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**ANÁLISE DETALHADA DA CRIOINJÚRIA NO TECIDO OVARIANO HUMANO
APÓS VITRIFICAÇÃO OU CONGELAMENTO LENTO**

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Trabalho de conclusão de curso de graduação apresentado ao Instituto de Ciências Básicas da Saúde da Universidade Federal do Rio Grande do Sul como requisito parcial para a obtenção do título de Bacharela em Biomedicina.

Orientadora: Prof^ª. Dra. Adriana Bos-Mikich

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RESUMO

A criopreservação do tecido ovariano humano representa um procedimento chave para a preservação da fertilidade. Os dois métodos de criopreservação amplamente mais usados para amostras de córtex ovariano humano são o congelamento lento\descongelamento (CL\D) e a vitrificação\aquecimento (V\A). O objetivo do presente estudo foi analisar os efeitos do CL\D e V\A em câmara metálica, em estruturas específicas do folículo e do oócito e na organização do estroma pós-criopreservação. Realizamos a análise histológica de fragmentos de ovário CL\D e V\A de nove indivíduos saudáveis. Os resultados gerais mostraram que os tecidos criopreservados apresentam taxas significativas de danos nos folículos primordiais e primários. A estrutura nuclear dos folículos primordiais e o desprendimento das células dos folículos primordiais e primários foram as principais lesões observadas após V\A e CL\D. Os componentes do estroma foram preservados de forma semelhante após a criopreservação. Concluimos que ambos os métodos de criopreservação podem ser usados para fins de preservação da fertilidade com resultados semelhantes em termos de integridade folicular e estromal. O desprendimento das células foliculares da membrana basal representa uma importante crioinjúria que merece mais investigação.

Palavras-chave: Tecido ovariano. Vitrificação. Congelamento. Estrutura de Folículos e oócitos. Integridade do estroma.

ABSTRACT

Cryopreservation of human ovarian tissue represents a key procedure for fertility preservation. The two most widely used cryopreservation methods for human ovarian cortex samples are slow freezing \Thawing (SF\T) and vitrification\warming (V\W). The aim of the present study was to analyze the effects of SF\T and V\W using a metal chamber, on specific follicle and oocyte structures and on the stromal organization post-cryopreservation. We did histology analysis of SF\T and V\W ovarian fragments from nine healthy subjects. Overall results showed that cryopreserved tissues presented significant rates of damage in primordial and primary follicles. Nuclear structure of primordial follicles and cell detachment from primordial and primary follicles were the main injuries observed after vitrification\rewarming and freezing\thawing. The stromal components were similarly well preserved after cryopreservation. We conclude that both cryopreservation methods may be used for fertility preservation purposes with similar outcomes in terms of follicular and stromal integrity. Detachment of follicle cells from basal membrane represents an important cryoinjury that deserves further investigation.

Keywords: Ovarian tissue. vitrification. Freezing. Follicle and oocyte structure. Stromal integrity.

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1. INTRODUÇÃO

No ano de 2020, segundo o Instituto Nacional do Câncer (INCA), a incidência de câncer ultrapassou o número de 309.750 e 316.280 casos diagnosticados em homens e em mulheres, respectivamente. Entretanto, nos dias de hoje, devido ao grande engajamento da ciência para conter essa doença, o número de sobreviventes encontra-se cada vez maior. As taxas de sobrevivência, para vários tipos de câncer, hoje estão acima de 80%, principalmente para pacientes jovens, como os casos de câncer na infância, na adolescência e no início da idade adulta. Apesar das altas taxas de sobrevivência, o câncer é uma doença que gera consequências permanentes em seus pacientes, entre elas a infertilidade. Os tratamentos extremamente agressivos usados para combater a doença, ocasionam danos irreparáveis nos folículos ovarianos e nas espermatogônias. Todavia, em homens, se uma pequena população de espermatogônias permanecer ativa após o tratamento do câncer, a espermatogênese pode voltar a ocorrer e a formação de espermatozoides é retomada. Em mulheres, por outro lado, a situação é mais complicada, uma vez que elas dispõem de uma quantidade limitada de gametas, pois o número de folículos primordiais que constituem a reserva ovariana não é renovável. Sendo assim, com o passar dos anos, a reserva ovariana da mulher vai diminuindo até chegar ao período da menopausa, quando ocorre o seu esgotamento. Por isso, mulheres com idade mais avançada apresentam risco maior de entrar em falência ovariana após os tratamentos oncológicos quando comparadas com mulheres mais jovens, principalmente na infância e na adolescência. Portanto, essas pacientes oncológicas, além de se tornarem inférteis, acabam entrando em menopausa precoce, sendo necessária a administração de terapia hormonal pós tratamento oncológico, fato que afeta principalmente crianças e adolescentes, que precisam carregar esse fardo por toda a vida. A idéia de infertilidade relacionada ao tratamento oncológico tende a causar sofrimento psicológico à paciente, podendo, inclusive, prejudicar o tratamento. As diretrizes internacionais recomendam que os médicos discutam o mais cedo possível com todos os pacientes em idade reprodutiva sobre os métodos de preservação da fertilidade.

Os métodos mais utilizados na clínica para a preservação da fertilidade são a criopreservação de gametas e de embriões, mas existem outras técnicas que têm recebido grande

atenção dos cientistas e médicos da área, entre elas a criopreservação de tecido ovariano humano.

O ovário é um órgão que se encontra ligado ao aparelho reprodutor pelo ligamento largo do útero e pelo mesovário, porém não há uma ligação contínua entre os ovários e as tubas uterinas, sendo os oócitos, portanto, captados pelas projeções da tuba uterina. Histologicamente, ele é revestido por epitélio simples pavimentoso ou cúbico, contínuo ao mesovário. Subjacentemente há uma camada de tecido conjuntivo denso não modelado, chamada de túnica albugínea. Esse órgão é dividido em duas zonas: a zona cortical e a zona medular. A zona medular é contínua ao hilo e é formada por tecido conjuntivo frouxo, sendo ricamente vascularizada. Na zona cortical se localizam os folículos ovarianos primordiais, formados por células germinativas e células foliculares planas, sendo assim, é nessa região que se encontra a reserva ovariana. Circundando as células foliculares encontra-se o estroma, formado por tecido conjuntivo denso, que tem como função dar sustentação e também dar suporte hormonal para os folículos. O número de folículos atinge entre seis a sete milhões em torno da vigésima semana de gestação, mas, devido ao processo de atresia folicular, apenas entre um a dois milhões de folículos alcançam o período neonatal. Os folículos primordiais são formados pelo oócito primário e uma camada de células pavimentosas, e, além disso, representam a reserva ovariana da mulher. Os folículos primários são folículos que iniciam seu crescimento e são formados pelo oócito primário e uma camada de células cúbicas. Os folículos secundários apresentam uma proliferação das células foliculares, sendo assim, formados pelo oócito primário e várias camadas de células cúbicas. Os folículos antrais apresentam acúmulo de flúidos que se acumulam e formam uma cavidade, chamada de antro folicular, e ao final de seu desenvolvimento ocorre a conclusão da meiose I do oócito, formando assim o oócito secundário. Por último, o folículo maduro ou de Graaf, tem cerca de 2 centímetros e é saliente na superfície do ovário, sendo formado por várias camadas de células foliculares cubóides e um oócito secundário pronto para ser ovulado. O crescimento folicular é iniciado no feto, de forma contínua, e está associado a massa total de folículos e a fatores liberados por folículos atrésicos (GASPARIN et al., 2015). O hormônio anti-Mulleriano (HAM), que está envolvido na diferenciação sexual do embrião de sexo masculino e é responsável pela regressão dos ductos de Muller, também é secretado em indivíduos do sexo feminino desde a 36^a semana de gestação. Esse hormônio é secretado apenas pelos folículos em crescimento: primários, secundários, pré-antrais e antrais pequenos; e tem como funções principais inibir o recrutamento inicial dos folículos primários a partir dos folículos primordiais, inibir a sensibilidade dos folículos antrais

ao hormônio folículo-estimulante (FSH) durante o recrutamento cíclico e prevenir a depleção prematura dos folículos. A reserva ovariana corresponde a quantidade de oócitos remanescentes nos ovários. A quantidade de folículos primordiais está relacionada com o número de folículos que entram em crescimento, portanto, como os folículos em crescimento secretam HAM, os níveis plasmáticos desse hormônio representam a quantidade de folículos primordiais presentes no ovário. Dessa forma, a dosagem de HAM no sangue de pacientes é uma das formas de estimar a sua reserva ovariana.

A criopreservação de tecido ovariano é uma técnica que se baseia na retirada, cirurgicamente, de fragmentos do córtex ovariano da paciente, por ser a região onde se encontra a reserva ovariana, e posterior criopreservação desses fragmentos e reimplante pós tratamento oncológico. Esse método é extremamente importante para a oncofertilidade, uma vez que, além de preservar os gametas, não necessita de estimulação ovariana, que pode atrasar o início do tratamento oncológico ou agravar o caso clínico em alguns tipos de tumores, e, após o reimplante do tecido, há o restabelecimento da função ovariana. Sendo assim, a técnica permite a retomada da produção hormonal e a restauração da fertilidade das pacientes. Além disso, essa técnica é a única maneira de preservar a fertilidade de crianças e jovens pré-puberes, uma vez que não há a possibilidade de estimular seus ovários para a captação de gametas. Após o reimplante do tecido, que pode ser feito em local ortotópico (cavidade pélvica, ovário ou região subperitoneal) ou heterotópico (região subcutânea), a retomada do ciclo ocorre entre 3 e 4 meses. O primeiro transplante de ovário com tecido criopreservado foi realizado em 1999 (OKTAY; KARLIKAYA, 2000) e o primeiro nascimento com sucesso ocorreu em 2004 (DONNEZ, 2004). Até hoje, essa técnica permitiu o nascimento de mais 130 bebês ao redor do mundo (DONNEZ; DOLMANS, 2017). As gestações pós retransplante podem ocorrer por concepção natural ou por meio de técnicas de reprodução assistida. Por muito tempo uma das preocupações sobre essa técnica foi a possível reintrodução de células malignas pelo enxerto, porém, diversos estudos foram realizados para assegurar o uso desse método de preservação da fertilidade. A maioria desses estudos concluiu que em pacientes com doença não disseminada, a criopreservação de tecido ovariano é segura.

Existem duas técnicas para a criopreservação de tecido ovariano: o congelamento lento e a vitrificação. A primeira se baseia na redução gradual de temperatura, ao passo que a segunda é um processo de resfriamento ultrarápido. O congelamento lento requer uma diminuição lenta

da temperatura, em torno de 2 °C por minuto, até que atinja uma temperatura de aproximadamente -9°C. O tecido permanece nessa temperatura por um período de tempo e em seguida a temperatura é reduzida novamente, até o ponto no qual ele pode ser armazenado em nitrogênio líquido (-196°C). Dessa forma, a técnica requer um equipamento específico, que é programado conforme a velocidade do resfriamento desejado, e leva em torno de 150 minutos para que o tecido esteja pronto para ser armazenado. Esse método, que foi usado pela primeira vez para preservar tecido ovariano em 1999 (OKTAY; KARLIKAYA, 2000), é ainda a técnica mais utilizada para esta finalidade, possuindo diversos relatos de sucessos em gestações após o retransplante. Em baixas velocidades de congelamento, ocorre a desidratação celular, ocasionando a concentração de solutos no meio intracelular e impedindo, assim, o super-resfriamento do citoplasma. Portanto, ocorre o surgimento de um meio intracelular hipertônico. Com isso, a água, em sua maioria, move-se para o meio extracelular e se solidifica externamente. Com a finalidade de reduzir a formação de cristais de gelo intracelular, pode-se induzir a formação de gelo extracelular, por meio do toque de um objeto de metal resfriado a -196°C na parede dos criotubos, processo conhecido como seeding. Além da formação de gelo, que pode ocasionar danos celulares, a concentração elevada dos eletrólitos intracelulares, pode também causar efeitos nocivos nas células. Outra maneira de criopreservar o tecido ovariano é por meio da vitrificação, que consiste em um processo de resfriamento ultrarápido e no emprego de concentrações elevadas de crioprotetores. O tecido, após preparação, é exposto diretamente ao NLiq e armazenado no mesmo. Essa técnica, devido a rápida queda de temperatura, produz a solidificação de células em forma de vidro por extrema elevação da viscosidade, evitando lesões celulares causadas pela formação de gelo. Além disso, outras duas vantagens da vitrificação, quando comparada ao congelamento lento, são a simplicidade de manejo e o custo bem mais baixo, pois não necessita de equipamento específico e o tecido é armazenado diretamente no nitrogênio líquido, economizando tempo para o profissional. Apesar de ser uma alternativa ao congelamento lento, proposta inicialmente como opção para a criopreservação de embriões, a vitrificação ainda é pouco utilizada para a criopreservação de tecido ovariano humano, obtendo apenas 2 nascidos vivos relatados até agora. Entretanto, muitos estudos vêm sendo feitos sobre as duas técnicas, mas ainda não se tem resultados suficientes para inferir se a vitrificação é ou não equivalente ao congelamento lento em termos de viabilidade tecidual pós-criopreservação.

2. JUSTIFICATIVA

A qualidade de vida de mulheres que sobrevivem ao câncer é de extrema importância tanto para a sua saúde física quanto psicológica. A infertilidade é a principal consequência vivida por pacientes sobreviventes dessa doença. O câncer de mama, por exemplo, é o que mais afeta mulheres em idade reprodutiva, sendo aproximadamente 10% das pacientes acometidas com idade inferior a 45 anos, segundo o National Cancer Institute – Surveillance, Epidemiology and End Results Program. O campo da oncofertilidade, que se baseia na preservação da fertilidade de pacientes oncológicas, têm crescido e ganhado grande notoriedade a partir dos últimos vinte anos (ATAMAN et al., 2016). Diversas técnicas têm sido estudadas para promover a preservação da fertilidade feminina, sendo as mais utilizadas na clínica a criopreservação de embriões e de oócitos (ANDERSON et al., 2015), porém outra técnica que tem proporcionado ótimos resultados é a criopreservação de tecido ovariano.

Existem duas técnicas possíveis para a realização da criopreservação de tecido ovariano: o congelamento lento e a vitrificação. O congelamento lento é um método que necessita de um equipamento específico e requer bastante tempo até que o tecido possa ser armazenado. A vitrificação, por outro lado, se baseia no resfriamento ultrarápido, portanto é um método mais simplificado, rápido e barato. Sendo assim, essa técnica é extremamente vantajosa para a utilização nas rotinas de criopreservação de tecido ovariano, pois requer menor orçamento e poupa tempo para o profissional, podendo assim, atender um número maior de pacientes. Essa técnica foi desenvolvida primeiramente para criopreservar oócitos e embriões, e devido ao sucesso obtido, pesquisadores passaram a estudá-la para criopreservar tecido ovariano, sendo o trabalho pioneiro publicado em 1995 (ZHANG et al., 1995). Já foi relatado que além de apresentar resultados semelhantes ao congelamento lento, a vitrificação tem a vantagem de preservar de maneira mais eficaz o estroma ovariano, normalmente afetado pelo congelamento lento (KEROS et al., 2009).

Nesse trabalho analisamos a preservação do estroma ovariano nas duas técnicas de criopreservação, com o intuito de demonstrar a eficiência da vitrificação nesse quesito, quando comparada com o congelamento lento, uma vez que a qualidade do estroma é um fator essencial a ser considerado, pois dele depende a neo-vascularização do implante. Além do estroma, analisamos danos celulares e danos nucleares encontrados nos folículos após o

descongelamento/desvitrificação dos tecidos, já que esses danos podem também estar relacionados com a perda folicular pós-criopreservação. A maior parte dos estudos publicados até agora utilizaram recipientes de plástico para conservar o tecido, o que pode ser causa de prejuízo nos resultados, uma vez que esse material não é um bom condutor de calor. Nesse trabalho utilizamos um recipiente de metal, por ser um melhor condutor de calor e apresentar resultados promissores em trabalhos anteriores (BOS-MIKICH et al., 2012).

Apesar de o congelamento lento ser a técnica mais bem estabelecida, diversos estudos têm mostrado o sucesso e as vantagens da vitrificação. Sendo assim, acredito que os resultados desse trabalho poderão contribuir para a valorização e implementação dessa técnica nas rotinas de criopreservação de tecido ovariano, com o intuito de ajudar diversas mulheres que lutam contra o câncer a serem capazes de obter sua prole da maneira mais natural possível, além de melhorar a sua qualidade de vida.

3. OBJETIVOS

3.1. OBJETIVOS PRIMÁRIOS:

Comparar duas técnicas de criopreservação de tecido ovariano, com ênfase em mostrar a qualidade e semelhança da vitrificação, em termos de viabilidade tecidual, quando comparada ao congelamento lento.

3.2. OBJETIVOS SECUNDÁRIOS

Analisar histologicamente a preservação do estroma, assim como os danos foliculares encontrados nas duas técnicas de criopreservação de tecido ovariano.

4. ARTIGO CIENTÍFICO

Revista: Human Fertility

Detailed analysis of cryoinjury in human ovarian tissue following vitrification or slow freezing.

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Abstract

Cryopreservation of human ovarian tissue represents a key procedure for fertility preservation. The two most widely used cryopreservation methods for human ovarian cortex samples are slow freezing \Thawing (SF\T) and vitrification\warming (V\W). The aim of the present study was to analyze the effects of SF\T and V\W using a metal chamber, on specific follicle and oocyte structures and on the stromal organization post-cryopreservation. We did histology analysis of SF\T and V\W ovarian fragments from nine healthy subjects. Overall results showed that cryopreserved tissues presented significant rates of damage in primordial and primary follicles. Nuclear structure of primordial follicles and cell detachment from primordial and primary follicles were the main injuries observed after V/W and SF/T. The stromal components were similarly well preserved after cryopreservation. We conclude that

both cryopreservation methods may be used for fertility preservation purposes with similar outcomes in terms of follicular and stromal integrity. Detachment of follicle cells from basal membrane represents an important cryoinjury that deserves further investigation.

Keywords: ovarian cryopreservation, vitrification-warming, vitrification in a metal container, stromal integrity

Introduction

Advances in cancer research and therapeutic management over the past years have led to longer survival and better quality of life following treatment. However, fertility preservation may be considered a major issue for young women, whose reproductive capacity may be compromised by an oncological treatment. In addition, recent reports have shown that fertility preservation is not only important for oncological patients, but also in other pathologic circumstances, in which the disease affects gonadal function or due to gonadotoxic treatments to the condition (Martinez et al., 2017). Female fertility preservation has gained much attention since the first birth after autotransplantation of frozen ovarian tissue in 2004 (Donnez et al., 2004).

Two major techniques have been applied for the cryopreservation of ovarian tissue: SF/T and V/W. SF/T was the first procedure employed for ovarian tissue cryopreservation (Hovatta et al., 1996; Newton et al., 1996; Rosendahl et al., 2011) and it has been the conventional technique employed for female fertility preservation for years. However, V/W has been gaining growing attention for cryopreservation of ovarian tissue after successful results obtained from experimental studies in rodents, non-human primates and domestic animals (Amorin et al., 2011; Santana et al., 2012).

Early work by our group demonstrated that V/W of mouse ovaries in a metal container made of aluminium foil resulted in high rates of primordial follicle survival and stromal integrity (Bös-Mikich et al., 2012; Marques et al., 2013). These findings impelled us to develop

a closed metal capsule, in which the biological specimens never directly contact the liquid nitrogen (LN₂) during the cryopreservation process and cryostorage (Aquino et al., 2014). High rates of primordial follicles survival, and no changes in antioxidant defence capacity and tissue viability were obtained after rewarming bovine ovarian samples vitrified using the metal capsule (Aquino et al., 2014; Massignam et al., 2018). The stromal components of the ovarian tissue represent key elements on the revascularization of the gonad fragments after retransplantation allowing thus, follicle survival and growth. The present study aimed to compare the efficacy of the metal capsule for V/W with the conventional SF/T for human ovarian tissue cryopreservation. Detailed histology analysis of primordial and primary follicles, oocytes and the ovarian stroma in V/W and SF/T ovarian samples were performed.

Methods

Ovarian tissue donors

Human ovarian samples were collected from nine women (median age: 34.6±3 y.o) who underwent laparoscopy due to different gynecological conditions. All patients signed an informed consent approved by Institutional Ethics Committee of Conceição Hospital Group, which also approved the present study.

Tissue preparation

Tissue samples were placed in 0.9% sterile saline and transported to the laboratory at room temperature, within 30 minutes from collection. The biopsies were transferred to a glass surface and cut in small pieces of about 2 (thick) x 5 x 5mm with scalpels. Tissues were kept immersed in HEPES-HTF-medium (Irvine) while working. Two to four small fragments of each biopsy were fixed in 4% paraformaldehyde (PFA) solution for light microscopy. The remaining tissue fragments were transferred to the treatment groups (SF/T or V/W) using sterile soft paintbrushes, to avoid tissue damage and to carry the least amount of medium in each transfer

during the cryopreservation procedures. After thawing or rewarming, samples were fixed for light microscopy. The ovarian tissue samples were stored in LN_i for three to eight weeks before thawing or rewarming.

Slow freezing and thawing procedures

The slow freezing procedure followed the method described by Rosendahl and colleagues (2011) for human ovarian tissue. Briefly, tissue pieces were transferred to a 50ml plastic tube containing ice-cold freezing solution composed of 0.1 M sucrose (Su) and 1.5 M ethylene glycol (EG) in PBS for equilibration in a tilting table for 30 minutes. Tissues were then transferred to 1.8 ml Nunc cryovials containing 1 ml of the cryoprotectant solution and frozen using a programmable freezer machine (Planer K10, UK). The slow-freezing program was: 2°C/min to 9°C, 5 minutes of soaking, manual seeding, 0.3°C/min to 40°C, 10°C/min to 140°C, where the vials were removed from the machine and plunged directly into LN_i (-196°C).

For thawing, the cryovials were removed from the LN_i and exposed to water bath at 37°C, until the cryoprotectant solution was liquid. Ovarian fragments were removed from the vials and placed in the first thawing solution (0.75 M EG and 0.25 M Su in PBS), moved to the second medium (0.25 M Su in PBS) and finally moved to PBS, at room temperature. All samples were fixed in PFA, as described above.

Vitrification and warming

The vitrification procedure followed the method described earlier (Aquino et al., 2014) with minor modifications. Ovarian fragments were equilibrated in two steps in solutions containing increasing concentrations of dimethyl sulfoxide (Me₂SO) and EG in HTF medium (Irvine). Tissues were exposed to vitrification solution 1 (VS1, 7.5% EG and 7.5% Me₂SO) for 25 min and then transferred to VS2 (15% EG and 15% Me₂SO) for 15 min before being loaded

with a minimum of cryoprotectant, into the metal chamber. The vessel lid was tightly screwed and the system was immersed in LN₂.

For warming, the metal capsules were removed from the LN₂ and exposed to tap water with the help of forceps. After opening, the bottom of the capsule was immersed in water bath at 37°C, for about 30 sec. Ovarian samples were removed from the metal chamber and transferred to three solutions containing decreasing concentrations of sucrose (1 M, 0,5 M and 0,25 M in PBS) for 1, 3 and 5 min in each, respectively. Samples were fixed in PFA, as described above.

Histology and staining procedure

Fresh, SF\T or V\W tissues were dehydrated by passing through ascending grades of alcohol and embedded in paraffin wax. Sections of 5µm thickness were prepared and every 10th section of each sample was mounted on glass slides and stained with haematoxylin & eosin. Primordial and primary follicles and oocytes were analyzed. Only follicles with a visible oocyte nucleus were analyzed. Structural normality was defined as a follicle with an even distribution of follicular cells, intact basal membrane and spherical oocyte presenting an even cytoplasm and nucleus with finely dispersed chromatin. Abnormalities were considered when the granulosa cells were detached from the basement membrane, the ooplasm was shrunken or presented vacuole and the chromatin was pyknotic. One extra slide of fresh control, V\W and SF\T tissues from each patient was taken at random for Gomori staining. The tissue was classified as < 10% damage when no damage or a small area of the tissue fragment slice presented alterations, such as gaps among the collagen bundles; 10-50% when the gaps covered up to half of the tissue slice and > 50% when the damage covered nearly all the tissue sample. Observations were performed on a Olympus microscope and the embryologist was not aware of the origin of the tissue present in the slide (blind analysis).

Statistics

Categorical variables for tissue quality were associated by the Mac Nemar test. Damage frequencies were compared by Chi-square test and Sidak's and Bonferroni's correction was applied for multiple comparisons. Statistical analysis were performed using SPSS 20.0 and the program WINPEPI 11.93 (Abranson, 2011). A P-value < 0.05 was considered statistically significant.

Results

Histological analysis by hematoxylin & eosin

Sections of human ovarian cortex fragments treated by SF/T and V/W and fresh controls were analyzed for morphological parameters of the primordial and primary follicles. The overall percentages of morphologically normal primordial and primary follicles from each treatment and control groups were compared (Table 1). The frequency of morphologically normal primordial and primary follicles in the fresh control groups was significantly higher than that of the treated cryopreserved groups. No difference was observed between primary and primordial follicles normal morphology for the SF/T and the V/W groups.

Table 1- Frequency of morphological normal human primordial and primary follicles in groups of SF/T, V/W and fresh control tissues.

| | N° primordial follicles | | N° primary follicles | |
|-------|-------------------------|-----------------------|----------------------|----------------------|
| | Total | Normal (%) | Total | Normal (%) |
| Fresh | 660 | 620 (94) ^a | 75 | 41 (55) ^a |
| SF/T | 59 | 44 (75) ^b | 50 | 40 (80) ^b |
| V/W | 106 | 86 (81) ^b | 60 | 50 (83) ^b |

*P<0,05 Chi-square test.

Frequency of damage in oocytes and follicular structure in V\W and SF\T ovarian tissue samples

Table 2 shows the frequency of damage in oocytes and follicular structure of primordial and primary follicles in fresh and cryopreserved ovarian tissue. Analysis of the nuclei of oocytes in primordial follicles revealed a significant higher incidence of abnormal pyknotic chromatin in the V/W and in the SF/T groups, when compared with controls. However, there was no difference in nuclear damage frequency between the two cryopreserved groups. Oocytes present in primary follicles did not show significant differences in nuclear structure between cryopreserved and control groups, nor between the SF/T and the V/W groups. The vast majority of cryopreserved oocytes presented a clearly visible euchromatic nucleus and nucleoli. The occurrence of cytoplasmic shrinkage was low and it was not significantly different among groups. The incidence of cytoplasmic vacuole was higher in V\W primordial follicles, compared to controls. However, primary follicles from fresh controls showed significantly higher incidence of vacuole than primary follicles present in V\W or SF\T ovarian fragments (Table 2). Detachment of follicular cells from basal membrane was the major damage caused by both cryopreservation techniques on primordial and primary follicles. Significantly increased frequencies of follicular cells detachment from basal membrane were observed in all

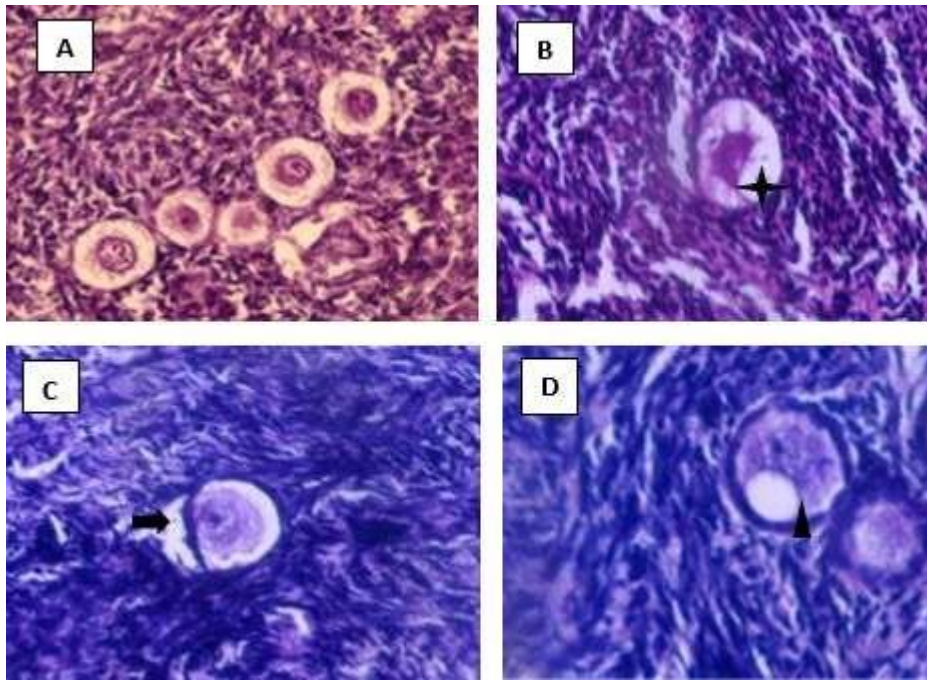
groups, compared to follicles in the fresh control samples (Table 2). Figure 1 shows some examples of oocyte and follicle intact and damaged structures after cryopreservation.

Table 2. Frequency of damage in oocytes and follicular structure in V\W and SF\T ovarian tissue sample

| Primordial follicles | | | | | Primary follicles | | | | |
|---|---------|------------------------|-------------------------|-----------------------|-------------------|---------|------------------------|-------------------------|-----------------------|
| Nuclear damage | | | | | | | | | |
| Group | N/total | P Fresh x V/W | P Fresh x SF/T | P V/W x SF/T | Group | N/Total | P Fresh x V/W | P Fresh x SF/T | P V/W x SF/T |
| Fresh | 0/660 | 0.014 | 0.005 | 0.912 | Fresh | 1/75 | 0.347 | 0.77 | 0.962 |
| V/W | 2/106 | | | | V/W | 2/60 | | | |
| SF/T | 2/59 | | | | SF/T | 1/50 | | | |
| Cytoplasmic shrinkage | | | | | | | | | |
| Group | N/total | P Fresh x V/W | P Fresh x SF/T | P V/W x SF/T | Group | N/Total | P Fresh x V/W | P Fresh x SF/T | P V/W x SF/T |
| Fresh | 12/660 | 0.163 | 0.999 | 0.387 | Fresh | 2/75 | 0.986 | 0.524 | 0.837 |
| V/W | 0/106 | | | | V/W | 1/60 | | | |
| SF/T | 1/59 | | | | SF/T | 2/50 | | | |
| Cytoplasmic vacuole | | | | | | | | | |
| Group | N/total | P Fresh x V/W | P Fresh x SF/T | P V/W x SF/T | Group | N/Total | P Fresh x V/W | P Fresh x SF/T | P V/W x SF/T |
| Fresh | 28/660 | 0.222 | 0.079 | 0.012 | Fresh | 30/75 | <0.001 | <0.001 | 1 |
| V/W | 9/106 | | | | V/W | 0/60 | | | |
| SF/T | 0/59 | | | | SF/T | 0/50 | | | |
| Follicle cells detachment from basal membrane | | | | | | | | | |
| Group | N/total | P Fresh x V/W | P Fresh x SF/T | P V/W x SF/T | Group | N/Total | P Fresh x V/W | P Fresh x SF/T | P V/W x SF/T |
| Fresh | 0/660 | <0.001 | <0.001 | 0.094 | Fresh | 1/75 | <0.001 | <0.001 | 0.977 |
| V/W | 9/106 | | | | V/W | 7/60 | | | |
| SF/T | 12/59 | | | | F/T | 7/50 | | | |

Figure 1: Examples of intact and damaged follicular and oocyte structures after cryopreservation.

(A) intact follicles, (B) cytoplasmic retraction ✦ (C) basal membrane detachment ➡ and (D) cytoplasmic vacuole ▲. (X 400)



Histological analysis of the ovarian stroma

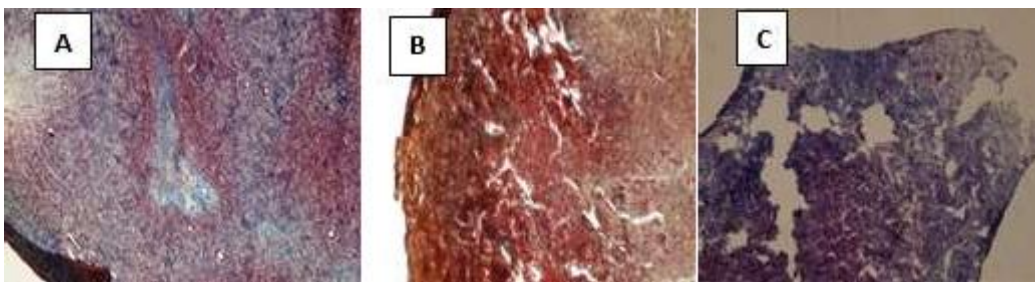
Figure 2 shows examples of histological sections of human ovarian cortex fragments stained using the trichrome Gomori technique, commonly used to identify collagen fibers, the main component of the ovarian stroma tissue. A qualitative analysis of slides presented in Table 3 revealed that the extent of stroma damage was similar in six out of the nine patients for V\W and SF\T samples. Among the samples that had discordant frequency of damage between the V\W and SF\T samples, SF\T tissues presented higher rates of damage than V\W. Fresh controls presented the highest rates of tissue damage after Gomori staining. (Table 3 and Figure 2 near here).

Table 3. Individual analysis of the extent of damage in the stroma of fresh, V\W and SF\T human ovarian tissue samples

| Patient | Fresh Control | Vitrified\warmed (V\W) | Frozen\thawed (SF\T) |
|---------|---------------|------------------------|----------------------|
| A | >50% | 10-50% | 10-50% |
| B | 10-50% | <10% | 10-50% |
| D | <10% | <10% | 10-50% |
| E | 10-50% | <10% | <10% |
| F | <10% | <10% | <10% |
| G | >50% | >50% | >50% |
| H | 10-50% | 10-50% | >50% |
| I | >50% | <10% | <10% |
| L | 10-50% | <10% | <10% |

Figure 2. Ovarian stroma morphology.

(A) <10%, (B) 10-50% and (C) >50% of tissue damaged after cryopreservation. (X 10).



Discussion

The present study showed that there is an overall decrease in the frequency of morphologically intact primordial and primary follicle structures in V/W and SF/T human ovarian samples, when compared with untreated controls. It is Important to point out that, primordial and primary follicles from both cryopreserved groups showed a similar degree of

damaged follicular morphology. Analysis of specific parameters related to follicular and oocyte structures revealed that the major damages caused by both cryopreservation processes affected the nuclear structure of oocytes of primordial follicles and follicular cells detachment from the basement membrane of primordial and primary follicles. Qualitative analysis of the ovarian stroma showed cryodamage of small entity indicating that stroma integrity is not dramatically altered by the cryopreservation processes.

Slow-freezing, is the conventional and first methodology employed for female fertility preservation and resulted in the majority of live births in patients with oncological pathologies. Vitrification, however, has been gaining increased interest for the cryopreservation of ovarian cortex, as it has successfully been applied to human oocytes and embryos. Regardless of the technique, the cryopreservation method should not only preserve the fertility potential of the patient, but also restore her endocrine function. To this aim, ovarian stroma integrity is a key factor for successful re-transplantation and graft survival.

Previous studies have compared the effects of SF/T and V/W for the cryopreservation of human ovarian tissue with contradictory outcomes (Huang et al., 2008; Isachenko et al., 2009; Keros et al., 2009; Klocke et al., 2015; Sanfilippo et al., 2015, Fabbri et al., 2016). In accordance with our findings, most of these studies found similar outcomes in terms of follicular integrity (Huang et al., 2008; Keros et al., 2009; Klocke et al., 2015; Sanfelippo et al., 2015), between both cryopreservation methodologies. However, two reports showed a higher proportion of follicular damage on V/W human ovarian samples, when compared to SF/T (Isachenko et al., 2009; Fabbri et al., 2016).

The scope of the present study was not limited to find putative alterations in follicular structure caused by SF/T and V/W on human ovarian samples, but we also intended to find out which follicular or oocyte component was being affected by cryopreservation. At the oocyte

level, data showed that vacuole were present predominantly in the ooplasm of oocytes of primordial follicles of V/W, when compared with SF/T tissue samples. These results are in accordance with early data reported by Fabbri and colleagues (2014), who suggested that this feature may indicate early signs of follicular degeneration in V/W samples. However, the highest prevalence of ooplasmic vacuole in our study was obtained in control non-cryopreserved samples, a finding that may suggest a histology processing effect, rather than a cryodamage. It is described that tissue-processing artifacts, such as vacuolation, may result from improper dehydration, particularly when the tissue sample is accidentally left for a too long period in lower dilution of alcohol (Margarone et al., 1985). These observations were strengthened by the analysis of stroma injury rates, which showed major tissue damage rates in non-cryopreserved ovarian samples. Despite careful handling of the materials throughout the methods, we cannot exclude the possibility of an accidentally longer exposure of a batch of tissues during dehydration procedures.

Results clearly showed that the major cryodamage observed in primordial and primary follicles was follicular cells detachment from the basal membrane. Except for one primary follicle in the control group, this feature was exclusively observed in SF/T and V/W tissue samples. Thus, follicular cells detachment from the basal membrane may represent a significant deleterious effect of cryopreservation affecting tissue survival post-thawing and post-rewarming. Basement membranes are highly specialized extracellular matrices. They act as formidable barriers that contain and separate dynamic and versatile environments that modulate cellular behaviors to regulate tissue development, function, and repair. Increasing evidence suggests that, in addition to providing structural support to neighboring cells, basement membranes serve as reservoirs of growth factors that direct and fine-tune cellular functions (Mao et al., 2015). Thus, follicular cells detachment from the basal membrane appears to be an important cause of follicle death after thawing or warming and transplantation. However,

follicular cells detachment from the basement membrane a morphological parameter not taken into account on previous studies of the effects of cryopreservation on the ovarian tissue integrity (Keros et al., 2009; Fabbri et al., 2016).

Another major aspect of ovarian tissue viability post-cryopreservation is the integrity of the stromal components. Not many studies analyzed the structural integrity of cryopreserved ovarian stroma post-thawing or rewarming. However, the maintenance of a functional stroma is fundamental for follicle survival post-transplantation as growth factors produced and released from stromal cells are responsible for revascularization and general metabolic activity of the ovarian structures including follicles. Our previous experiments using cow ovaries as experimental model, showed that the stromal structure is well preserved after cryopreservation using the metal capsule for V/W (Marques et al., 2013). Similar results were obtained in the present study, where the stromal components were better preserved after V\W than after SF\T in the vast majority of samples taken from individual patients. These observations are in accordance with a previous study that did not detect a significant destructive effect of V/W and SF/T for stromal cells by the TUNEL assay (Huang et al., 2008). In addition, in accordance with present observations, two studies (Keros et al., 2009; Fabbri et al., 2016) reported a significantly better morphological integrity of stromal components after vitrification, than after controlled-rate freezing of ovarian cortex. Interesting to note the clear difference in the extent of tissue injury among samples taken from the nine patients. Keros and colleagues (2009) described that the integrity of the tissue after cryopreservation depends on the condition of non-frozen tissue used in the experiment. According to the authors, there appeared to be clear patient to patient variation, a relevant factor to be taken into account when interpreting morphological parameters and subsequent clinical application of cryopreservation for fertility preservation.

Despite the fact that morphology represents an important parameter to assess tissue viability, recovery of normal physiological function signifies an additional challenge for tissues

post-cryopreservation. One study performed functional imaging analysis by Laser Scanning Confocal Microscopy showing that the mitochondrial activity and intracellular reactive oxygen species levels were reduced both in SF/T and in V/W samples compared with fresh samples, being that V/W samples presented significantly lower values than SF/T ones (Fabbri et al., 2016). In another study, the viability and proliferative capacity of SF/T and V/W tissue were assessed by *in vitro* production of hormones, development of follicles, and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene expression. Results showed no difference between the two techniques in respect to hormonal activity and follicle viability (Klocke et al., 2015; Isachenko et al., 2009). However, *GAPDH* gene expression was significantly decreased after V/W, in contrast to conventional SF/T (Isachenko et al., 2009).

In conclusion, the present results show that both, V/W and SF/T affect the overall normal morphology of human ovarian tissue samples. However, there are no differences between the two cryopreservation methods. The significant incidence of follicle cells detachment from the basal membrane following cryopreservation is an important finding that deserves further investigation given the fundamental role of the basal membrane on follicle survival and function. It is our understanding that either V/W or SF/T may be applied for female fertility preservation with similar viability outcomes, provided that the tissue samples are submitted to careful handling and precise cryopreservation methodology.

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5. CONCLUSÃO E PERSPECTIVAS

Os resultados apresentados nesse trabalho indicaram uma equivalência quanto a diminuição geral na frequência de estruturas foliculares morfológicamente intactas nas amostras dos grupos vitrificados e congelados ao compará-los com o grupo controle. Quanto aos danos foliculares, encontramos uma prevalência de vacúolos presentes nas amostras pós-vitrificação. Entretanto, também encontramos uma grande prevalência de vacúolos nos oócitos de amostras do grupo controle não criopreservado. Sendo assim, pode-se sugerir que o aparecimento desse dano no ooplasma de tecidos criopreservados pode decorrer do processo histológico, de modo que a desidratação inadequada, por exemplo, pode resultar no aparecimento de artefatos, como os vacúolos. Ademais, com os resultados obtidos nesse trabalho, identificamos que o principal dano encontrado nas amostras de tecidos criopreservados foi o desprendimento de células foliculares da membrana basal, de modo que o aparecimento foi equivalente nas duas técnicas de criopreservação. Sendo assim, esse dano pode ser capaz de afetar a sobrevivência dos tecidos pós-criopreservação.

A preservação do estroma é um aspecto extremamente importante para a sobrevivência do tecido, uma vez que fatores de crescimento produzidos pelas células do estroma são responsáveis pela revascularização e pelo metabolismo dos folículos ovarianos. Neste trabalho identificamos que os componentes do estroma foram melhor preservados no grupo vitrificado, quando comparados com o grupo congelado.

Logo, nosso trabalho mostrou que as duas técnicas de criopreservação, congelamento lento e vitrificação, afetam a morfologia das amostras de tecido ovariano. Entretanto, não foram encontradas diferenças significativas capazes de afirmar qual dos métodos é mais eficaz para ser utilizado nas rotinas de criopreservação de tecido ovariano humano. Portanto, pode-se dizer que as duas técnicas são viáveis para aplicação na preservação da fertilidade feminina, sendo a vitrificação um método mais vantajoso, uma vez que, além de apresentar resultados semelhantes ao congelamento lento, é um procedimento mais rápido e barato.

Sendo assim, é necessária uma continuidade de estudos na área, demonstrando a eficiência e as vantagens da vitrificação. Uma avaliação do tempo necessário de incubação pós reaquecimento seria interessante, uma vez que pode ser, também, requisito para uma maior sobrevivência tecidual.

Dessa maneira, com a comprovação da eficácia da vitrificação para a criopreservação de tecido ovariano humano, essa técnica poderá ser usada nas rotinas de preservação da fertilidade feminina, assim como, futuramente, abrir portas para o desenvolvimento de um banco de tecido ovariano humano, que poderá amparar milhares de pacientes, principalmente aquelas que necessitam de algum tratamento oncológico e optam por preservar sua fertilidade.

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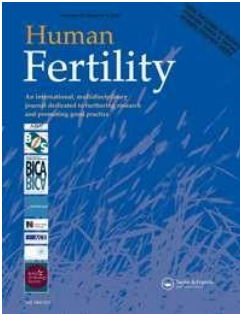
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ANEXO A – NORMAS DE PUBLICAÇÃO DA REVISTA HUMAN FERTILITY



Journal Human Fertility

An international, multidisciplinary journal dedicated to furthering research and promoting good practice

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All authors submitting to medicine, biomedicine, health sciences, allied and public health journals should conform to the Uniform Requirements for Manuscripts Submitted to Biomedical Journals, prepared by the International Committee of Medical Journal Editors (ICMJE).

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3. **Graphical abstract** (optional). This is an image to give readers a clear idea of the content of your article. It should be a maximum width of 525 pixels. If your image is narrower than 525 pixels, please place it on a white background 525 pixels wide to ensure the dimensions are maintained. Save the graphical abstract as a .jpg, .png, or .tiff. Please do not embed it in the manuscript file but save it as a separate file, labelled GraphicalAbstract1.
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5. No more than 6 **keywords**. Read making your article more discoverable, including information on choosing a title and search engine optimization.
6. **Funding details.** Please supply all details required by your funding and grant-awarding bodies as follows:

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This work was supported by the [Funding Agency #1] under Grant [number xxxx]; [Funding Agency #2] under Grant [number xxxx]; and [Funding Agency #3] under Grant [number xxxx]

7. **Disclosure statement.** This is to acknowledge any financial interest or benefit that has arisen from the direct applications of your research. Further guidance on what is a conflict of interest and how to disclose it.
8. **Biographical note.** Please supply a short biographical note for each author. This could be adapted from your departmental website or academic networking profile and should be relatively brief (e.g. no more than 200 words).
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11. **Tables.** Tables should present new information rather than duplicating what is in the text. Readers should be able to interpret the table without reference to the text. Please supply editable files.
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All authors are required to follow the ICMJE requirements on privacy and informed consent from patients and study participants. Please confirm that any patient, service user, or participant (or that person’s parent or legal guardian) in any research, experiment, or clinical trial described in your paper has given written consent to the inclusion of material pertaining

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