3. Réduction de l'apoptose liée à la cryopréservation

3.1. Introduction

Comme déjà mentionné à plusieurs reprises dans ce manuscrit, la cryopréservation ovarienne est une technique prometteuse mais qui nécessite encore d'être améliorée à plusieurs niveaux. En effet, une perte folliculaire massive est observée suite à la réimplantation du fragment qui est avasculaire, mais la congélation joue également un rôle dans l'altération tissulaire. En effet, la congélation lente de type *slow freezing*, utilisée en clinique pour la congélation tissulaire, est connue pour induire une fibrose tissulaire (Nisolle 2000) ainsi que pour altérer la viabilité des cellules, tant stromales que folliculaires (Keros 2009, Silber 2012). L'apoptose semble jouer un rôle important dans l'apparition de ces lésions tissulaires liées à la congélation, notamment via l'activation des caspases et du système FAS (Stroh 2002, Rimon 2005, Xiao 2010).

Parmi les anti-apoptotiques déjà étudiés dans la littérature lors de la cryopréservation ovarienne, nous retrouvons le Z-VAD-FMK, qui est un inhibiteur pan-caspase ainsi que le S1P, un lipide bioactif possédant notamment des propriétés anti-apoptotiques par l'intermédiaire du système Fas. Utilisé dans le milieu de vitrification d'ovaires murins, le Z-VAD-FMK a permis de réduire l'apoptose ainsi que le nombre de jours nécessaires au rétablissement d'un cycle œstral après réimplantation ovarienne chez la souris (Zhang 2009). Le S1P ajouté au milieu de vitrification d'ovaires murins permet également une protection de la réserve en follicules primordiaux après la transplantation (Jee 2010, Tsai 2013).

Nous avons séparé nos recherches en 2 axes. Le premier axe concerne l'évaluation et la comparaison des effets de ces deux anti-apoptotiques, Z-VAD-FMK et S1P, ajoutés aux milieux de transport et de congélation de tissu ovarien ovin, sur la survie folliculaire après culture *in vitro* des fragments après décongélation. Cette culture permet d'étudier les effets de la congélation puisque l'intégrité tissulaire analysée directement après décongélation ne reflète pas réellement l'état tissulaire, et ce sans faire intervenir la transplantation (Keros 2009, Maffei 2013, Sanfilippo 2013).

Le deuxième axe vise à étudier *in vitro* l'effet protecteur du Z-VAD-FMK sur des cultures de 3 lignées différentes de cellules de la granulosa (GC1a, HGL5 et COV434), dans des conditions pouvant induire une mort cellulaire. Pour améliorer la survie folliculaire lors de la cryopréservation ovarienne, il est primordial d'avoir un effet protecteur sur l'entièreté

du follicule afin de conserver l'ovocyte ainsi que les cellules folliculaires. Les cellules folliculaires de la granulosa, qui entourent l'ovocyte, jouent un rôle indispensable dans le développement et la maturation folliculaire. En condition d'hypoxie, les cellules de la granulosa peuvent accroître leur synthèse de VEGF (Koos 1995), indispensable pour réduire la période hypoxique suivant la réimplantation de fragments de cortex ovarien après cryopréservation. Il a par ailleurs été démontré que les cellules de la granulosa, plus que les ovocytes, sont affectées par les processus de congélation/décongélation (Siebzehnrubl 2000). Etant donné la difficulté d'approvisionnement ainsi que la durée de vie limitée des cultures primaires de cellules de la granulosa, des lignées cellulaires humaines de la granulosa ont été développées pour faciliter la recherche (Havelock 2004).

3.2. Addition d'anti-apoptotiques lors du processus de cryopréservation

Publication n° 5

"Supplementation of transport and freezing media with anti-apoptotic drugs improves ovarian cortex survival."

Henry L, Fransolet M, Labied S, Blacher S, Masereel MC, Foidart JM, Noel A, Nisolle M and Munaut C.

J Ovarian Res. 2016;9(1):4. doi: 10.1186/s13048-016-0216-0.

3.2.1. Résumé des résultats

Lors de l'évaluation de la densité folliculaire du tissu mis en culture, nous avons observé une chute progressive de la densité en follicules primordiaux au cours du temps. Nous avons démontré que l'ajout de Z-VAD-FMK permet de réduire significativement la perte de follicules primordiaux par rapport au contrôle, avec une densité folliculaire qui se maintient jusqu'à 6 jours de culture.

Nous avons analysé la qualité folliculaire en nous basant sur l'analyse de la morphologie des follicules primordiaux et de la prolifération des cellules de la granulosa. Nous avons observé que l'ajout de S1P permet de limiter la diminution de la densité des follicules morphologiquement normaux par rapport au groupe contrôle, dans le tissu frais ainsi qu'après 2 jours de culture. Les effets du Z-VAD-FMK semblent se refléter plus tardivement avec, après 6 jours de culture, une densité en follicules morphologiquement normaux plus élevée que dans le groupe contrôle. Cette amélioration de la qualité folliculaire a été constatée avec l'étude de la prolifération des cellules de la granulosa puisque le S1P

augmente leur nombre après 2 jours de culture et le Z-VAD-FMK après 6 jours.

Nous avons également analysé la prolifération cellulaire globale au sein du tissu (cellules stromales et cellules folliculaires) qui reflète la qualité et la survie tissulaire après la congélation. Nous avons mis en évidence que cette prolifération n'est pas modifiée par rapport au contrôle avec l'ajout de S1P mais est améliorée, tant après 2 jours que 6 jours, par l'adjonction de Z-VAD-FMK.

3.2.2. Discussion

Une des limitations de la cryopréservation ovarienne est la déplétion folliculaire importante liée à la cryopréservation et à la transplantation des fragments ovariens, aboutissant à une durée de vie réduite des greffons. Ce phénomène est lié au nombre de follicules primordiaux présents au sein du fragment et aux nombres de cycles ovulatoires possibles qui en découlent. La congélation des fragments est à l'origine d'une partie de cette perte folliculaire, et est en partie due à l'activation de l'apoptose (Liu 2002, Rimon 2005). Chez l'homme, l'utilisation de molécules anti-apoptotiques au niveau du site de transplantation est difficile et, surtout limitée éthiquement chez des patients au contexte néoplasique. Nous avons dès lors décidé de traiter les fragments ovariens uniquement *ex vivo*, lors du transport, de la préparation et de la congélation du cortex ovarien. Nous avons utilisé un système de culture *in vitro* jusqu'à 6 jours afin de pouvoir évaluer le tissu ainsi que l'intégrité et la viabilité folliculaire. En effet, l'analyse du fragment directement après sa décongélation ne permet pas d'évaluer l'état réel du tissu.

Nos résultats confirment l'effet cryoprotecteur du Z-VAD-FMK déjà obtenu lors de son utilisation dans les milieux de congélation d'ovaires murins, d'embryons porcins et d'autres types cellulaires (Stroh 2002, Men 2006, Zhang 2009). Néanmoins, notre étude a l'avantage, par rapport à ces dernières, de concerner une espèce plus proche de l'homme, la brebis. Nous confirmons également l'effet bénéfique, bien que dans une moindre mesure que pour le Z-VAD-FMK, du lipide bioactif S1P dont l'effet est controversé dans la littérature.

Henry *et al. Journal of Ovarian Research* (2016) 9:4
DOI 10.1186/s13048-016-0216-0

Journal of Ovarian Research

RESEARCH Open Access

Supplementation of transport and freezing media with anti-apoptotic drugs improves ovarian cortex survival

Laurie Henry[1,2], Maïté Fransolet[1], Soraya Labied[1,2], Silvia Blacher[1], Marie-Caroline Masereel[1,2], Jean-Michel Foidart[1], Agnès Noel[1], Michelle Nisolle[1,2] and Carine Munaut[1*]

Abstract

Background: Ovarian tissue preservation is proposed to patients at risk of premature ovarian failure, but this procedure still needs to be optimized. To limit injury during ovarian tissue cryopreservation, anti-apoptotic drugs were added to the transport and freezing media of ovarian cortex tissue.

Methods: Sheep ovaries were transported, prepared and frozen in solutions containing vehicle or anti-apoptotic drugs (Z-VAD-FMK, a pan-caspase inhibitor, or sphingosine-1-phosphate (S1P), a bioactive lipid). After the tissue was thawed, the ovarian cortex was cultured for 2 or 6 days. Follicular quantification and morphological and proliferation analyses were performed on histological sections.

Results: After 2 days of culture, S1P improved the quality of primordial follicles; higher densities of morphologically normal and proliferative primordial follicles were found. Z-VAD-FMK displayed similar effects by preserving global primordial follicular density, but this effect was evident after 6 days of culture. This drug also improved cell proliferation after 2 and 6 days of culture.

Conclusions: Our results showed that the addition of S1P or Z-VAD-FMK to the transport and freezing media prior to ovarian tissue cryopreservation improves primordial follicular quality and therefore improves global tissue survival. This should ultimately lead to improved fertility restoration after auto-transplantation.

Keywords: Ovarian cryopreservation, anti-apoptotic drugs, slow-freezing, fertility preservation

Background

Currently, patients with cancer clearly benefit from aggressive chemotherapy, radiotherapy and bone marrow transplantation. However, these treatments can induce premature ovarian failure (POF) in girls or young women. Ovarian cryopreservation should be proposed to these patients before they begin treatment. This procedure has already allowed the birth of more than 40 babies across the world [1–3], but it still needs to be optimized. Indeed, ovarian cortex cryopreservation followed by auto-transplantation after cancer remission affects the quality of the ovarian tissue. The primordial follicular reserve decreases, which consequently impairs the survival lifespan of the grafted tissue fragments, limiting the possibility for procreation.

Ovarian transplantation is avascular, which results in tissue ischemia over the 5 first days after grafting and is sometimes associated with reperfusion injury [4, 5]. This transient hypoxic period is followed by gradual oxygenation, leading to reperfusion of the ovarian transplant. This process represents the main origin of ischemia and follicular loss [6, 7]. The freezing procedure also induces tissue damage. In fact, slow freezing is the gold standard for ovarian tissue cryopreservation. However, this technique is known to lead to tissue fibrosis [8] and to alter the viability of both stromal and follicular cells within the ovarian samples [9, 10]. Apoptosis also plays an important role in cryo-injuries, mainly by the activation caspases and the Fas system [11–13]. Indeed, caspase

* Correspondence: c.munaut@ulg.ac.be
[1]Laboratory of Tumor and Development Biology, Groupe Interdisciplinaire de Génoprotéomique Appliquée (GIGA-R), Université de Liège, Tour de Pathologie (B23) Sart-Tilman, B-4000 Liège, Belgium
Full list of author information is available at the end of the article

© 2016 Henry et al. **Open Access** This article is distributed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The Creative Commons Public Domain Dedication waiver (http://creativecommons.org/publicdomain/zero/1.0/) applies to the data made available in this article, unless otherwise stated.

activation was observed in frozen-thawed tissue with preserved architecture [14].

Several anti-apoptotic drugs have already been proposed to improve fertility preservation. They were studied along with preventive treatment during oncologic therapy rather than during the ovarian tissue cryopreservation process, including during freezing or transplantation. Among these drugs, imatinib, a kinase inhibitor that mainly targets the ABL family members KIT and PDGFR, protects ovarian tissue during chemotherapy by inhibiting cisplatin-induced Tap63-α phosphorylation [15]. Sphingosine-1-phosphate (S1P) is a bioactive lipid in follicular fluid that has two interesting properties: it is anti-apoptotic and pro-angiogenic [16]. In several studies, S1P has been shown to protect the follicle reserve by decreasing apoptosis during chemotherapy and radiotherapy [17–22].

On the other hand, anti-apoptotic drugs have been used during the cryopreservation process to prevent the activation of apoptotic pathways and subsequently improve tissue survival during freezing and grafting. The addition of Z-VAD-FMK, a pan-caspase inhibitor, to the freezing and thawing media has been shown to decrease the apoptosis rate and the number of days before the estrous cycle is resumed following auto-transplantation of cryopreserved mouse ovaries [23]. S1P has also been used in the vitrification media before the transplantation of mouse ovaries and conferred significant protection of primordial follicles after grafting [24, 25].

Anti-apoptotic drugs have also been evaluated during tissue transplantation. In an auto-transplantation model of fresh sheep ovarian fragments into the abdominal wall, S1P did not show a beneficial effect [26]. However, the use of the same drug in xenografts of fresh human ovarian cortex in immunodeficient mice improved angiogenesis and decreased follicular apoptosis [27].

Meanwhile, these anti-apoptotic drugs could have a protective effect on ovaries during oncologic treatment or transplantation in mice, but the use of these anti-apoptotic drugs in vivo during oncologic treatment is only possible in animal models. However, their use in cryopreservation media alone appears to be safer and could be applicable to humans.

In vitro culture of thawed ovarian tissue is an important approach to use to analyze tissue after the cryopreservation process because the integrity of the tissue immediately after thawing may not reflect its true state [10, 28, 29]. It also enables observation of the effects of cryopreservation without transplantation.

The purpose of our study was to analyze and compare the effects of two anti-apoptotic drugs, Z-VAD-FMK and S1P, when added to the transport and freezing media for sheep ovarian cortex pieces, on survival during in vitro culture of the tissue immediately after thawing.

Methods

Ovarian tissue sampling

This study was approved by the Animal Ethics Committees of the Universities of Liège and Namur. Our experimental design is detailed in Fig. 1. Four ewes, 4 and 5 months old, were obtained from the Ovine Research Center of Namur University, and their ovaries were collected immediately after the ewes were euthanized. The ovaries were cut into 2 pieces, and one piece of each ovary was immersed in transport media composed of Leibovitz L-15 medium (Lonza, Verviers, Belgium, BE12-700 F) supplemented with 10 % normal sheep serum (Hormonology Laboratory, Belgium) and anti-apoptotic drugs. The anti-apoptotic drugs included 10 μM Z-VAD-FMK (R&D, United Kingdom, FMK001), which was diluted in DMSO, and 10 μM S1P (Biomol, Germany, Cay62570-10), which was diluted in 0.3 M NaOH. In the control groups, the anti-apoptotic drugs were replaced with the appropriate vehicles, namely, DMSO for Z-VAD-FMK and NaOH for S1P.

The ovaries were further prepared by removing medullary tissue, and the cortex was cut in 2-mm-diameter round pieces with a disposable biopsy punch (Miltex, Germany, 33-31-P/25). Twenty-four pieces per condition were obtained for the cryopreservation process, and 6 were immediately fixed in 4 % formaldehyde and embedded in paraffin for histological analysis (fresh condition, F). Throughout the entire process of ovarian cortex preparation, the ovaries were kept at 4 °C.

Freezing and thawing of ovarian tissue

Cryopreservation was performed by slow freezing according to the technique first described by Gosden [30, 31]. Z-VAD-FMK (10 μM) or S1P (10 μM) was added to the cryopreservative medium. For thawing, cryovial tubes were removed from liquid nitrogen, left at room temperature for 2 min and then immersed in a 37 °C water bath at until the tissue was completely thawed. The ovarian pieces were subsequently washed three times in 37 °C culture medium without serum to remove the cryoprotective agents. Each wash lasted for 5 min. For each group, 6 pieces were fixed in 4 % formaldehyde and embedded in paraffin for histological analysis (frozen-thawed condition, F-T).

In vitro culture of ovarian pieces

Thawed ovarian fragments were individually transferred to a Cellstar® 96-well plate U-Bottom (Greiner Bio-One, Italy, 650 185). Each well was filled with 100 μL of Dulbecco's Modified Eagle Medium (Life Technologies, Belgium, 31053-028) supplemented with 200 mM L-Glutamine (Life Technologies, Belgium, 25030024), 100 μg/ml ascorbic acid, 1 μl/ml Insulin-Transferrin-Selenium solution (Life Technologies, Belgium, 41400-045), 1 μl/ml penicillin-streptomycin

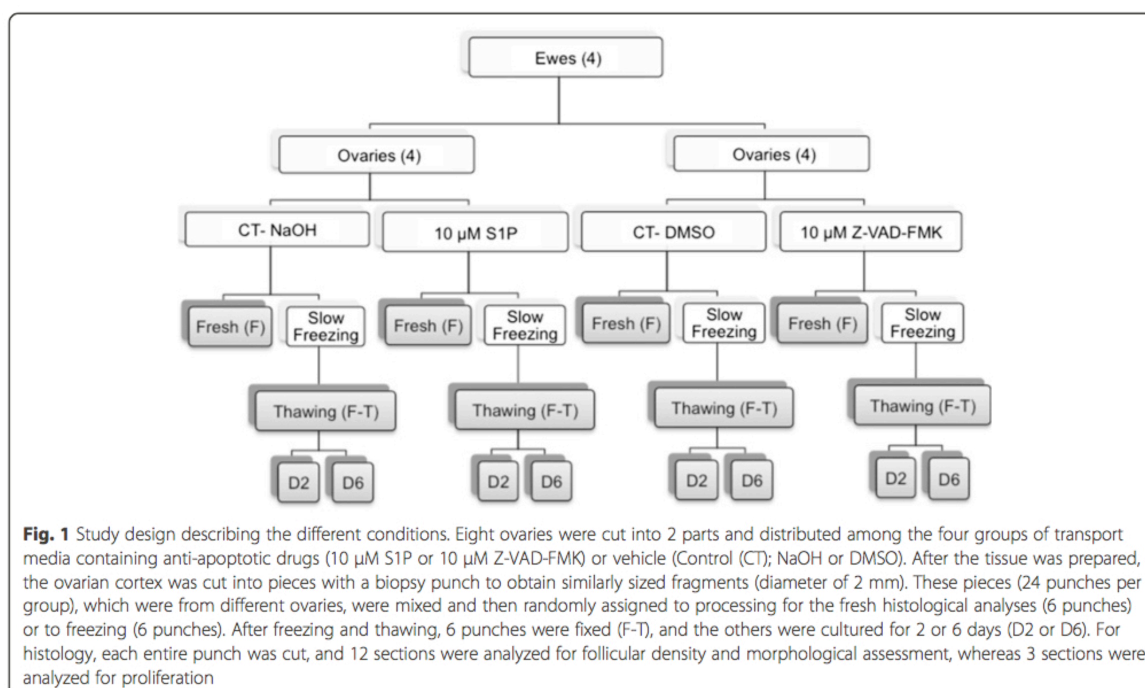

Fig. 1 Study design describing the different conditions. Eight ovaries were cut into 2 parts and distributed among the four groups of transport media containing anti-apoptotic drugs (10 μM S1P or 10 μM Z-VAD-FMK) or vehicle (Control (CT); NaOH or DMSO). After the tissue was prepared, the ovarian cortex was cut into pieces with a biopsy punch to obtain similarly sized fragments (diameter of 2 mm). These pieces (24 punches per group), which were from different ovaries, were mixed and then randomly assigned to processing for the fresh histological analyses (6 punches) or to freezing (6 punches). After freezing and thawing, 6 punches were fixed (F-T), and the others were cultured for 2 or 6 days (D2 or D6). For histology, each entire punch was cut, and 12 sections were analyzed for follicular density and morphological assessment, whereas 3 sections were analyzed for proliferation

(10,000 U/mL; Life Technologies, Belgium, 15140-122), 25 mIU/ml recombinant FSH (Merck, Germany, Gonal-F®) and 5 % normal sheep serum. The tissue fragments were cultured for 2 (D2 condition) or 6 days (D6 condition) at 37 °C in a humidified incubator with 5 % CO_2 and 5 % O_2 as described by Sanfilippo [28]. The ovarian punches were fixed in 4 % formaldehyde, embedded in paraffin and cut into 5-μm serial sections for histological analysis.

Histological analysis

Virtual images were acquired as previously described with an automatic digital slide scanner NanoZoomer 2.0HT (Hamamatsu, Belgium) [32].

To limit the effect of the heterogeneous distribution of the follicular pool within the ovarian cortex, 12 sections per ovarian piece, which covered the entire fragment, were analyzed as previously described [33]. Follicular quantification and morphological evaluation were performed as previously described [34]. Briefly, primordial follicles were considered degenerated if they contained disorganized granulosa cells, shrunken ooplasm or pyknotic oocytes.

Cell proliferation was evaluated by Ki-67 immunolabeling using MIB-1, a monoclonal mouse anti-human Ki-67 antigen clone (Dako, Denmark, M7240), at 1/100. Dako EnVision + HRP anti-mouse (Dako, Denmark, K4001) and DAB+ (K3468, Dako, Belgium) were used to view the immunolabeling.

Follicles were considered proliferative if at least one Ki-67-positive granulosa cell was observed at ×200 magnification (Leica ICC50 HD Camera, Belgium).

Stromal cell proliferation was automatically quantified using the image analysis toolbox of MATLAB 8.1.0.604 (R2013a) (MathWorks, Inc.). Because cell detection was mainly based on color segmentation, contrast was first enhanced by determining the excess of the red component (two times red value minus blue value minus green value). Then, based on the enhanced red component of the resulting color image, binary images of the cells (i.e., pixels belonging to cells were assigned an intensity of 1, whereas background pixels were assigned an intensity of 0) were obtained using an automatic entropy threshold [35]. To eliminate small artifacts, morphological filters [36] were applied on the resultant images. Binary images of the total tissue sections were obtained by applying an appropriate threshold to the blue component of the images. Lastly, cell density was defined as the area occupied by cells divided by the total area of the section.

Statistical analyses

After the outcomes were logarithmically transformed, a linear mixed model was fit to the data to test for differences between the treatments and the number of days in culture. In this model, the ovarian fragment was introduced as a random factor. To correct for multiple comparisons and to avoid type I errors, the level of statistical significance was set at $p = 0.01$. Calculations were always

carried out on the maximum amount of data available. The data analysis was carried out using the SAS (version 9.3 for Windows) statistical package.

Results

Follicular density

The investigation of follicular density in H&E stained sections revealed a very low number of primary and secondary follicles, which precluded statistical analysis. The analysis was therefore performed only on primordial follicles.

Evaluation of the primordial follicular density demonstrated that supplementation with S1P did not completely prevent primordial follicular loss during culture. Nevertheless, follicular density was higher in the S1P-treated group compared to the control group (Fig. 2b, Table 1 and Additional file 1: Tables S1 and S2).

Z-VAD-FMK significantly decreased follicular loss after cryopreservation compared to vehicle (Fig. 2b). Indeed, post-thawing follicular density was maintained after up to 6 days of culture after Z-VAD-FMK treatment, while there was a constant decrease in the control group (Table 1 and Additional file 1: Table S1). Therefore, after 6 days of culture, more follicles were observed in the treated group than in the control group (Fig. 2a and Additional file 1: Table S2).

Table 1 Primordial follicle density (number of primordial follicles/mm^2 (log(X + 1))

	F	F-T	D2	D6
Control NaOH	1.4111	1.7764	0.6482**	0.1584***
10 μM S1P	1.6012	1.6200	0.9070**	0.4799***
Control DMSO	1.6609	1.3464*	1.0350**	0.4099***
10 μM Z-VAD-FMK	1.6209	1.2348*	1.1145	0.9636

*$p < 0.01$ between the frozen-thawed (FT) and the fresh (F) tissue
**$p < 0.01$ between tissue after 2 days of culture (D2) and frozen-thawed tissue
***$p < 0.01$ between 6 (D6) and 2 days of culture
All p values are provided in Additional file 1: Tables S1 and S2

Follicular quality

Morphology of primordial follicles

Primordial follicle morphology was evaluated in H&E sections (Fig. 3a). Morphologically normal follicles were distinguished from degenerated follicles. This analysis showed that the freezing process decreased the density of morphologically normal primordial follicles. This reduction continued progressively throughout the culture duration.

Supplementation with S1P preserved follicular morphology; compared to the density of normal follicles found in the control tissues, the density of normal follicles was greater in the fresh tissue and after 2 days of culture, whereas this difference faded after 6 days in culture (Fig. 3b, Table 2 and Additional file 1: Tables S3 and S4).

Compared to the effects of S1P, the effects of Z-VAD-FMK seemed to occur later. Compared to vehicle, Z-VAD-FMK significantly preserved the density of morphologically normal follicles between 2 and 6 days of culture (Fig. 3c, Table 2 and Additional file 1: Table S3). Therefore, the density of normal primordial follicles that was observed after 6 days of culture

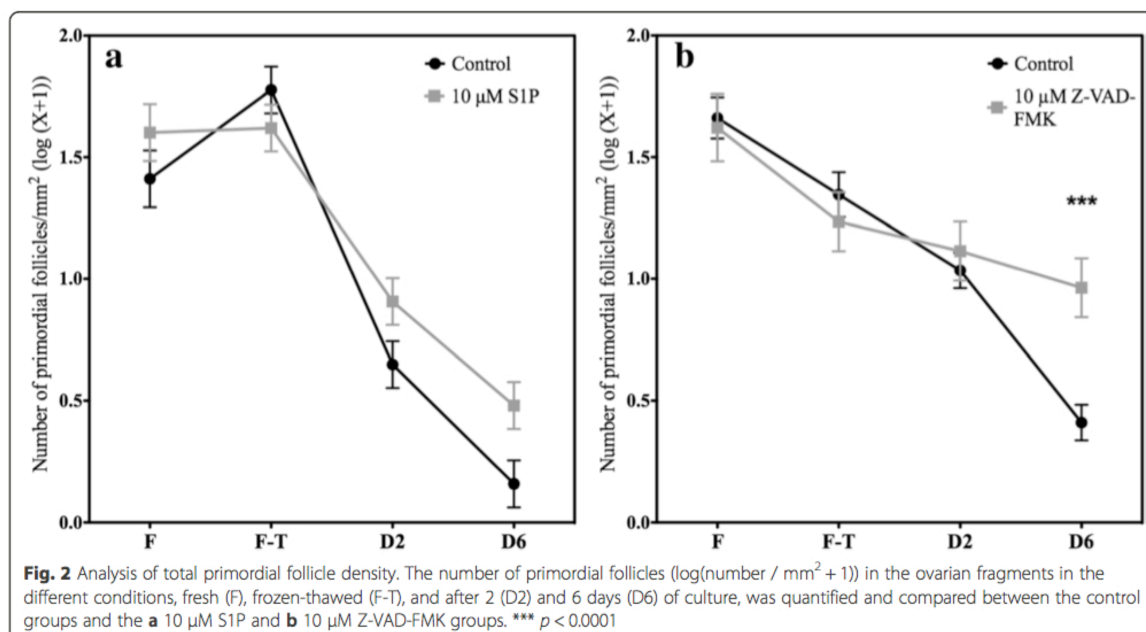

Fig. 2 Analysis of total primordial follicle density. The number of primordial follicles (log(number / mm^2 + 1)) in the ovarian fragments in the different conditions, fresh (F), frozen-thawed (F-T), and after 2 (D2) and 6 days (D6) of culture, was quantified and compared between the control groups and the **a** 10 μM S1P and **b** 10 μM Z-VAD-FMK groups. *** $p < 0.0001$

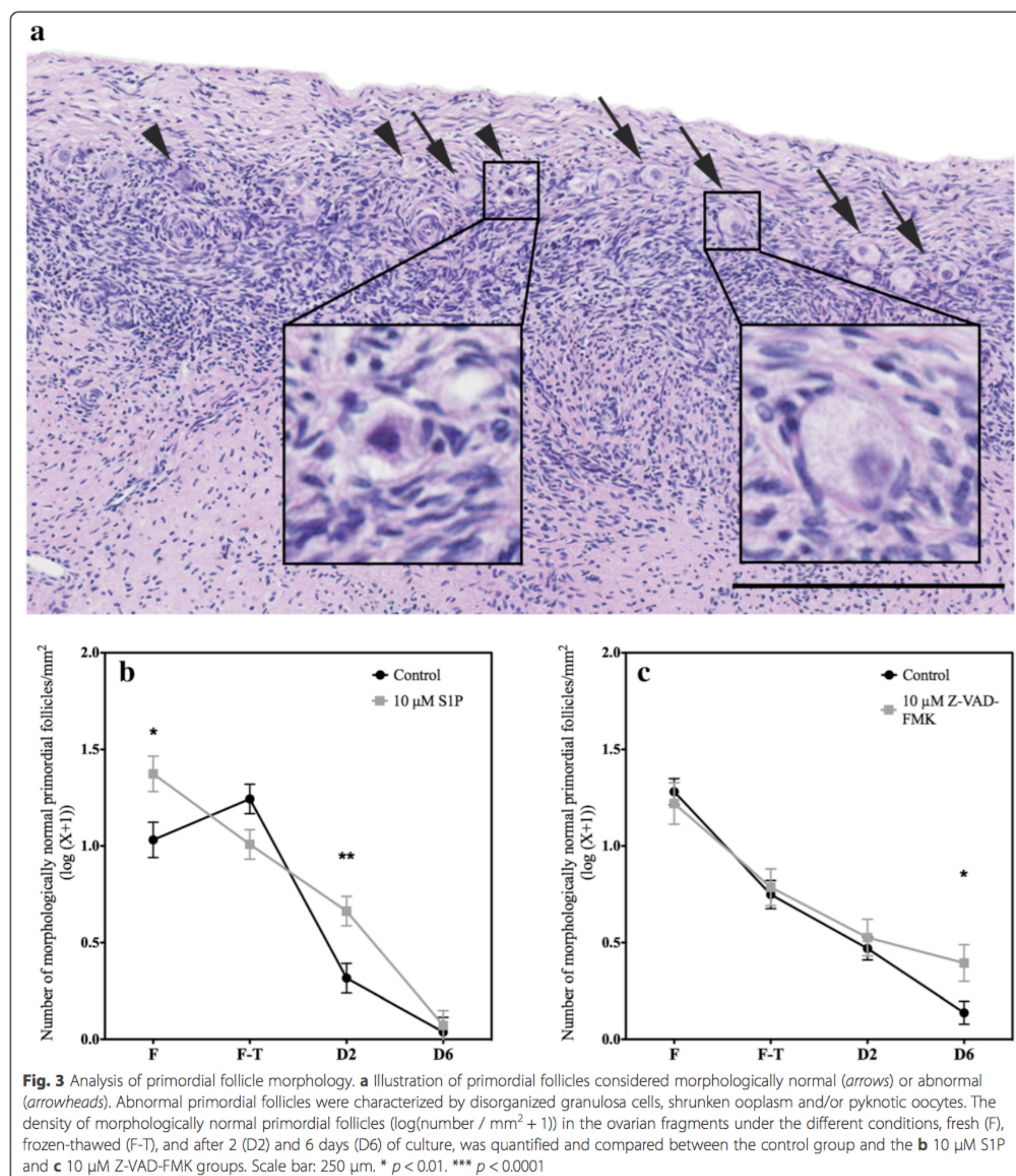

Fig. 3 Analysis of primordial follicle morphology. **a** Illustration of primordial follicles considered morphologically normal (*arrows*) or abnormal (*arrowheads*). Abnormal primordial follicles were characterized by disorganized granulosa cells, shrunken ooplasm and/or pyknotic oocytes. The density of morphologically normal primordial follicles (log(number / mm^2 + 1)) in the ovarian fragments under the different conditions, fresh (F), frozen-thawed (F-T), and after 2 (D2) and 6 days (D6) of culture, was quantified and compared between the control group and the **b** 10 μM S1P and **c** 10 μM Z-VAD-FMK groups. Scale bar: 250 μm. * $p < 0.01$. *** $p < 0.0001$

was higher in the treated group than in the control group (Fig. 3c and Additional file 1: Table S4).

Granulosa cell proliferation

Granulosa cell proliferation, as analyzed by Ki-67 immunostaining, was used as a second marker of follicular health (Figs. 4 and 5). Granulosa cells that were associated with healthy follicles were able to proliferate as soon as 2 days of culture; thereafter, proliferation seemed to decrease, as observed after 6 days (Fig. 6a and b). Supplementation with anti-apoptotic drugs resulted in a higher density of proliferative granulosa cells

Table 2 Morphologically normal primordial follicle density (number of primordial follicles/mm² (log(X + 1))

	F	F-T	D2	D6
Control NaOH	1.0316	1.2435	0.3161**	0.03793***
10 μM S1P	1.3735	1.0081*	0.6635**	0.07230***
Control DMSO	1.2810	0.7495*	0.4699**	0.1374***
10 μM Z-VAD-FMK	1.2200	0.7870*	0.5263**	0.3946

*$p < 0.01$ between the frozen-thawed (FT) and the fresh (F) tissue
**$p < 0.01$ between tissue after 2 days of culture (D2) and frozen-thawed tissue
***$p < 0.01$ between 6 (D6) and 2 days of culture
All p values are provided in Additional file 1: Tables S3 and S4

(which were defined as proliferative primordial follicles) after 2 days of culture with S1P (Figs. 4 and 6a and Additional file 1: Table S5) and after 6 days of culture with Z-VAD-FMK (Figs. 5 and 6b and Additional file 1: Table S5), confirming the beneficial effect of the 2 anti-apoptotic drugs on follicular health.

Cell proliferation

Global proliferation (granulosa and stroma) in ovarian punches, as revealed by Ki-67 immunostaining was used to evaluate the quality of the ovarian tissue. The proliferation of follicles together with the presence of mitosis in granulosa cells serves as an indication of the viability of follicles that are grown in culture. These measures also indicate that frozen/thawed follicles can remain functional over the culture period.

Supplementation with S1P did not affect proliferation (Fig. 6c and Additional file 1: Table S6), whereas increased proliferative cell density was found after 2 and 6 days of culture after supplementation with Z-VAD-FMK compared to vehicle treatment, demonstrating that this anti-apoptotic drug improved tissue survival (Fig. 6d, Additional file 1: Table S6).

Discussion

In the present study, ovarian tissues treated with anti-apoptotic drugs before and during cryopreservation showed significantly higher primordial follicle density and quality after 2 or 6 days of culture. Two different drugs, Z-VAD-FMK and SIP, were added in the transport and freezing media used for the sheep ovarian tissue. For SIP, these positive effects were evident over the short term, after 2 days in culture, whereas for Z-VAD-FMK, they were evident over the long term, after 6 days in culture. Our results demonstrated that Z-VAD-FMK supplementation during transport and slow freezing improved the survival of the ovarian tissue, as demonstrated by a higher rate of cell proliferation after 2 and 6 days of culture compared to that of the control. In our study, primary and more mature follicles were not analyzed due to their low numbers, and transitional follicles were considered primordial. Only immature follicles enable sustainable restoration of ovarian function after transplantation because antral follicles are not cryoresistant [2].

Ovarian tissue cryopreservation followed by autotransplantation is a promising method that has produced good results. However, this technique still needs to be

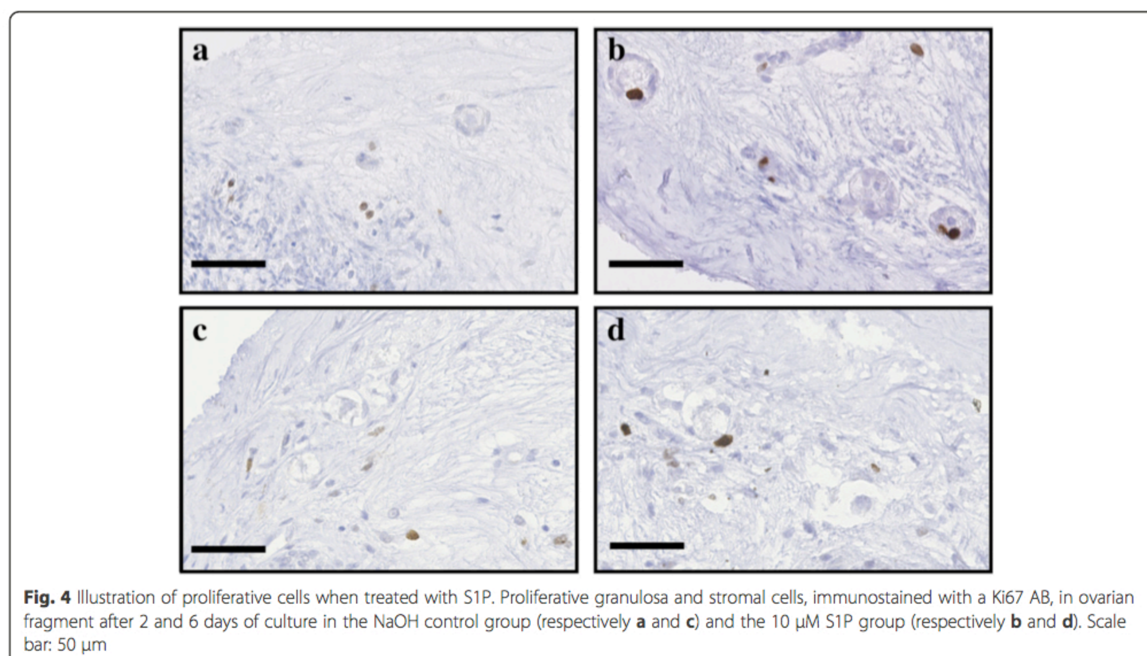

Fig. 4 Illustration of proliferative cells when treated with S1P. Proliferative granulosa and stromal cells, immunostained with a Ki67 AB, in ovarian fragment after 2 and 6 days of culture in the NaOH control group (respectively **a** and **c**) and the 10 μM S1P group (respectively **b** and **d**). Scale bar: 50 μm

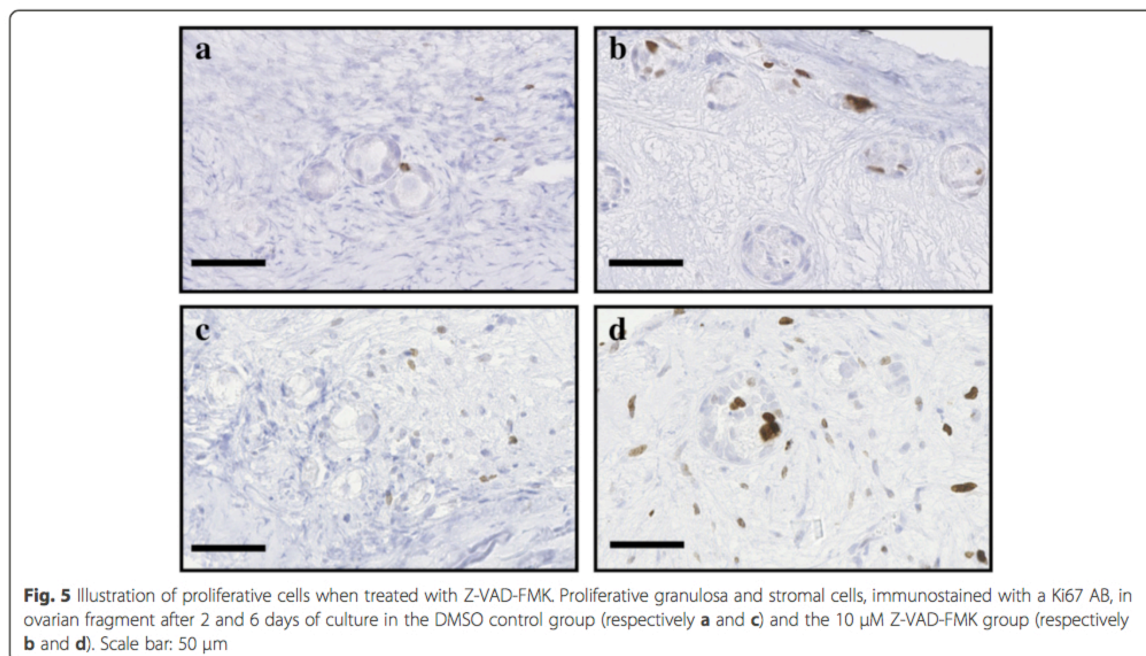

Fig. 5 Illustration of proliferative cells when treated with Z-VAD-FMK. Proliferative granulosa and stromal cells, immunostained with a Ki67 AB, in ovarian fragment after 2 and 6 days of culture in the DMSO control group (respectively **a** and **c**) and the 10 μM Z-VAD-FMK group (respectively **b** and **d**). Scale bar: 50 μm

improved, including both the freezing and transplantation processes. Indeed, significant apoptotic loss of primordial follicles occurs during cryopreservation [13, 37]. In humans, the use of anti-apoptotic drugs within the site of transplantation is technically difficult and also raises ethical issues. We therefore decided to treat ovarian samples only ex vivo. Our aim was to assess if supplementation of the transport, preparation and cryopreservation media before transplantation of the ovarian cortex could be beneficial. The in vitro tissue culture system that was utilized was previously used by others to test the effects of drugs on ovarian tissue survival [10, 28, 29] and allowed us to evaluate tissue and follicle viability and integrity. Indeed, the analysis of cortex samples immediately after thawing may not reflect their real state [10, 28]. We therefore performed tissue culture for up to 6 days after the tissue was thawed.

The potential benefit of anti-apoptotic drugs such as Z-VAD-FMK was based on previous studies that described significant apoptotic loss of primordial follicles during slow freezing-based cryopreservation [13, 37] and by studies that described the role of caspases in the activation of apoptosis in granulosa cells via activation of the Fas system [12, 14]. This anti-apoptotic drug was already identified as a cryoprotective agent that improves the recovery and the survival of cryopreserved cells and vitrified porcine embryos [11, 38]. The bioactive lipid, S1P, has been demonstrated to limit ovarian toxicity during both chemo- and radiotherapy [17–21] (see Additional file: 2 FigureS1) . Addition of S1P in granulosa cells culture has shown to prevent apoptosis induced by oxidative stress [39]. Its protective effects during ovarian tissue cryopreservation and transplantation are more controversial. When used during autotransplantation of fresh ovine tissue, no beneficial effect was observed [26]. The same effect was described when SIP was added to the freezing media of whole ovine ovaries [40] or to the culture media of ovarian tissue after slow freezing or vitrification [41]. S1P supplementation during vitrification of mouse ovaries improved the number of morphologically intact primordial follicles present after grafting [24, 25]. In our study, S1P did not preserve follicle density after cryopreservation but did improve follicle morphology after transport (fresh tissue) and after 2 days in culture, when increased granulosa cell proliferation was detected. We did not observe an S1P-mediated improvement in cell proliferation. However, S1P has already been shown to improve stromal cell proliferation, although these effects were observed for a higher concentration of S1P and after a different supplementation method [27].

Conclusions

Our results showed that the addition of S1P or Z-VAD-FMK to the transport and freezing media prior to ovarian tissue cryopreservation improved primordial follicular quality and therefore improved global tissue survival.

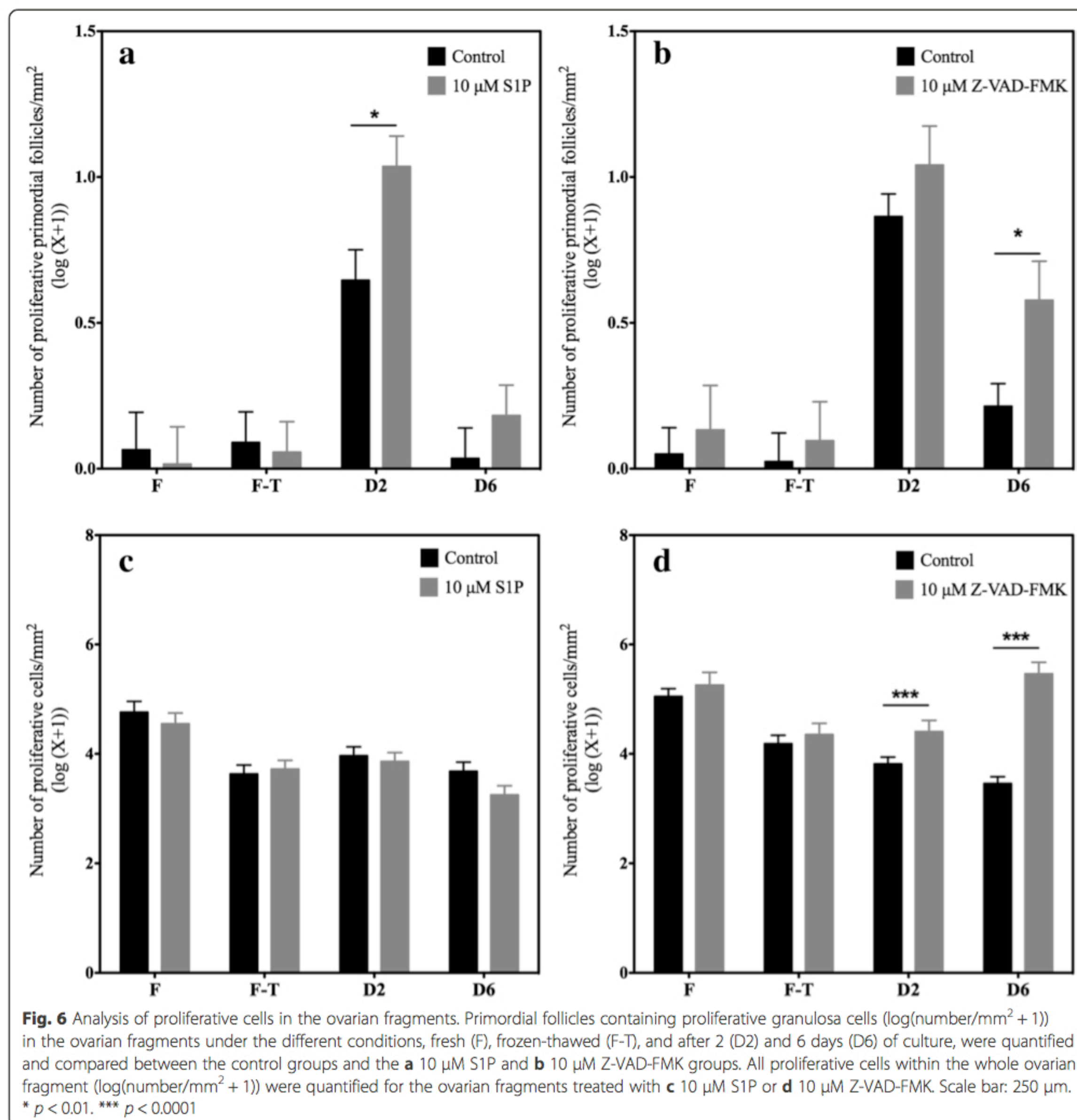

Fig. 6 Analysis of proliferative cells in the ovarian fragments. Primordial follicles containing proliferative granulosa cells (log(number/mm^2 + 1)) in the ovarian fragments under the different conditions, fresh (F), frozen-thawed (F-T), and after 2 (D2) and 6 days (D6) of culture, were quantified and compared between the control groups and the **a** 10 μM S1P and **b** 10 μM Z-VAD-FMK groups. All proliferative cells within the whole ovarian fragment (log(number/mm^2 + 1)) were quantified for the ovarian fragments treated with **c** 10 μM S1P or **d** 10 μM Z-VAD-FMK. Scale bar: 250 μm. * $p < 0.01$. *** $p < 0.0001$

However, the beneficial effects of these two compounds over 2–6 days in culture do not necessarily imply that this approach will improve engraftment and/or maintenance of primordial follicle health after transplantation. Eventually, it will be necessary to show that this approach improves the potential for conception by demonstrating normal follicular development. Similarly, improved oocyte health and fertilization will need to be demonstrated via healthy, live-born pregnancies and other supporting in vivo experimental evidence. Thus, at present, this model only provides a surrogate marker to encourage additional work in this area.

Additional files

Additional file 1: Statistical analyses between the different conditions and treatments. **Table S1** and **Table S2**. Primordial follicular density. **Table S3** and **Table S4**. Density of morphologically normal primordial follicules. **Table S5.** Density of proliferative granulosa cells

between treatments. **Table S6.** Density of global proliferative cells between treatments. (XLSX 38 kb)

Additional file 2: Figure S1. Cellular anti-apoptotic mechanisms of S1P and Z-VAD-FMK. The effects of extracellular S1P are primarily mediated through S1P receptors (mainly S1PR-1 and −3), which are G protein-coupled receptors that activate the PI3K/Akt pathway. This activation inhibits caspase activation and, consequently, inhibits the execution of apoptosis in granulosa cells (Nakahara [39]). Z-VAD-FMK, which is cell-permeable, is known to inhibit the activity of caspase-3, −8 and −9 by binding to the catalytic sites of these caspases. Inhibition of these caspases prevents the execution of apoptosis (Men [38]). (DOCX 1386 kb)

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
LH performed experiments, interpreted data and wrote the manuscript. MF performed experiments and interpreted data. SL designed the study, interpreted data and revised the manuscript. SB performed the computer images analysis. MCM contributed to computer images analysis. JMF conceived the study and corrected the manuscript. AN revised the manuscript. MN conceived and designed the study and corrected the manuscript. CM conceived and designed the study, interpreted data, corrected the manuscript and substantially contributed to critical revisions. All authors read and approved the final manuscript.

Acknowledgments
The authors thank I. Dasoul and E. Feyereisen for their excellent histology and immunostaining assistance. The authors are very grateful to Dr. N. Kirshvink (Ovine Research Center, University of Namur) for help with collection of sheep ovaries and to Dr. AF. Donneau (Public Health Department, University of Liège) for statistical analyses. This work was supported by grants from the Fonds de la Recherche Scientifique Médicale, the Fonds de la Recherche Scientifique - FNRS (F.R.S.-FNRS, Belgium), the Foundation against Cancer (foundation of public interest, Belgium), the Fonds spéciaux de la Recherche (University of Liège), the Centre Anticancéreux près l'Université de Liège, the Fonds Léon Fredericq (University of Liège).

Author details
[1]Laboratory of Tumor and Development Biology, Groupe Interdisciplinaire de Génoprotéomique Appliquée (GIGA-R), Université de Liège, Tour de Pathologie (B23) Sart-Tilman, B-4000 Liège, Belgium. [2]Department of Gynecology, University of Liège, Boulevard du XIIème de Ligne, B-4000 Liège, Belgium.

Received: 12 August 2015 Accepted: 4 February 2016
Published online: 12 February 2016

References
1. Meirow D, Ra'anani H, Biderman H. Ovarian tissue cryopreservation and transplantation: a realistic, effective technology for fertility preservation. Methods Mol Biol. 2014;1154:455–73. doi:10.1007/978-1-4939-0659-8_21.
2. Donnez J, Dolmans MM, Pellicer A, Diaz-Garcia C, Sanchez Serrano M, Schmidt KT, et al. Restoration of ovarian activity and pregnancy after transplantation of cryopreserved ovarian tissue: a review of 60 cases of reimplantation. Fertil Steril. 2013;99(6):1503–13. doi:10.1016/j.fertnstert.2013.03.030.
3. Anderson RA, Mitchell RT, Kelsey TW, Spears N, Telfer EE, Wallace WH. Cancer treatment and gonadal function: experimental and established strategies for fertility preservation in children and young adults. Lancet Diabetes Endocrinol. 2015;3(7):556–67. doi:10.1016/S2213-8587(15)00039-X.
4. Aubard Y, Piver P, Cogni Y, Fermeaux V, Poulin N, Driancourt MA. Orthotopic and heterotopic autografts of frozen-thawed ovarian cortex in sheep. Hum Reprod. 1999;14(8):2149–54.
5. Jassem W, Heaton ND. The role of mitochondria in ischemia/reperfusion injury in organ transplantation. Kidney Int. 2004;66(2):514–7. doi:10.1111/j.1523-1755.2004.761_9.x.
6. Van Eyck AS, Jordan BF, Gallez B, Heilier JF, Van Langendonckt A, Donnez J. Electron paramagnetic resonance as a tool to evaluate human ovarian tissue reoxygenation after xenografting. Fertil Steril. 2009;92(1):374–81. doi:10.1016/j.fertnstert.2008.05.012.
7. David A, Van Langendonckt A, Gilliaux S, Dolmans MM, Donnez J, Amorim CA. Effect of cryopreservation and transplantation on the expression of kit ligand and anti-Mullerian hormone in human ovarian tissue. Hum Reprod. 2012;27(4):1088–95. doi:10.1093/humrep/des013.
8. Nisolle M, Casanas-Roux F, Qu J, Motta P, Donnez J. Histologic and ultrastructural evaluation of fresh and frozen-thawed human ovarian xenografts in nude mice. Fertil Steril. 2000;74(1):122–9.
9. Silber SJ. Ovary cryopreservation and transplantation for fertility preservation. Mol Hum Reprod. 2012;18(2):59–67. doi:10.1093/molehr/gar082.
10. Keros V, Xella S, Hultenby K, Pettersson K, Sheikhi M, Volpe A, et al. Vitrification versus controlled-rate freezing in cryopreservation of human ovarian tissue. Hum Reprod. 2009;24(7):1670–83. doi:10.1093/humrep/dep079.
11. Stroh C, Cassens U, Samraj A, Sibrowski W, Schulze-Osthoff K, Los M. The role of caspases in cryoinjury: caspase inhibition strongly improves the recovery of cryopreserved hematopoietic and other cells. FASEB J. 2002;16(12):1651–3. doi:10.1096/fj.02-0034fje.
12. Xiao Z, Wang Y, Li L, Li SW. Cryopreservation of the human ovarian tissue induces the expression of Fas system in morphologically normal primordial follicles. Cryo Letters. 2010;31(2):112–9.
13. Rimon E, Cohen T, Dantes A, Hirsh L, Amit A, Lessing JB, et al. Apoptosis in cryopreserved human ovarian tissue obtained from cancer patients: a tool for evaluating cryopreservation utility. Int J Oncol. 2005;27(2):345–53.
14. Fauque P, Ben Amor A, Joanne C, Agnani G, Bresson JL, Roux C. Use of trypan blue staining to assess the quality of ovarian cryopreservation. Fertil Steril. 2007;87(5):1200–7. doi:10.1016/j.fertnstert.2006.08.115.
15. Gonfloni S, Di Tella L, Caldarola S, Cannata SM, Klinger FG, Di Bartolomeo C, et al. Inhibition of the c-Abl-TAp63 pathway protects mouse oocytes from chemotherapy-induced death. Nat Med. 2009;15(10):1179–85. doi:10.1038/nm.2033.
16. Hannun YA, Obeid LM. Principles of bioactive lipid signalling: lessons from sphingolipids. Nat Rev Mol Cell Biol. 2008;9(2):139–50. doi:10.1038/nrm2329.
17. Morita Y, Perez GI, Paris F, Miranda SR, Ehleiter D, Haimovitz-Friedman A, et al. Oocyte apoptosis is suppressed by disruption of the acid sphingomyelinase gene or by sphingosine-1-phosphate therapy. Nat Med. 2000;6(10):1109–14. doi:10.1038/80442.
18. Hancke K, Strauch O, Kissel C, Gobel H, Schafer W, Denschlag D. Sphingosine 1-phosphate protects ovaries from chemotherapy-induced damage in vivo. Fertil Steril. 2007;87(1):172–7. doi:10.1016/j.fertnstert.2006.06.020.
19. Kaya H, Desdicioglu R, Sezik M, Ulukaya E, Ozkaya O, Yilmaztepe A, et al. Does sphingosine-1-phosphate have a protective effect on cyclophosphamide- and irradiation-induced ovarian damage in the rat model? Fertil Steril. 2008;89(3):732–5. doi:10.1016/j.fertnstert.2007.03.065.
20. Zelinski MB, Murphy MK, Lawson MS, Jurisicova A, Pau KY, Toscano NP, et al. In vivo delivery of FTY720 prevents radiation-induced ovarian failure and infertility in adult female nonhuman primates. Fertil Steril. 2011;95(4):1440–5 e1-7. doi:10.1016/j.fertnstert.2011.01.012.
21. Li F, Turan V, Lierman S, Cuvelier C, De Sutter P, Oktay K. Sphingosine-1-phosphate prevents chemotherapy-induced human primordial follicle death. Hum Reprod. 2014;29(1):107–113. doi:10.1093/humrep/det391.
22. Paris F, Perez GI, Fuks Z, Haimovitz-Friedman A, Nguyen H, Bose M, et al. Sphingosine 1-phosphate preserves fertility in irradiated female mice without propagating genomic damage in offspring. Nat Med. 2002;8(9):901–2. doi:10.1038/nm0902-901.
23. Zhang JM, Li LX, Yang YX, Liu XL, Wan XP. Is caspase inhibition a valid therapeutic strategy in cryopreservation of ovarian tissue? J Assist Reprod Genet. 2009;26(7):415–20. doi:10.1007/s10815-009-9331-9.
24. Jee BC, Lee JR, Youm H, Suh CS, Kim SH, Moon SY. Effect of sphingosine-1-phosphate supplementation on follicular integrity of vitrified-warmed mouse ovarian grafts. Eur J Obstet Gynecol Reprod Biol. 2010;152(2):176–80. doi:10.1016/j.ejogrb.2010.06.019.
25. Tsai YC, Tzeng CR, Wang CW, Hsu MI, Tan SJ, Chen CH. Antiapoptotic agent sphingosine-1-phosphate protects vitrified murine ovarian grafts. Reprod Sci. 2014;21(2):236–243. doi:10.1177/1933719113493515.
26. Hancke K, Walker E, Strauch O, Gobel H, Hanjalic-Beck A, Denschlag D. Ovarian transplantation for fertility preservation in a sheep model: can follicle loss be prevented by antiapoptotic sphingosine-1-phosphate administration? Gynecol Endocrinol. 2009;25(12):839–43. doi:10.3109/09513590903159524.

27. Soleimani R, Heytens E, Oktay K. Enhancement of neoangiogenesis and follicle survival by sphingosine-1-phosphate in human ovarian tissue xenotransplants. PLoS One. 2011;6(4):e19475. doi:10.1371/journal.pone.0019475.
28. Sanfilippo S, Canis M, Romero S, Sion B, Dechelotte P, Pouly JL, et al. Quality and functionality of human ovarian tissue after cryopreservation using an original slow freezing procedure. J Assist Reprod Genet. 2013;30(1):25–34. doi:10.1007/s10815-012-9917-5.
29. Maffei S, Pennarossa G, Brevini TA, Arav A, Gandolfi F. Beneficial effect of directional freezing on in vitro viability of cryopreserved sheep whole ovaries and ovarian cortical slices. Hum Reprod. 2014;29(1):114–24. doi:10.1093/humrep/det377.
30. Gosden RG, Baird DT, Wade JC, Webb R. Restoration of fertility to oophorectomized sheep by ovarian autografts stored at −196 degrees C. Hum Reprod. 1994;9(4):597–603.
31. Labied S, Delforge Y, Munaut C, Blacher S, Colige A, Delcombel R, et al. Isoform 111 of vascular endothelial growth factor (VEGF111) improves angiogenesis of ovarian tissue xenotransplantation. Transplantation. 2013;95(3):426–33. doi:10.1097/TP.0b013e318279965c.
32. Fransolet M, Henry L, Labied S, Masereel MC, Blacher S, Noel A, et al. Influence of mouse strain on ovarian tissue recovery after engraftment with angiogenic factor. J Ovarian Res. 2015;8(1):14. doi:10.1186/s13048-015-0142-6.
33. Fransolet M, Labied S, Henry L, Masereel MC, Rozet E, Kirschvink N, et al. Strategies for using the sheep ovarian cortex as a model in reproductive medicine. PLoS One. 2014;9(3):e91073. doi:10.1371/journal.pone.0091073.
34. Henry L, Labied S, Fransolet M, Kirschvink N, Blacher S, Noel A, et al. Isoform 165 of vascular endothelial growth factor in collagen matrix improves ovine cryopreserved ovarian tissue revascularisation after xenotransplantation in mice. Reprod Biol Endocrinol. 2015;13(1):15. doi:10.1186/s12958-015-0015-2.
35. Kapur JN, Sahoo PK, Wong AKC. A new method for gray-level picture thresholding using the entropy of the histogram. Comput Vis Graph Image Process. 1985;29(3):273–85. http://dx.doi.org/10.1016/0734-189X(85)90125-2.
36. Soille P. Morphological image analysis: principles and applications. 1st ed. Berlin: Springer; 1999.
37. Liu J, Van der Elst J, Van den Broecke R, Dhont M. Early massive follicle loss and apoptosis in heterotopically grafted newborn mouse ovaries. Hum Reprod. 2002;17(3):605–11.
38. Men H, Agca Y, Riley LK, Critser JK. Improved survival of vitrified porcine embryos after partial delipation through chemically stimulated lipolysis and inhibition of apoptosis. Theriogenology. 2006;66(8):2008–16. doi:10.1016/j.theriogenology.2006.05.018.
39. Nakahara T, Iwase A, Nakamura T, Kondo M, Bayasula, Kobayashi H, et al. Sphingosine-1-phosphate inhibits H2O2-induced granulosa cell apoptosis via the PI3K/Akt signaling pathway. Fertil Steril. 2012;98(4):1001–8 e1. doi:10.1016/j.fertnstert.2012.06.008.
40. Onions VJ, Mitchell MR, Campbell BK, Webb R. Ovarian tissue viability following whole ovine ovary cryopreservation: assessing the effects of sphingosine-1-phosphate inclusion. Hum Reprod. 2008;23(3):606–18. doi:10.1093/humrep/dem414.
41. Klocke S, Bundgen N, Koster F, Eichenlaub-Ritter U, Griesinger G. Slow-freezing versus vitrification for human ovarian tissue cryopreservation. Arch Gynecol Obstet. 2015;291(2):419–26. doi:10.1007/s00404-014-3390-6.

Submit your next manuscript to BioMed Central and we will help you at every step:

- We accept pre-submission inquiries
- Our selector tool helps you to find the most relevant journal
- We provide round the clock customer support
- Convenient online submission
- Thorough peer review
- Inclusion in PubMed and all major indexing services
- Maximum visibility for your research

Submit your manuscript at www.biomedcentral.com/submit

3.3. Etude d'un anti-apoptotique sur des lignées de cellules de la granulosa

Publication n° 6

« In vitro evaluation of the anti-apoptotic drug Z-VAD-FMK on human ovarian granulosa cell lines for further use in ovarian tissue transplantation »

Fransolet M, **Henry L**, Labied S, Noel A, Nisolle M and Munaut C.

J Assist Reprod Genet. 2015. doi:10.1007/s10815-015-0536-9.

3.3.1. Résumé des résultats

Nous avons mis en évidence, par notre étude de l'activité métabolique et des analyses FACS en conditions normoxiques, que le Z-VAD-FMK protège les cellules de la granulosa contre l'apoptose induite par l'étoposide, un inhibiteur des topoisomérases de type II. Lorsque les cellules sont exposées à une hypoxie et à une carence en sérum, afin de mimer les conditions *in vivo* de post-transplantation, leur activité métabolique est réduite mais le Z-VAD-FMK n'a pas démontré d'effet protecteur car nous n'avons pas mis en évidence d'apoptose cellulaire dans notre modèle d'hypoxie.

3.3.2. Discussion

Dans cette étude, nous avons pu mettre en évidence que le Z-VAD-FMK est capable de prévenir l'apoptose induite par l'étoposide dans les 3 lignées de cellules de la granulosa. Lorsque nous avons étudié les voies de signalisation de l'apoptose, nous n'avons pas mis en évidence de modulation de ces voies en condition d'hypoxie et de carence en sérum. Les cellules n'ont pas été sensibles à ces conditions de culture. En effet, en FACS, seules les HGL5 ont été sensibles à ces conditions avec un taux de cellules viables plus faible. Par conséquent, le modèle d'ischémie tissulaire que nous avons utilisé *in vitro* n'est pas adéquat et ne peut nous permettre de prédire l'effet du Z-VAD-FMK *in vivo*. Le faible taux de mort cellulaire observé pourrait être lié au développement d'une forte résistance au stress, consécutive aux modifications subies par les cellules de la granulosa pour les immortaliser (Rainey 1994). Nous avons vérifié cette hypothèse en observant que seules les cellules où le p53, facteur clé de la stabilité génomique, n'était pas détecté (à savoir dans les HGL5) ont une sensibilité à l'ischémie entrainant une apoptose. Les différentes lignées ne répondent pas de la même manière au stress, ce qui est expliqué par leurs propriétés propres. Une culture primaire de cellules de la granulosa serait donc un modèle plus adapté que ces lignées cellulaires, pour étudier les effets de l'hypoxie et par conséquent l'effet protecteur du

Z-VAD-FMK.

En outre, nous avons mis en évidence une mort cellulaire dans la lignée HGL5 dans notre modèle d'ischémie alors qu'il n'y avait pas d'activation de l'apoptose, suggérant qu'un autre type de mort cellulaire serait impliqué. Il pourrait s'agir d'autophagie, processus par lequel la cellule dégrade une partie de son cytoplasme, des protéines ou organelles dysfonctionnelles ou non nécessaires, par ses propres lysosomes. Il s'agit d'un processus qui a déjà été mis en évidence en cas de carence, afin de promouvoir la survie cellulaire en attendant le réapprovisionnement en nutriments mais ce processus peut aboutir à la mort cellulaire si le support nutritionnel n'est pas restauré (Maiuri 2007). Par conséquent, il faudrait explorer le rôle de l'autophagie et des différents mécanismes de mort cellulaire dans la perte folliculaire liée à la transplantation de fragments de cortex ovarien afin d'en améliorer la technique.

En conclusion, le modèle d'ischémie cellulaire utilisé dans notre étude n'a pas pu induire la mort des cellules de la granulosa. Par conséquent, l'effet protecteur du Z-VAD-FMK *in vitro* sur la survie des cellules de la granulosa n'a pu être mis en évidence.

J Assist Reprod Genet
DOI 10.1007/s10815-015-0536-9

FERTILITY PRESERVATION

In vitro evaluation of the anti-apoptotic drug Z-VAD-FMK on human ovarian granulosa cell lines for further use in ovarian tissue transplantation

Maïté Fransolet[1] · Laurie Henry[1,2] · Soraya Labied[2] · Agnès Noël[1] · Michelle Nisolle[1,2] · Carine Munaut[1]

Received: 16 April 2015 / Accepted: 2 July 2015
© Springer Science+Business Media New York 2015

Abstract
Purpose Because ovarian granulosa cells are essential for oocyte survival, we examined three human granulosa cell lines as models to evaluate the ability of the pan-caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (Z-VAD-FMK) to prevent primordial follicle loss after ovarian tissue transplantation.
Methods To validate the efficacy of Z-VAD-FMK, three human granulosa cell lines (GC1a, HGL5, COV434) were treated for 48 h with etoposide (50 μg/ml) and/or Z-VAD-FMK (50 μM) under normoxic conditions. To mimic the ischemic phase that occurs after ovarian fragment transplantation, cells were cultured without serum under hypoxia (1 % O_2) and treated with Z-VAD-FMK. The metabolic activity of the cells was evaluated by WST-1 assay. Cell viability was determined by FACS analyses. The expression of apoptosis-related molecules was assessed by RT-qPCR and Western blot analyses.
Results Our assessment of metabolic activity and FACS analyses in the normoxic experiments indicate that Z-VAD-FMK protects granulosa cells from etoposide-induced cell death. When cells are exposed to hypoxia and serum starvation, their metabolic activity is reduced. However, Z-VAD-FMK does not provide a protective effect. In the hypoxic experiments, the number of viable cells was not modulated, and we did not observe any modifications in the expressions of apoptosis-related molecules (p53, Bax, Bcl-xl, and poly (ADP-ribose) polymerase (PARP)).
Conclusion The death of granulosa cell lines was not induced in our ischemic model. Therefore, a protective effect of Z-VAD-FMK in vitro for further use in ovarian tissue transplantation could not be directly confirmed. It will be of interest to potentially use Z-VAD-FMK in vivo in xenograft models.

Keywords Ovarian granulosa cells · Z-VAD-FMK · Etoposide · Hypoxia · Ovarian transplantation · Fertility preservation

Capsule Granulosa cell lines are resistant to low oxygen concentration (1 % O_2) and the caspase inhibitor Z-VAD-FMK could ensure follicular maintenance after ovarian tissue auto-transplantation.

Electronic supplementary material The online version of this article (doi:10.1007/s10815-015-0536-9) contains supplementary material, which is available to authorized users.

✉ Carine Munaut
c.munaut@ulg.ac.be

[1] Laboratory of Tumor and Developmental Biology, GIGA-R, University of Liège, Tour de Pathologie (B23), Sart Tilman, B-4000 Liège, Belgium

[2] Department of Obstetrics and Gynecology, Hôpital de la Citadelle, University of Liège, B-4000 Liège, Belgium

Published online: 14 July 2015

Introduction

In the last decade, the removal, cryopreservation, and subsequent grafting of ovarian strips after cancer treatment has been successfully used to re-establish female fertility. To date, more than 40 live births after using this technique have been reported worldwide. However, the pregnancy rate after the autografting of frozen-thawed ovarian tissue is approximately 30 % [1, 2]. The absence of vascular anastomoses between host and grafted tissues is responsible for post-transplantation ischemic injuries that jeopardize long-term fecundity. The primordial follicle pool in ovarian tissue represents the ovarian reserve and is closely correlated with the life span of the graft. Previous studies have demonstrated a major primordial follicular loss after grafting rather than after the freeze/thaw step [3–5]. Multiple researches have attempted to determine the primary reason for accelerated follicular loss after transplantation.

Several explanations that have been identified include fibrosis [6], ischemia [4, 5], the production of reactive oxygen species [7], and, more recently, follicular activation after disruption of the Hippo signaling pathway and the activation of the PI3K-PTEN-AKT-FOXO3 pathway following transplantation [8, 9]. Grafting studies have also indicated an apoptosis of the follicles shortly after transplantation, which leads to a 50 to 80 % depletion of the initial pool of primordial follicles [10, 11].

In the ovary, the layer of granulosa cells surrounding the oocyte plays an essential role in its development and maturation [12]. Therefore, rescuing granulosa cells from death is crucial to favor follicular survival. Moreover, in a previous study, granulosa cells, more so than oocytes, were identified as the main cells affected by the freeze/thaw processing of ovarian tissue [13]. Granulosa cells seem to be able to respond to hypoxia by increasing their synthesis of vascular endothelial growth factor (VEGF) [14], which is an important angiogenic factor that could reduce the hypoxic period post-transplantation, as previously shown in ovarian grafting studies [11, 15–20]. Because of the restricted availability and relatively short life span of primary cultures of granulosa cells, human granulosa cell lines were developed to facilitate research [21].

As a consequence of exposure to stressors, apoptotic responses can be activated within follicles and granulosa cells. Apoptosis is a physiological "self-killing" process crucial for the maintenance of tissue homeostasis during development, and the disruption of the apoptotic pathway is involved in numerous pathologies [22]. The caspase family of cysteine proteases is a key regulator of the apoptotic pathway within the ovary. Their activation leads to the cleavage of a specific subset of cellular polypeptides and the modification of expression of Bcl-2 family members. Bcl-2 protein family members can be pro- or anti-apoptotic, such as Bax or Bcl-xl, respectively. At the end of the apoptotic signaling cascade, one of the main targets of caspase-3, the poly (ADP-ribose) polymerase (PARP), is cleaved. Similarly, p53 is involved in the detection of cellular damage, and its activation induces the expression of target genes leading to cell cycle arrest and apoptosis [23, 24]. Together, these molecules serve as important markers of cells undergoing apoptosis.

To improve follicular preservation after transplantation, anti-apoptotic molecules were tested in different models. In the field of fertility preservation, sphingosine-1-phosphate (S1P) and its agonists are largely studied for their combined anti-apoptotic and pro-angiogenic properties. While the ability of S1P to protect follicles from chemotherapy and radiation-induced damages has been well demonstrated [25–29], the protective effects of S1P in cryopreserved tissue grafting studies remain controversial [30–33]. Another alternative, a permeable synthetic peptide pan-caspase inhibitor, benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (Z-VAD-FMK), has only been studied once in a xenograft model of cryopreserved ovarian tissue after producing encouraging results as a therapeutic option [34]. Z-VAD-FMK has also been identified as an efficient caspase inhibitor to prevent ischemia/reperfusion injuries in the liver [35], brain [36], and pancreas [37]. Moreover, successful cryopreservation and recovery of cells were obtained using Z-VAD-FMK as a cryoprotective agent [38]. Collectively, these results indicate the potential suitability of Z-VAD-FMK to improve ovarian tissue cryopreservation and the recovery rate of transplanted tissue.

In this study, our aim was to determine whether human granulosa cell lines could be protected by Z-VAD-FMK from induced cell death in vitro. Our experiments were performed on three human granulosa-derived cell lines displaying different morphologies and properties (GC1a, HGL5, and COV434) [21]. Two of them were generated from human luteinized granulosa cells isolated from follicular fluids and further immortalized through oncogenic transformation (with SV40 large T antigen for GC1a [39, 40] or with HPV 16 for HGL5 [41]). The COV434 cell line was derived from a metastatic granulosa cell tumor [42].

In a more global context, we hypothesize that a broad-spectrum caspase inhibitor, such as Z-VAD-FMK, could act to prevent apoptosis in transplanted ovarian tissue.

Materials and methods

Cell culture and treatment

The GC1a cell line was provided by Dr. Takashi Ohba (Department of Obstetrics and Gynecology of Kumamoto University School of Medicine, Kumamoto, Japan), and the HGL5 cell line was a gift from Dr. Bruce Carr (Department of Obstetrics and Gynecology of University of Texas Southwestern Medical Center, Dallas, TX, USA). We purchased the COV434 human granulosa cell line from the European Collection of Cell Cultures (ECACC cat. No. 07071909, Sigma-Aldrich, St. Louis, MO, USA). GC1a and HGL5 cells were maintained in Dulbecco's modified Eagle's medium/F-12 and COV434 cells in Dulbecco's modified Eagle's medium with GlutaMAX (Gibco, Grand Island, NY, USA), supplemented with 10 % heat-inactivated fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin. The cell culture was maintained in a traditional humidified incubator supplied with room air (20 % oxygen and 75 % nitrogen) buffered with 5 % CO_2 and set to 37 °C. All culture reagents were purchased from Invitrogen (Merelbeke, Belgium). To induce apoptosis, cells were treated with etoposide at 50 μg/ml (Sigma-Aldrich, St. Louis, MO, USA). The etoposide and doxorubicine (another apoptotic agent tested) dose-response curves of granulosa cells are provided in Online Resource 1. To induce hypoxia, cells were cultured in tri-gas (CO_2, atmospheric O_2, and N_2), where nitrogen supply is used to displace and reduce O_2 levels to 1 %

(Heracell™ 150, Thermo Scientific, Waltham, MA, USA) for 48 h at 37 °C. The anti-apoptotic Z-VAD-FMK was added at a concentration of 50 μM (R&D Systems, Abingdon, UK). The time and dose-response curves of granulosa cells after Z-VAD-FMK and other anti-apoptotic drugs treatment are given in Online Resource 2. The etoposide and anti-apoptotic drugs time-response curves of granulosa cell metabolic activity are provided in Online Resource 3.

WST-1 metabolic activity assay

The cell metabolic activity was estimated using a WST-1 assay (Roche, Mannheim, Germany). Granulosa cells were seeded in 96-well plates at a density of 1.0×10^4 for GC1a and HGL5 cells and 2.5×10^4 for COV434 cells in 100 μl of medium per well. After 24 h of incubation at 37 °C, duplicate wells were treated as previously described. Experiments were repeated at least three times. After treatment, a total of 10 μl of WST-1 solution was added to each well before a second identical incubation of approximately 3 h. Sample absorbance was measured at 450 nm (OD_{450}) and 620 nm (OD_{620}) according to the manufacturer's instructions. Colorimetric analysis was performed using a microplate spectrophotometer (Multiskan FC, Thermo Scientific, Waltham, MA, USA). Cell metabolic activity was calculated using the following equation:

$$\text{Metabolic activity} = \frac{\left[(OD_{450}-OD_{620})_{test}-(OD_{450}-OD_{620})_{blank}\right]}{\left[(OD_{450}-OD_{620})_{control}-(OD_{450}-OD_{620})_{blank}\right]} \quad (1)$$

Flow cytometry

The apoptosis of granulosa cells was analyzed by flow cytometry after annexin V-FITC and propidium iodide double staining using a BD Pharmingen™ Annexin V FITC apoptosis detection kit (BD Pharmingen, San Jose, CA, USA). Granulosa cells were seeded at a density of 6.0×10^5 for GC1a and HGL5 cells and 1.25×10^6 for COV434 cells in 6-cm petri dishes. Experiments were repeated at least three times. After treatment (see above), cells were harvested with trypsin, washed twice with PBS, and resuspended in binding buffer at a concentration of 1.0×10^6 cells/ml. Then, 100 μl of the cell suspension was incubated with 5 μl of annexin V-FITC and 5 μl of PI in dark for 10 min at room temperature according to the manufacturer's instructions. At the end of the incubation, 400 μl of binding buffer was added to the solution. Viable and apoptotic cells were detected using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). Subsequently, the results were analyzed using the BD CellQuest™ Pro software (BD Biosciences, San Jose, CA, USA).

RT real-time-PCR for mRNA quantification

Total RNA from treated granulosa cell lines was extracted using the High Pure RNA Isolation Kit (Roche, Mannheim, Germany), following the manufacturer's protocol. One microgram of RNA was then reverse transcribed into cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Mannheim, Germany). RT and real-time quantitative PCR were performed using specific primers and Brilliant SYBR GREEN QPCR master mix (Roche, Mannheim, Germany) on a LightCycler® 480 (Roche, Mannheim, Germany). Sequence primers for the target genes were B2M forward 5′-TGCTGT CTCCATGTTTGATGTATCT-3′ and reverse 5′-TCTCTGCT CCCCACCTCTAAGT-3′; RPL13A forward 5′-CCTGGAGG AGAAGAGGAAAGAGA-3′ and reverse 5′-TTGAGGACCT CTGTGTATTTGTCAA-3′; TBP forward 5′-TGCACAGGAG CCAAGAGTGAA-3′ and reverse 5′-CACATCACAGCTCC CCACCA-3′; p53 forward 5′-TCCGAGTGGAAGGAAATT TGCGTG-3′ and reverse 5′-GAGTCTTCCAGTGTGATGAT GGTG-3′; Bax forward 5′-TGCCAGCAAACTGGTGCTCA A-3′ and reverse 5′-GCCCATCTTCTTCCAGATGGT-3′; and Bcl-xl forward 5′-GCGTGGAAAGCGTAGACAAG-3′ and reverse 5′-AAAAGTATCCCAGCCGCCGT-3′. Gene expression values were normalized to the geometric mean of three housekeeping genes (B2M, L13A, and TBP), and mRNA expression levels were quantified using the ΔCT method [43].

Western blot analysis

For the preparation of total cell extracts, samples were washed three times with cold PBS and lysed in an appropriate amount of radio immunoprecipitation assay (RIPA) buffer containing phosphatase and protease inhibitors (Roche, Mannheim, Germany). The lysate was collected and the protein concentration was determined using a DC protein assay kit (Bio-Rad Laboratories, Hercules, CA). Equal amounts of protein were denatured and separated by electrophoresis on 10 or 12 % SDS-polyacrylamide gels and then transferred onto a polyvinylidene difluoride membrane (PerkinElmer) at 100 V for 1 h. After blocking, proteins were incubated with respective primary antibodies in blocking solution according to the manufacturer's protocol. The appropriate horseradish peroxidase-conjugated secondary antibody was added to the membrane followed by a 1-h incubation at room temperature. After sequential washing of the membranes to remove excess secondary antibody, signals were detected using an enhanced chemiluminescence (ECL) kit (PerkinElmer Life Sciences, Boston, MA, USA) according to the manufacturer's instructions in a LAS4000 imager (Fujifilm, Tokyo, Japan). Blocking solution, primary antibody, and their appropriate secondary antibodies are given in Table 1.

Table 1 Primary and secondary antibodies used for Western blotting

Blocking solutions	Primary antibodies	Secondary antibodies
BSA 2 %	P53 (Calbiochem, OP53) 1/2000 ON 4 °C	Horse anti-mouse 1/2000 (Cell Signaling #7076)
Milk 5 %	Bax (Santa Cruz, sc-7480) 1/500 ON 4 °C	Horse anti-mouse 1/2000 (Cell Signaling #7076)
Milk 5 %	Bcl-xl (Cell Signaling, #2764) 1/1000 ON 4 °C	Goat anti-rabbit 1/2000 (Cell Signaling #7074)
Milk 5 %	PARP (Cell Signaling, #9542) 1/1000 ON 4 °C	Swine anti-rabbit 1/1000 (Dako P0399)
Milk 5 %	Actine (Sigma, A2066) 1/1000 1 h RT	Swine anti-rabbit 1/1000 (Dako P0217)

Statistical analysis

All quantitative data are expressed as means±S.E.M. of three independent experiments. Statistical analyses were conducted with the GraphPad Prism software (La Jolla, CA, USA) using a Mann–Whitney test for comparisons between two groups. A $p<0.05$ was considered to be significant.

Results

Cell viability assessment in etoposide and Z-VAD-FMK-treated cells

To evaluate the anti-apoptotic effect of the pan-caspase inhibitor Z-VAD-FMK on the three granulosa cell lines, apoptosis was induced by etoposide, a topoisomerase II inhibitor that stabilizes DNA double strand breaks. The WST-1 assays indicate that etoposide significantly decreased the metabolic activity of cells. These observations were confirmed, as the number of viable cells was reduced by etoposide as shown by FACS analyses after annexin V-FITC and propidium iodide double staining. Z-VAD-FMK treatment inhibited the etoposide-induced decrease in metabolic activity of the granulosa cells. Flow cytometry experiments also indicated a protective effect of Z-VAD-FMK against etoposide by showing a higher number of viable cells when the cells were treated with both drugs compared with treatment with etoposide alone (Fig. 1a–f).

Expression of apoptosis-related molecules in etoposide and Z-VAD-FMK-treated cells

The effect of etoposide and Z-VAD-FMK treatment on the apoptotic pathway was studied at the mRNA and protein

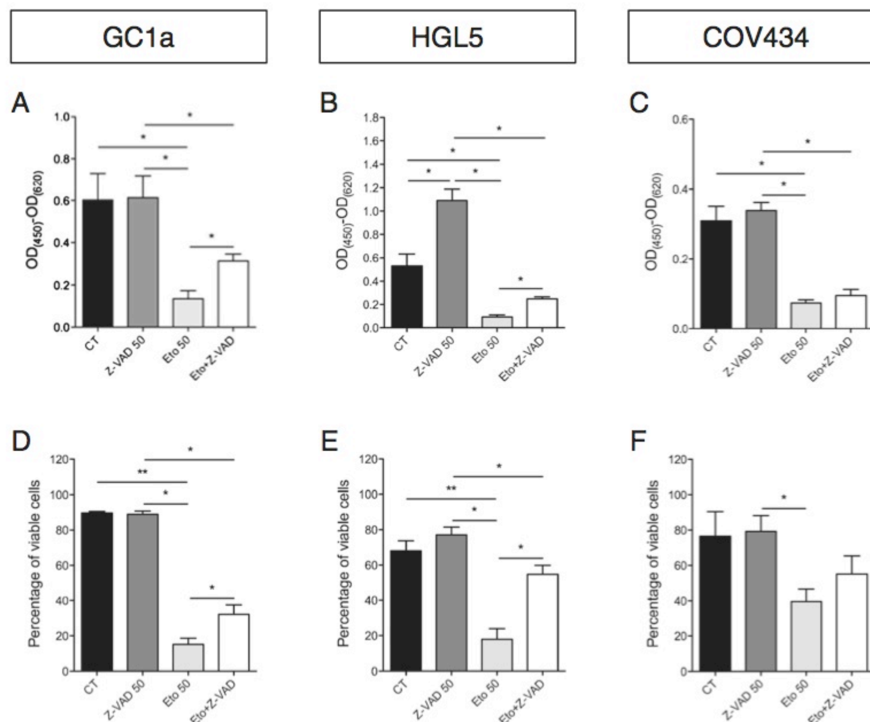

Fig. 1 Granulosa cell metabolic activity and viability after pro- and anti-apoptotic drug treatment. Evaluation of the metabolic activity as measured by WST-1 assay (**a**–**c**), and the percentage of viable cells as shown by flow cytometry analyses (**d**–**f**), of the three granulosa cell lines after etoposide and Z-VAD-FMK treatments. $*p<0.05$; $**p<0.01$

levels. As shown by RT-qPCR, the p53 mRNA expression levels were increased by etoposide in the GC1a and COV434 cell lines. In contrast, in the HGL5 cells, the p53 mRNA expression levels decreased under etoposide treatment. No protective effects from Z-VAD-FMK treatment were detected (Fig. 2a–c). In the GC1a cells, the ratio between pro- and anti-apoptotic molecules (Bax and Bcl-xl, respectively) was increased by etoposide but was not modulated in the other granulosa cell lines. Anti-apoptotic treatment had no effect on Bax/Bcl-xl mRNA expression levels in all cell lines (Fig. 2d–f).

Using Western blotting, we studied the expression levels of the apoptosis-related proteins. In the GC1a cells, p53 expression was decreased by etoposide, whereas the opposite was observed in the COV434 cells. The addition of Z-VAD-FMK decreased p53 expression in the COV434 cells but had no effect in the GC1a cells. p53 was not detectable in HGL5 cells. Under etoposide treatment, Bax expression was decreased in

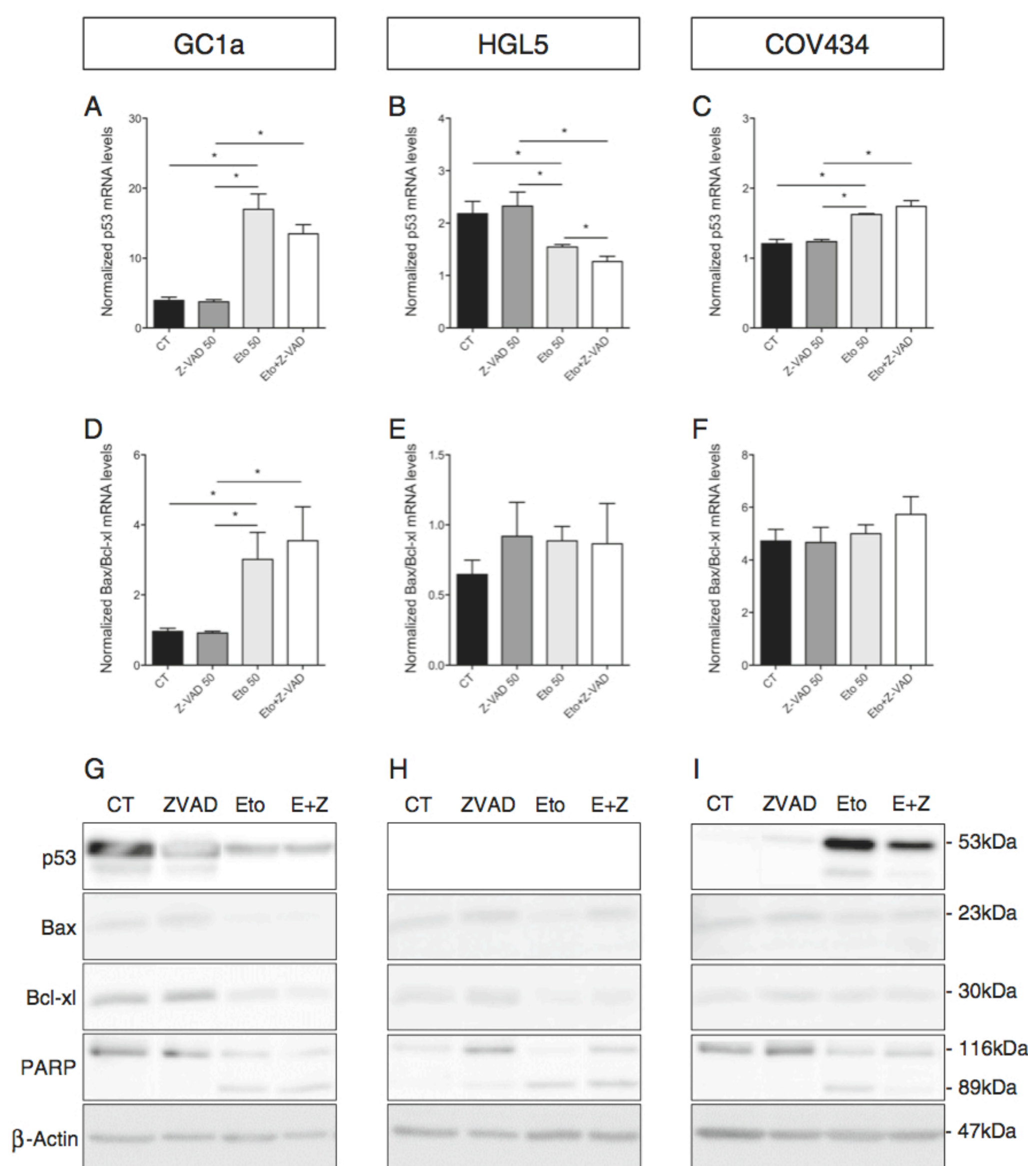

Fig. 2 mRNA and protein expression levels of pro- and anti-apoptotic molecules after etoposide and Z-VAD-FMK treatment. Expression levels of p53 mRNA (**a**–**c**). The ratio between mRNA expression levels of pro- and anti-apoptotic (Bax and Bcl-xl, respectively) molecules (**d**–**f**). Representative images of Western blot highlighting the expression of proteins involved in the apoptosis pathway (**g**–**i**). ß-Actin was used as a loading control. *$p<0.05$

all cell lines and Bcl-xl was decreased in the GC1a and HGL5 cells. For all cell lines, treatments with both Z-VAD-FMK and etoposide did not have any effects on Bax and Bcl-xl expression levels compared with etoposide treatments alone. When the cells were treated with both etoposide and Z-VAD-FMK, full-length PARP expression was increased in the HGL5 cells but the expression of the cleaved form was not modulated. In the COV434 cells, cleaved PARP expression was decreased and there was an increase in the expression of the full-length form. In the GC1a cells, Z-VAD-FMK did not modulate the effects of etoposide (Fig. 2g–i).

Cell viability assessment under hypoxia

To reproduce the early ischemic phase that occurs after ovarian fragment transplantation, cells were cultured without serum at 1 % O_2. Under these hypoxic conditions, the metabolic activity of the granulosa cells was decreased due to serum starvation. Flow cytometry analyses confirmed that this deprivation induced HGL5 cell death. However, through these two analytical methods, we did not observed any protective effects attributable to Z-VAD-FMK (Fig. 3a–f).

Expression of apoptosis-related molecules under hypoxia

In all cell lines, no variations in the mRNA expression levels of p53 were found (Fig. 4a–c). A decrease in the ratio of Bax and Bcl-xl was observed under serum starvation in GC1a cells but no effect is detected in other cell lines (Fig. 4d–f).

In the GC1a cells, the p53 expression level was not modulated by hypoxia, serum starvation, or Z-VAD-FMK. There was a slight decrease in the level of p53 under serum starvation in the COV434 cells; however, anti-apoptotic drug treatment increased the level of p53. In the HGL5 cells, p53 protein remained undetectable. The expressions of pro- and anti-apoptotic molecules were not modulated under any of our experimental conditions or in our cell lines. PARP was never cleaved when the cells were under hypoxia or serum starvation. However, full-length PARP expression levels increased in the GC1a and HGL5 cells after Z-VAD-FMK treatment (Fig. 4g–i).

Discussion

In this in vitro study, we used three human granulosa cell lines (GC1a, HGL5, and COV434) at low oxygen concentration (1 % O_2) without serum to mimic post-grafting ischemic conditions. Our goal was to evaluate the efficacy of the pan-caspase inhibitor Z-VAD-FMK to prevent ovarian granulosa cell death. Indeed, in vivo granulosa cells are of paramount importance for follicle survival due to their influence on oocyte maturation. Moreover, the apoptosis of granulosa cells has been shown to play an important role in follicular atresia [44, 23].

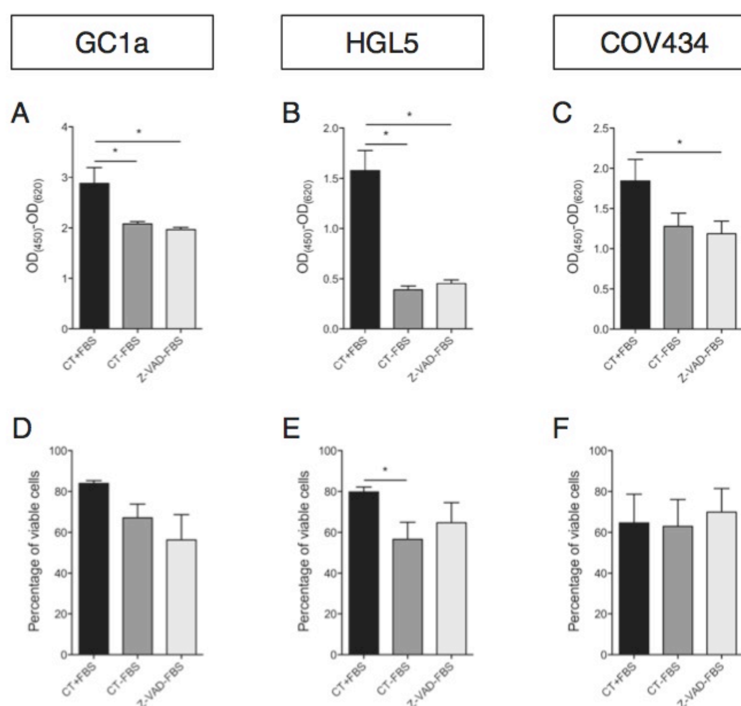

Fig. 3 Granulosa cell metabolic activity and viability under hypoxia (1 % O_2). Evaluation of the metabolic activity (**a**–**c**) and the percentage of viable cells (**d**–**f**) maintained in hypoxia with or without serum and with Z-VAD-FMK treatment. *$p<0.05$

J Assist Reprod Genet

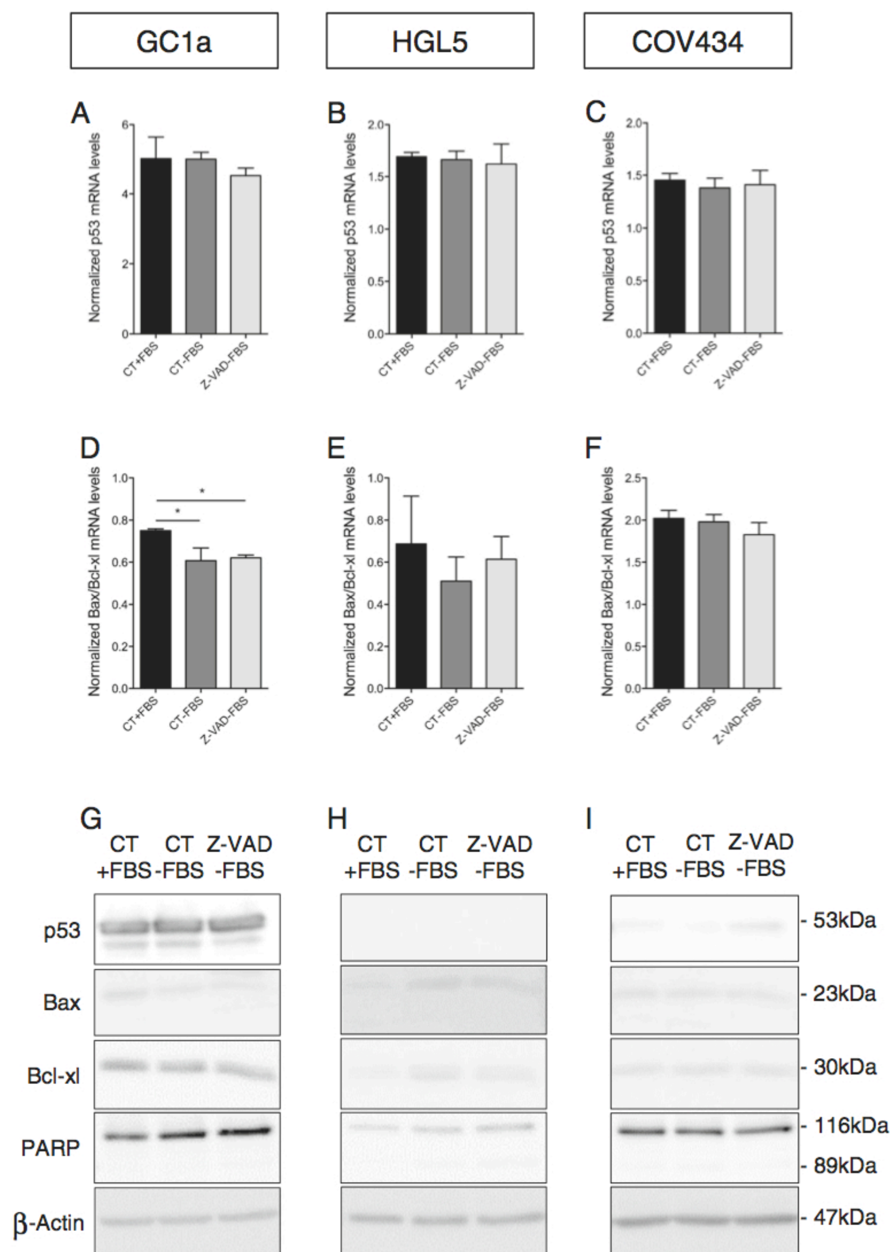

Fig. 4 mRNA and protein expression levels of pro- and anti-apoptotic molecules under hypoxia (1 % O_2). The evolution of mRNA expression levels of p53 (**a**–**c**) and Bax/Bcl-xl (**d**–**f**) in the three granulosa cell lines. Representative images of Western blot experiments showing apoptosis-related protein expression in granulosa cells 48 h after the initiation of hypoxia (**g**–**i**). ß-Actin was used as a loading control. $^{*}p<0.05$

In previous studies, it was demonstrated that the short-term culture of pancreatic islets and mice ovarian cortex cryopreserved with Z-VAD-FMK reduced post-transplantation apoptosis and subsequently improved the outcome of grafts [37, 34]. Moreover, Z-VAD-FMK has been found to abolish the ischemia-mediated activation of caspases [35]. Based on these data, Z-VAD-FMK appears to be an interesting compound to treat ovarian biopsy at the time of transplantation in order to minimize the effects of ischemic damages and to improve ovarian tissue recovery.

The present study demonstrates that Z-VAD-FMK was able to prevent etoposide-induced apoptosis in three different human granulosa cell lines. We further tested this protective effect against cell death under hypoxia and serum starvation. However, our data suggest that the apoptotic pathway was not activated by hypoxic or serum starvation culture conditions in the selected granulosa cell lines. We found no modulation in the expression of apoptosis-related molecules under low oxygen conditions. However, modifications in the expression levels of hypoxia-sensitive genes, such as VEGF and PAI-1,

were confirmed (data not shown), which indicated that granulosa cells do respond to hypoxia.

Even though the apoptotic pathway was not activated in our ischemic model, these cells were also not sensitive to low oxygen concentrations or serum starvation. Indeed, as shown by the FACS analyses, the percentage of viable cells was significantly reduced only in the HGL5 cells under stress conditions. Therefore, the in vitro ischemic model used in our study is not suitable for our purposes. However, these data do not collectively indicate that Z-VAD-FMK will be inefficient in vivo. The low rate of cell death could be attributed to the development of high resistance to stress following the modifications that granulosa cells underwent to become immortal [41]. To test this hypothesis, we observed that cell death in the ischemic model was only observed in the HGL5 cell line in which p53, a key factor in the maintenance of genomic stability, was not detected. In vivo, granulosa cells displayed different properties related to their localization and function around the oocyte [45]. Therefore, it is not very surprising that the three different cell lines display different responses to stress injuries. Primary granulosa cell culture in ischemic conditions could be more appropriate for the study of the effectiveness of Z-VAD-FMK in vitro.

In addition, HGL5 cell death was demonstrated in the ischemic model despite the quiescence of the apoptotic pathway, suggesting that other cell death pathways were involved. In the case of ovarian damage due to toxicant exposure, there was some evidence that follicular loss can occur via non-apoptotic mechanisms, such as autophagy [46]. In contrast to apoptosis, autophagy is a “self-eating” process in which autophagosome vesicles envelop dysfunctional or unnecessary proteins and organelles in the cytoplasm before elimination by lysosomal proteases. It has been demonstrated that during starvation, cellular components can be degraded to promote cell survival and maintain cellular energy levels but if the nutrient supply was not restored, cell death was induced [47]. Thus, examination of the role of autophagy in follicular loss after ovarian tissue transplantation should be explored. It has been well-established that several causes of follicular loss after transplantation coexist. However, the mechanisms regulating follicular death in grafted tissue are poorly understood. A better comprehension of these processes would result in better treatments of the ovarian fragments and improve ovarian tissue recovery and subsequently follicular preservation.

In conclusion, the ischemic model used in our study failed to induce ovarian granulosa cell death. The potential protective effect of Z-VAD-FMK in vitro for application in ovarian tissue transplantation could not be shown. Because several studies have already identified Z-VAD-FMK as an efficient caspase inhibitor to prevent ischemia/reperfusion injuries in different tissues, it will be interesting to investigate its effect in vivo in a xenograft model. Anti-apoptotic drugs should be combined with pro-angiogenic and anti-oxidative molecules to act on the previously mentioned conditions that are responsible for accelerated follicular loss after transplantation.

Acknowledgments The authors acknowledge Erika Konradowski and Nathalie Lefin for their excellent technical assistance. The authors also thank Dr. S. Ormenese and R. Stephan from the GIGA-Imaging and Flow Cytometry facility for their support with FACS analyzes.

M.F. is Televie granted PhD students (F.R.S.-FNRS, Belgium). C.M. is Research Associate from the F.R.S.-FNRS (Belgium). This work was supported by grants from the Fonds de la Recherche Scientifique Médicale, the Fonds de la Recherche Scientifique-FNRS (F.R.S.-FNRS, Belgium), the Foundation against Cancer (foundation of public interest, Belgium), the Fonds spéciaux de la Recherche (University of Liège), the Centre Anticancéreux près l’Université de Liège, the Fonds Léon Fredericq (University of Liège), the Direction Générale Opérationnelle de l’Economie, de l’Emploi et de la Recherche from the S.P.W. (Région Wallonne, Belgium), and the Plan National Cancer (Service Public Fédéral).

References

1. Donnez J, Dolmans MM. Transplantation of ovarian tissue. Best Pract Res Clin Obstet Gynaecol. 2014;28(8):1188–97. doi:10.1016/j.bpobgyn.2014.09.003.
2. Imbert R, Moffa F, Tsepelidis S, Simon P, Delbaere A, Devreker F et al. Safety and usefulness of cryopreservation of ovarian tissue to preserve fertility: a 12-year retrospective analysis. Hum Reprod. 2014. doi:10.1093/humrep/deu158
3. Nugent D, Meirow D, Brook PF, Aubard Y, Gosden RG. Transplantation in reproductive medicine: previous experience, present knowledge and future prospects. Hum Reprod Update. 1997;3(3):267–80.
4. Aubard Y, Piver P, Cogni Y, Fermeaux V, Poulin N, Driancourt MA. Orthotopic and heterotopic autografts of frozen-thawed ovarian cortex in sheep. Hum Reprod. 1999;14(8):2149–54.
5. Baird DT, Webb R, Campbell BK, Harkness LM, Gosden RG. Long-term ovarian function in sheep after ovariectomy and transplantation of autografts stored at −196 C. Endocrinology. 1999;140(1):462–71. doi:10.1210/en.140.1.462.
6. Nisolle M, Casanas-Roux F, Qu J, Motta P, Donnez J. Histologic and ultrastructural evaluation of fresh and frozen-thawed human ovarian xenografts in nude mice. Fertil Steril. 2000;74(1):122–9.
7. Nugent D, Newton H, Gallivan L, Gosden RG. Protective effect of vitamin E on ischaemia-reperfusion injury in ovarian grafts. J Reprod Fertil. 1998;114(2):341–6.
8. Kawamura K, Cheng Y, Suzuki N, Deguchi M, Sato Y, Takae S, et al. Hippo signaling disruption and Akt stimulation of ovarian follicles for infertility treatment. Proc Natl Acad Sci U S A. 2013;110(43):17474–9. doi:10.1073/pnas.1312830110.
9. Hsueh AJ, Kawamura K, Cheng Y, Fauser BC. Intraovarian control of early folliculogenesis. Endocr Rev. 2015;36(1):1–24. doi:10.1210/er.2014-1020.
10. Liu J, Van der Elst J, Van den Broecke R, Dhont M. Early massive follicle loss and apoptosis in heterotopically grafted newborn mouse ovaries. Hum Reprod. 2002;17(3):605–11.
11. Yang H, Lee HH, Lee HC, Ko DS, Kim SS. Assessment of vascular endothelial growth factor expression and apoptosis in the ovarian graft: can exogenous gonadotropin promote angiogenesis after ovarian transplantation? Fertil Steril. 2008;90(4):1550–8. doi:10.1016/j.fertnstert.2007.08.086.
12. Buccione R, Schroeder AC, Eppig JJ. Interactions between somatic cells and germ cells throughout mammalian oogenesis. Biol Reprod. 1990;43(4):543–7.

13. Siebzehnrubl E, Kohl J, Dittrich R, Wildt L. Freezing of human ovarian tissue–not the oocytes but the granulosa is the problem. Mol Cell Endocrinol. 2000;169(1–2):109–11.
14. Koos RD. Increased expression of vascular endothelial growth/permeability factor in the rat ovary following an ovulatory gonadotropin stimulus: potential roles in follicle rupture. Biol Reprod. 1995;52(6):1426–35.
15. Shikanov A, Zhang Z, Xu M, Smith RM, Rajan A, Woodruff TK et al. Fibrin Encapsulation and Vascular Endothelial Growth Factor Delivery Promotes Ovarian Graft Survival in Mice. Tissue engineering Part A. 2011. doi:10.1089/ten.TEA.2011.0204
16. Abir R, Fisch B, Jessel S, Felz C, Ben Haroush A, Orvieto R. Improving posttransplantation survival of human ovarian tissue by treating the host and graft. Fertil.Steril. 2011; p. 1205–10.
17. Labied S, Delforge Y, Munaut C, Blacher S, Colige A, Delcombel R, et al. Isoform 111 of vascular endothelial growth factor (VEGF111) improves angiogenesis of ovarian tissue xenotransplantation. Transplantation. 2013;95(3):426–33. doi:10.1097/TP.0b013e318279965c.
18. Wang L, Ying YF, Ouyang YL, Wang JF, Xu J. VEGF and bFGF increase survival of xenografted human ovarian tissue in an experimental rabbit model. J Assist Reprod Genet. 2013;30(10):1301–11. doi:10.1007/s10815-013-0043-9.
19. Henry L, Labied S, Fransolet M, Kirschvink N, Blacher S, Noel A, et al. Isoform 165 of vascular endothelial growth factor in collagen matrix improves ovine cryopreserved ovarian tissue revascularisation after xenotransplantation in mice. Reprod Biol Endocrinol. 2015;13(1):15. doi:10.1186/s12958-015-0015-2.
20. Fransolet M, Henry L, Labied S, Masereel MC, Blacher S, Noel A, et al. Influence of mouse strain on ovarian tissue recovery after engraftment with angiogenic factor. J Ovarian Res. 2015;8(1):14. doi:10.1186/s13048-015-0142-6.
21. Havelock JC, Rainey WE, Carr BR. Ovarian granulosa cell lines. Mol Cell Endocrinol. 2004;228(1–2):67–78. doi:10.1016/j.mce.2004.04.018.
22. Goldar S, Khaniani MS, Derakhshan SM, Baradaran B. Molecular mechanisms of apoptosis and roles in cancer development and treatment. Asian Pac J Cancer Prev. 2015;16(6):2129–44.
23. Hussein MR. Apoptosis in the ovary: molecular mechanisms. Hum Reprod Update. 2005;11(2):162–77. doi:10.1093/humupd/dmi001.
24. Hutt KJ. The role of BH3-only proteins in apoptosis within the ovary. Reproduction. 2015;149(2):R81–9. doi:10.1530/rep-14-0422.
25. Morita Y, Perez GI, Paris F, Miranda SR, Ehleiter D, Haimovitz-Friedman A, et al. Oocyte apoptosis is suppressed by disruption of the acid sphingomyelinase gene or by sphingosine −1-phosphate therapy. Nat Med. 2000;6(10):1109–14.
26. Hancke K, Strauch O, Kissel C, Gobel H, Schafer W, Denschlag D. Sphingosine 1-phosphate protects ovaries from chemotherapy-induced damage in vivo. Fertil Steril. 2007;87(1):172–7. doi:10.1016/j.fertnstert.2006.06.020.
27. Kaya H, Desdicioglu R, Sezik M, Ulukaya E, Ozkaya O, Yilmaztepe A, et al. Does sphingosine-1-phosphate have a protective effect on cyclophosphamide- and irradiation-induced ovarian damage in the rat model? Fertil Steril. 2008;89(3):732–5. doi:10.1016/j.fertnstert.2007.03.065.
28. Zelinski MB, Murphy MK, Lawson MS, Jurisicova A, Pau KY, Toscano NP, et al. In vivo delivery of FTY720 prevents radiation-induced ovarian failure and infertility in adult female nonhuman primates. Fertil Steril. 2011;95(4):1440–5. doi:10.1016/j.fertnstert.2011.01.012. **e1-7**.
29. Meng Y, Xu Z, Wu F, Chen W, Xie S, Liu J et al. Sphingosine-1-phosphate suppresses cyclophosphamide induced follicle apoptosis in human fetal ovarian xenografts in nude mice. Fertility and sterility. 2014. doi:10.1016/j.fertnstert.2014.05.040
30. Hancke K, Walker E, Strauch O, Gobel H, Hanjalic-Beck A, Denschlag D. Ovarian transplantation for fertility preservation in a sheep model: can follicle loss be prevented by antiapoptotic sphingosine-1-phosphate administration? Gynecol Endocrinol. 2009;25(12):839–43. doi:10.3109/09513590903159524.
31. Jee BC, Lee JR, Youm H, Suh CS, Kim SH, Moon SY. Effect of sphingosine-1-phosphate supplementation on follicular integrity of vitrified-warmed mouse ovarian grafts. Eur J Obstet Gynecol Reprod Biol. 2010;152(2):176–80. doi:10.1016/j.ejogrb.2010.06.019.
32. Soleimani R, Heytens E, Oktay K. Enhancement of neoangiogenesis and follicle survival by sphingosine-1-phosphate in human ovarian tissue xenotransplants. PLoS One. 2011;6(4):e19475.
33. Tsai YC, Tzeng CR, Wang CW, Hsu MI, Tan SJ, Chen CH. Antiapoptotic Agent Sphingosine-1-Phosphate Protects Vitrified Murine Ovarian Grafts. Reproductive sciences. 2013. doi:10.1177/1933719113493515
34. Zhang JM, Li LX, Yang YX, Liu XL, Wan XP. Is caspase inhibition a valid therapeutic strategy in cryopreservation of ovarian tissue? J Assist Reprod Genet. 2009;26(7):415–20. doi:10.1007/s10815-009-9331-9.
35. Cursio R, Gugenheim J, Ricci JE, Crenesse D, Rostagno P, Maulon L, et al. Caspase inhibition protects from liver injury following ischemia and reperfusion in rats. Transpl Int. 2000;13(1):S568–72.
36. Himi T, Ishizaki Y, Murota S. A caspase inhibitor blocks ischaemia-induced delayed neuronal death in the gerbil. Eur J Neurosci. 1998;10(2):777–81.
37. Montolio M, Tellez N, Biarnes M, Soler J, Montanya E. Short-term culture with the caspase inhibitor z-VAD.fmk reduces beta cell apoptosis in transplanted islets and improves the metabolic outcome of the graft. Cell Transplant. 2005;14(1):59–65.
38. Stroh C, Cassens U, Samraj AK, Sibrowski W, Schulze-Osthoff K, Los M. The role of caspases in cryoinjury: caspase inhibition strongly improves the recovery of cryopreserved hematopoietic and other cells. The FASEB Journal. 2002
39. Nitta M, Katabuchi H, Ohtake H, Tashiro H, Yamaizumi M, Okamura H. Characterization and tumorigenicity of human ovarian surface epithelial cells immortalized by SV40 large T antigen. Gynecol Oncol. 2001;81(1):10–7. doi:10.1006/gyno.2000.6084.
40. Okamura H, Katabuchi H, Ohba T. What we have learned from isolated cells from human ovary? Mol Cell Endocrinol. 2003;202(1–2):37–45.
41. Rainey WH, Sawetawan C, Shay JW, Michael MD, Mathis JM, Kutteh W, et al. Transformation of human granulosa cells with the E6 and E7 regions of human papillomavirus. J Clin Endocrinol Metab. 1994;78(3):705–10. doi:10.1210/jcem.78.3.8126145.
42. Zhang H, Vollmer M, De Geyter M, Litzistorf Y, Ladewig A, Dürrenberger M, et al. Characterization of an immortalized human granulosa cell line (COV434). Mol Hum Reprod. 2000;6(2):146–53.
43. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C (T)) Method. Methods. 2001;25(4):402–8. doi:10.1006/meth.2001.1262.
44. Hsueh AJ, Billig H, Tsafriri A. Ovarian follicle atresia: a hormonally controlled apoptotic process. Endocr Rev. 1994;15(6):707–24. doi:10.1210/edrv-15-6-707.
45. Dzafic E, Stimpfel M, Virant-Klun I. Plasticity of granulosa cells: on the crossroad of stemness and transdifferentiation potential. J Assist Reprod Genet. 2013;30(10):1255–61. doi:10.1007/s10815-013-0068-0.
46. Gannon AM, Stampfli MR, Foster WG. Cigarette smoke exposure leads to follicle loss via an alternative ovarian cell death pathway in a mouse model. Toxicol Sci. 2012;125(1):274–84. doi:10.1093/toxsci/kfr279.
47. Maiuri MC, Zalckvar E, Kimchi A, Kroemer G. Self-eating and self-killing: crosstalk between autophagy and apoptosis. Nat Rev Mol Cell Biol. 2007;8(9):741–52. doi:10.1038/nrm2239.