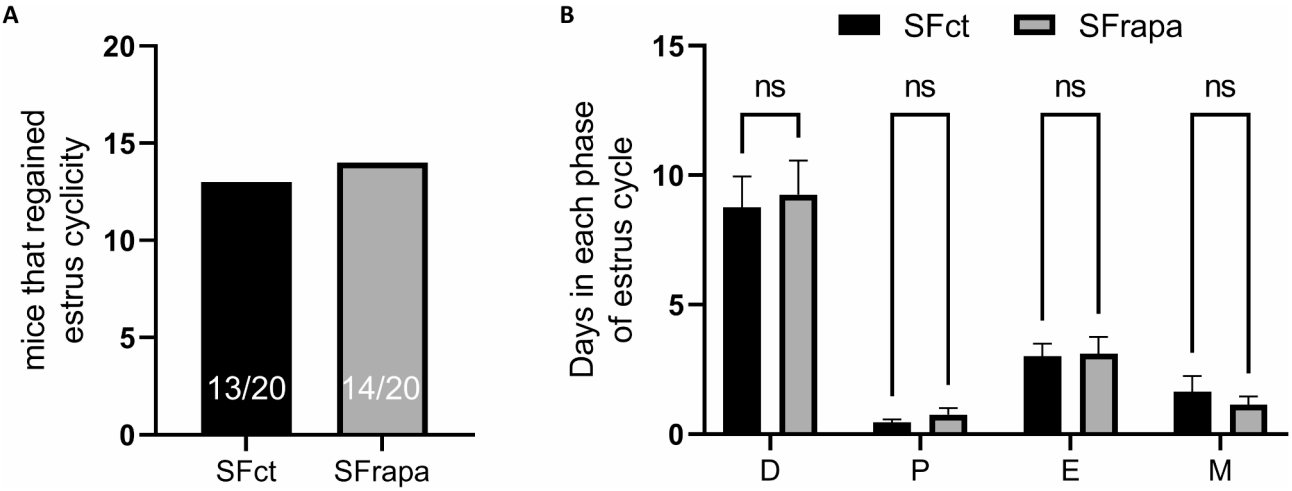

Fig. 2. Estrous cycle recovery after autotransplantation in mice. (**A**) Number of mice that recovered their estrous cycle after autotransplantation in both the control and rapamycin group. (**B**) Mean time (days) that mice were in each phase of the estrous cycle during the 3 weeks post-ovarian-transplantation (mean ± SEM). SFct = ovaries slow-frozen in control medium, SFrapa = ovaries slow-frozen in medium supplemented with rapamycin, D = diestrus, P = proestrus, E = estrus, M = metestrus. Two-way ANOVA with šídák's multiple comparisons: ns = non-significant. $n = 20$ mice per group.

	SFct	SFrapa	Statistics
Total females	20	20	NA
No. of mice giving birth	8 (40%)	13 (65%)	$P = 0.2049^a$
No. of pups born	48	102	NA
Live birth rate	20/48 (41.7%)	69/102 (67.7%)	$P = 0.0025^a$
No. of litter per mouse (minimum 1) (mean ± SEM)	2.38 ± 0.46	2.31 ± 0.31	$P = 0.9739^b$
Litter size (mean ± SEM)	2.53 ± 0.35	3.43 ± 0.37	$P = 0.0837^b$

Table 1. Effects of adding rapamycin to the slow-freezing medium on the fertility of mice after orthotopic ovarian autotransplantation and subsequent mating. NA = not applicable, SFct = ovaries slow-frozen in control medium, SFrapa = ovaries slow-frozen in medium supplemented with rapamycin. [a] = Chi-square test, [b] = Mann-Whitney test.

Rapamycin did not affect oocyte numbers after superovulation

At the end of the experiment when the females were ten months old, 8 control mice and 8 mice in the rapamycin group were super-ovulated and oocytes were collected. Three mice in the control group ovulated at least 1 oocyte after superovulation, compared to one mouse in the rapamycin group (Fig. 3A). No significant difference in the mean number of ovulated oocytes was observed between the two experimental groups (Fig. 3B).

Ovarian cysts impact live birth rate regardless of rapamycin treatment

During the recovery of oocytes post-superovulation, the presence of ovarian cysts was examined. Rapamycin did not significantly affect the number of ovaries containing cysts. Specifically, 6 (75%) mice in the control group had ovaries with cysts, compared to 5 (62.5%) mice in the rapamycin group (figure S2). As the presence of ovarian cysts could impair fertility, a correlation assay was performed[36]. No correlation was observed between the presence of cysts and the possibility for mice to deliver offspring ($P = 0.1951$) (Fig. 4A). However, the presence of cysts was significantly correlated to the live birth rate ($^{**}P = 0.0032$) (Fig. 4B). Furthermore, the mean number of pups per dam was significantly lower in mice with cysts present (4 ± 1.29), compared to mice without cysts (10 ± 1.10, $^{*}P = 0.0119$) (Fig. 4C). Data from both control and rapamycin groups were pooled to perform the correlation tests and to calculate the mean number of pups per dam with/without cysts in the ovary.

Rapamycin preserved primordial follicle pool in mice

The use of rapamycin resulted in a higher number of mice still having a primordial pool at the end of the experiment compared to control mice. Besides the superovulation, 20 other females were sacrificed at the end of the experiment and their ovaries were collected to analyze whether they had any remaining primordial follicles. A significant correlation between adding rapamycin to the freezing medium and the presence of primordial follicles at the end of the experiment was found ($^{*}P = 0.0397$). Indeed, 8 mice whose ovaries were slow-frozen with rapamycin still had remaining primordial follicles compared to 3 mice in the control group (Fig. 5A–B).

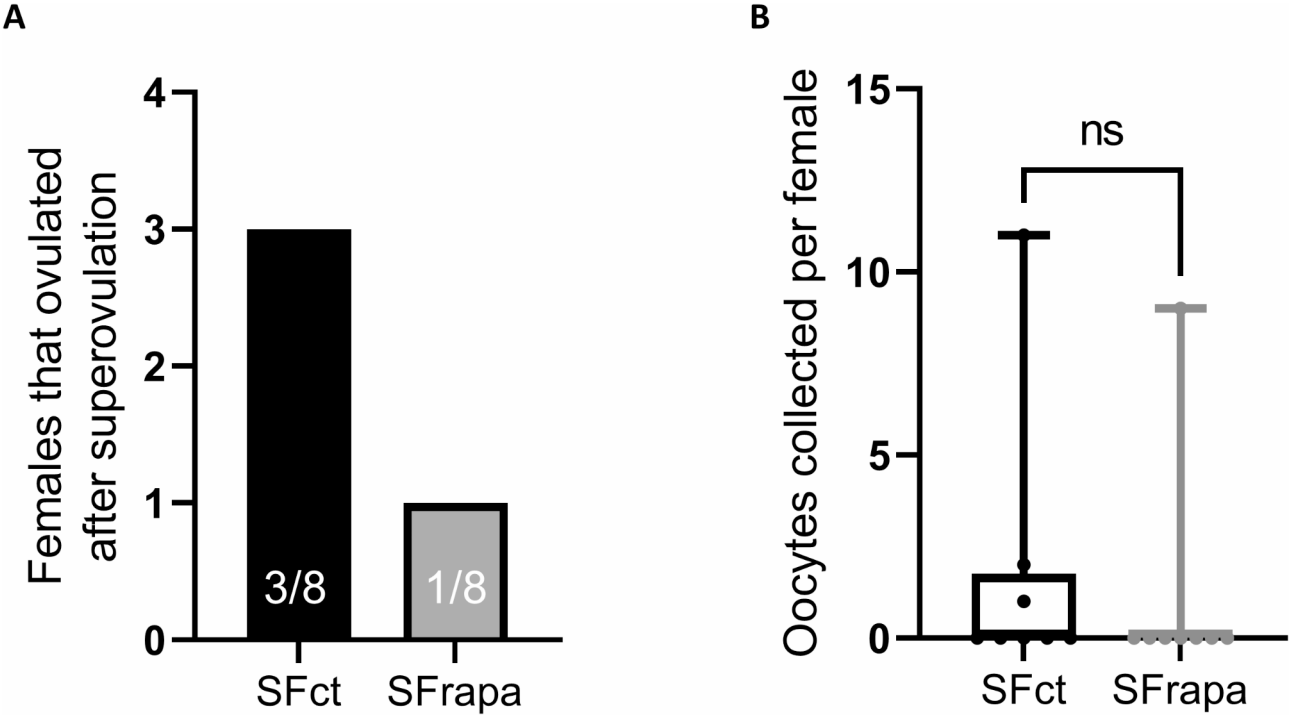

Fig. 3. Superovulation outcome in mice ± 6 months after orthotopic autotransplantation with ovaries slow-frozen in control medium or with ovaries slow-frozen in medium supplemented with rapamycin. (**A**) Comparison of the number of mice (SFct versus SFrapa groups) that ovulated at least one oocyte after stimulation. (**B**) The mean number of oocytes collected per female after superovulation (median + min to max). SFct = ovaries slow-frozen in control medium, SFrapa = ovaries slow-frozen in medium supplemented with rapamycin. Mann-Whitney test: ns = non-significant. $n = 8$ mice per group.

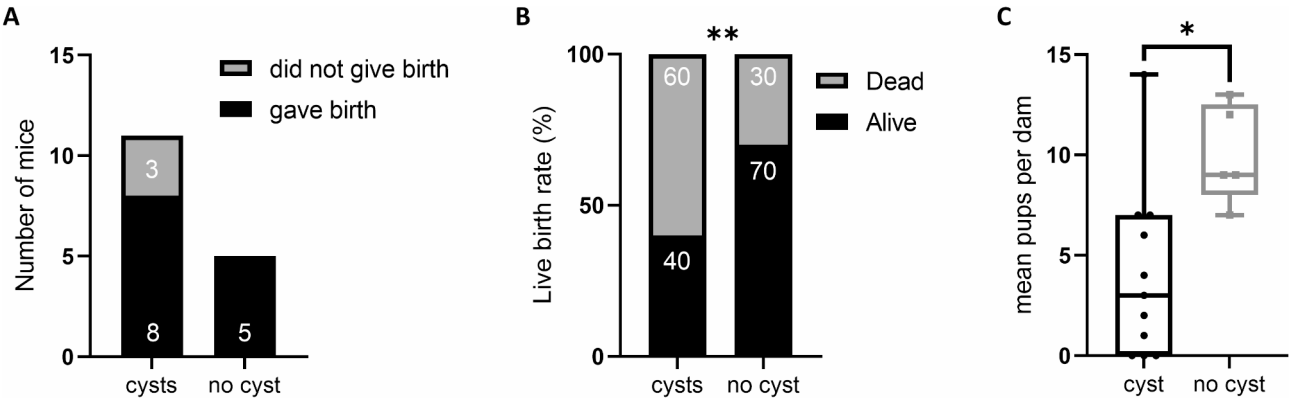

Fig. 4. Correlation between the presence of cysts and various fertility parameters in mice orthotopically autotransplanted with slow-frozen ovaries. The correlation between the presence of ovarian cysts and various fertility parameters was analyzed, including the ability to give birth (**A**), the live birth rate (**B**), and the mean number of pups per dam (**C**) (median + min to max). SFct = ovaries slow-frozen in control medium, SFrapa = ovaries slow-frozen in medium supplemented with rapamycin. Chi-square test (**A** and **B**) or Mann-Whitney test (**C**): * $P \leq 0.05$, ** $P \leq 0.01$. $n = 16$ total mice. Results contain pooled data from the control and rapamycin groups.

We then quantified the number of primordial follicles in ovaries from those mice. No difference in primordial follicle density was observed between the two experimental groups (figure S3).

Discussion

Female mice whose ovaries were cryopreserved with rapamycin in the freezing medium prior to chemically disabling the remaining ovary, and subsequent orthotopic autotransplantation gave birth to more pups with

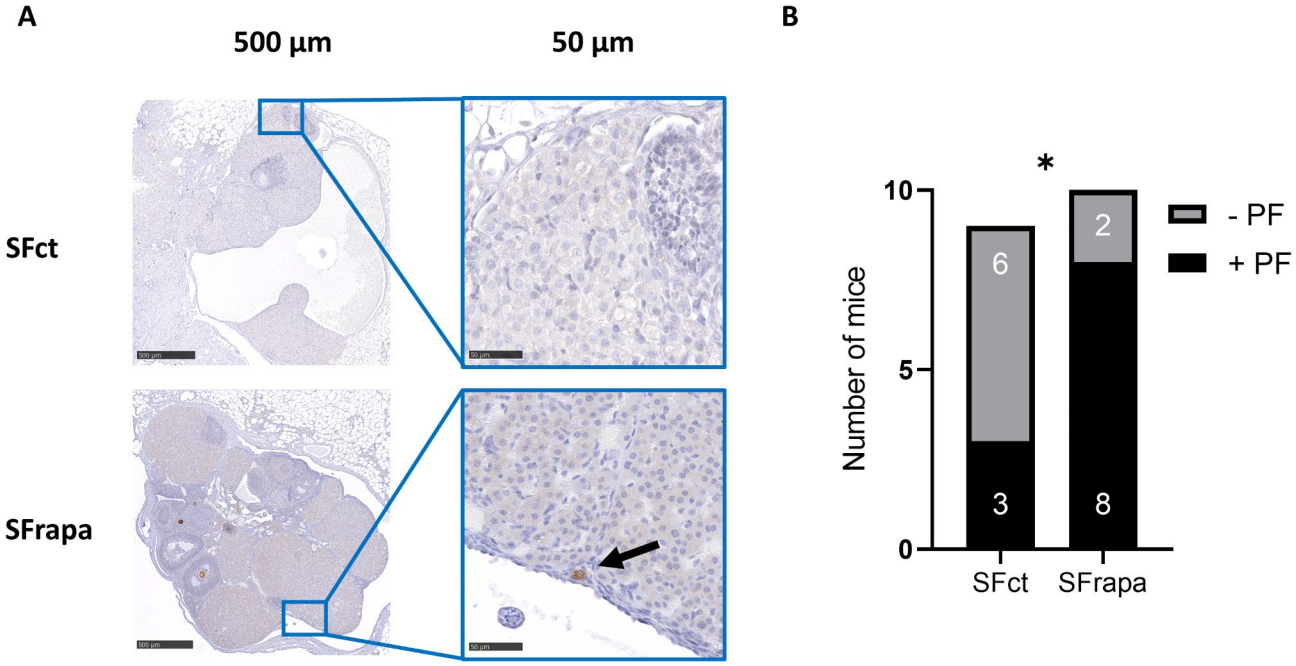

Fig. 5. Evaluation of the presence of primordial follicles at the end of the experiment in mice ovaries orthotopically autotransplanted to the remaining ovarian bursa of C57BL/6 mice for ± 6 months. (**A**) Representative images of DDX4 staining of SFct and SFrapa mice ovaries orthotopically transplanted. The arrow indicates primordial follicle. (**B**) Immunohistochemistry-assisted comparison of the number of mice still having primordial follicles at the time of sacrifice between the SFct and the SFrapa groups, including correlation analysis. SFct = ovaries slow-frozen in control medium, SFrapa = ovaries slow-frozen in medium supplemented with rapamycin, - PF = mice without primordial follicles at the end of the experiment, + PF = mice with primordial follicles left at the end of the experiment. Chi-square test: * $P \leq 0.05$. $n = 9–10$ mice per group.

a significantly greater live birth rate after 4 months of mating compared to mice whose ovaries were frozen in control medium. Additionally, a significantly higher number of mice in the rapamycin group still had primordial follicles at the end of the experiment, and there was a trend indicating that more mice in the rapamycin group gave birth with a higher average litter size. However, no differences were observed in estrous cycle recovery, although a significant association between the restoration of estrous cyclicity and the capacity to produce offspring was found. Furthermore, the number of oocytes collected after superovulation and the number of litters per mouse were similar between the groups, as well as ovarian cyst formation. While rapamycin did not influence cyst formation, we found that mice with ovarian cysts gave birth to significantly fewer pups per dam and had significantly lower live birth rates compared to mice without ovarian cysts. However, no correlation was observed between the presence of cysts and the possibility for mice to deliver offspring.

Autotransplantation of ovarian tissue successfully restored estrous cyclicity in a portion of the mice, regardless of the use of rapamycin during the freezing process. This finding aligns with other studies that have evaluated the effectiveness of ovarian transplantation in restoring hormonal cyclicity. For instance, subcutaneous transplantation of ovarian tissue in bilaterally oophorectomized mice showed approximately 75% of the females regaining cyclicity within 3 weeks[37]. Another study found that orthotopic transplantation of cryopreserved/thawed ovaries could restore the estrous cycle within 2 weeks after grafting in all rats whose normal cyclicity was disrupted by chemotherapy treatment[38].

Interestingly, although we found no statistically significant difference in the proportion of mice recovering estrous cyclicity between the control and rapamycin groups, our results did reveal a significant correlation between restored cyclicity and improved fertility outcomes. This suggests that while returning to normal cyclicity is a key indicator of ovarian function, it is not the sole determinant of successful fertility restoration. Factors such as the quality and quantity of the primordial follicle pool, robust graft revascularization, and the prevention of rapid follicle "burn-out" also play critical roles. Our evidence indicates that rapamycin's protective effect on the primordial follicle reserve likely contributed to better long-term fertility outcomes, even if estrous cycle recovery itself was not significantly different between the groups.

Our previous work has demonstrated that adding rapamycin to the cryopreservation protocol can counteract slow-freezing-induced follicle activation in a heterotopic ovarian transplantation model, providing mechanistic insights into how rapamycin influences the Akt/mTOR pathway[24]. These earlier findings indicated that rapamycin modulates key signaling components involved in follicle survival and quiescence. However, we did not present these mechanistic data in the current manuscript because the experiments were conducted in a different transplantation model and at earlier timepoints more suitable for detecting transient molecular

changes. We chose to avoid conflating two distinct experimental settings and to maintain our focus on the long-term orthotopic transplantation outcomes described herein.

Performing additional molecular analyses in this long-term model would have required sacrificing mice at earlier timepoints to capture the immediate effects of rapamycin on the mTOR pathway. Given the ethical considerations and the study's primary objective of assessing long-term fertility restoration, we opted not to conduct these analyses. Thus, while we acknowledge that direct mechanistic data (e.g., Western blot or qPCR analyses for mTOR and apoptosis markers) would further strengthen the understanding of rapamycin's action, we emphasize that mechanistic aspects have been previously established. Future research could build upon both our earlier mechanistic studies and the current findings by integrating additional timepoints and interventions aimed at delineating the molecular changes across the transplantation timeline.

Our present results showed that adding rapamycin during the cryopreservation of ovaries prior to transplantation to mice with ovarian failure improved fertility preservation and increased graft survival. This was evident from the presence of primordial follicles approximately 6 months after grafting. To the best of our knowledge, the effect of including rapamycin during the slow-freezing process before orthotopic autotransplantation has not yet been studied. Recent studies have looked at various applications of rapamycin, such as injecting the inhibitor after ovarian transplantation, which resulted in higher primordial follicle numbers[19,39]. So far, only one in vivo study showed that the rapamycin pre-treatment before vitrification diminished the loss of follicles after grafting and increased graft survival[40]. All these studies show the potential of rapamycin to protect the follicle pool during cryopreservation, which is in line with the improved fertility preservation we found in our study.

While not significant, we found that the rapamycin group had a higher average litter size (3.43) compared to the control group (2.53). For C57BL/6 mice, the average number of pups per litter is approximately 7[41,42]. Thus, a litter size half of this, as observed in the rapamycin group, is close to the normal condition, considering that the mice have only one functioning ovary after transplantation.

At the end of the experiment superovulation was induced. Considering that our transplanted ovaries were ± 7.5 months old (time without storage in nitrogen), we obtained an average of 1.75 oocytes in the control and 1.13 in the rapamycin group. This superovulation efficiency is low, but aligns with the age-related decline reported in mice, where the oocyte number decreases from an average of 22.5 ± 3.8 oocytes/oviduct in 3-month-old females, to only 2.1 ± 0.2 oocytes/oviduct by 15 months[43]. Additionally, the OTCTP procedure itself can shorten the reproductive lifespan and thereby reduce the superovulatory response. Although rapamycin helps preserve the primordial follicle pool, it was not intended to improve superovulation efficiency. Moreover, we did not conduct direct oocyte quality assessments (e.g., in vitro maturation or fertilization tests) to determine whether rapamycin influenced oocyte competence. Future studies will be necessary to assess younger graft ages and incorporate developmental competence assays to determine whether altered superovulation responses or oocyte quality might be observed under different conditions.

Ovarian manipulation, such as ovarian transposition in women, often leads to cyst formation[28]. Consistent with this, one side effect observed in our study was the formation of ovarian cysts after whole ovary transplantation, irrespective of rapamycin treatment. Indeed, cyst incidence was similar between the groups, occurring in 6 out of 8 mice in the control group, compared to 5 out of 8 in the rapamycin group. This finding aligns with previous reports of cyst formation following subcutaneous/subfascial autotransplantation of fresh whole rat ovaries[29], suggesting that the OTCTP technique itself, rather than the brief exposure to rapamycin, is the primary contributor to cystogenesis. As the presence of ovarian cysts could lead to infertility, we performed a correlation analysis between the presence of cysts and different aspects of fertility[36,44]. No correlation was found between the presence of cysts and the ability to give birth. Females that developed ovarian cysts had fewer pups and a lower live birth rate compared to mice without ovarian cysts. Nonetheless, as the occurrence of cysts was similar in both the control and rapamycin groups, it is unlikely that cyst formation solely accounts for the observed differences in fertility outcomes. Thus, we remain confident that rapamycin's protective effects on the follicle pool are the main driver of the improved fertility measures observed in this study.

One of the strengths of our study is that rapamycin is only added during the cryopreservation process, meaning that there will be no contact between the inhibitor and the graft recipient, minimizing the possibility of side effects. The use of rapamycin during the OTCTP procedure in humans should be relatively simple to implement, as it is already used in clinical settings for preventing organ transplant rejection[21,45]. Additionally, both mice and humans have similar reproductive cycles exhibiting periodic fluctuations in progesterone and estrogen levels. These similarities make mice models suitable for addressing specific research questions about reproduction[46]. To further enhance translatability, a xenograft model, in which human ovarian tissue is transplanted into mice for several months, could be employed in future studies[47]. Such a model would allow for direct evaluation of the long-term safety and potential toxicological effects of rapamycin on human follicles, for instance by examining oocyte morphology. One concern is the potential for toxicity of rapamycin towards oocytes, potentially impairing offspring health. Conversely, rapamycin may also improve oocyte quality. Since we did not thoroughly assess these possibilities here, additional research is required. A future follow-up study could compare the fertility of pups born from females that underwent OTCTP with rapamycin-treated grafts to those from control groups[48,49]. Similar fertility indexes between pups born from mice that underwent OTCTP with rapamycin and those from the control group suggest that rapamycin is not toxic to oocytes and does not cause developmental issues in the pups. Furthermore, to determine whether rapamycin can enhance oocyte competence, in vitro maturation assays could be conducted, analyzing indicators such as first polar body morphology[50]. Because oocyte yield in our current study was low—likely due to the advanced age of the transplanted ovaries—superovulation and oocyte collection at an earlier time point might be necessary to obtain sufficient oocytes for detailed quality assessments.

In conclusion, our findings show that adding the mTOR inhibitor rapamycin during the OTCTP procedure significantly improved fertility restoration in mice. By addressing the main issue of the OTCTP technique - the excessive activation of primordial follicles after grafting - this method shows promise in prolonging the lifespan of the ovarian graft quality. This advancement could extend the window during which recovered patients can conceive, thereby increasing the likelihood of successful pregnancies following OTCTP usage.

Data availability

Data is provided within the manuscript or supplementary information file.

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Author contributions

C.M. and J.B. designed the study and methodology.; J.B. performed the experiments and statistical analysis with input and assistance from M.S. and L.B.; J.B. and C.M. drafted the manuscript.; J.B., C.M., M.S., L.B., and M.N. reviewed and edited the manuscript.; C.M. and M.N. acquired the funding.; C.M. supervised the project. All authors have read and agreed to the published version of the manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Ethical approval

This study was approved by the Animal Ethics Committee of the University of Liège (#2270). We confirmed that all experiments in this study were performed in accordance with the relevant guidelines and regulations. All the procedure of the study is followed by the ARRIVE guidelines.

Additional information

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