Universidade Federal do Rio Grande do Sul

Faculdade de Medicina

Programa de Pós-Graduação em Ciências da Saúde: Ginecologia e Obstetrícia

VITRIFICAÇÃO *VERSUS* CONGELAMENTO LENTO NÃO AUTOMATIZADO EM TECIDO OVARIANO DE CAMUNDONGOS CF1

Paula Barros Terraciano

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SUMÁRIO

1. RESUMO

Introdução: a alta prevalência do câncer e o aumento significativo da sobrevivência em longo prazo geraram interesse quanto à preservação da fertilidade em mulheres jovens expostas a quimioterapia e radioterapia. Neste sentido estudos de congelamento de tecido ovariano para posterior transplante, abriram uma nova perspectiva de aplicação no tratamento e prevenção da infertilidade feminina. **Objetivos:** comparar dois protocolos de congelamento de tecido ovariano, um lento não automatizado e um por vitrificação, com o intuito de avaliar a viabilidade dos tecidos para posterior transplante autólogo. **Método:** Foram utilizadas 30 camundongos fêmea CF1 com aproximadamente 8 semanas e pesando 29,29g±2,9. •Os ovários extraídos foram vitrificados ou congelados, mantidos em nitrogênio líquido por 30 dias e descongelados. Após o descongelamento, o ovário esquerdo foi destinado às análises histológicas e caracterização por imuno histoquímica para o marcador mouse vasa homologue (MVH) e o ovário direito foi utilizado para os testes de viabilidade celular com exclusão por azul de trypan. **Resultados:** Nas análises de Hematoxilina e Eosina (HE) foram contados folículos primordiais, primários, pré-antrais e antrais. Não houve diferença significativa na proporção de folículos primordiais, primários e pré-antrais após descongelamento entre os grupos testados. A contagem de folículos antrais foi significativamente maior no grupo de vitrificação (p = 0,004). No ensaio de imunohistoquímica para o marcador MVH, folículos MVH + e MVH- foram contados e comparados com o número total de folículos. O grupo congelamento lento apresentou maior número de células não marcadas (p = 0,012). Conclusão: Embora ambos os protocolos tenham apresentado resultados semelhantes na análise histológica das contagens

foliculares, o protocolo de vitrificação foi significativamente melhor para preservar a população de células tronco ovarianas.

Palavras-chave: criopreservação, infertilidade, tecido ovariano, célula germinativa primordial.

2. ABSTRACT

Introduction: The high prevalence of cancer and the significant increase in long-term survival have generated interest as the preservation of fertility in young women exposed to chemotherapy and radiotherapy. Experimental techniques have been tried in an attempt to reverse the ovarian failure induced by these treatments. In this regard studies of ovarian tissue freezing for subsequent transplantation disclose a new application perspective in the treatment and prevention of female infertility. **Objective:** two ovarian tissue freezing protocols were tested, a non-automated slow-freezing and by vitrification, in order to assess the viability of the tissues for subsequent autologous transplantation. **Methods:** as ovaries donors, were used 30 female CF1 mice approximately 8 weeks and weighing 29,29g±2,9. • The ovaries were vitrified or frozen, stored in liquid nitrogen for 30 days and thawed. After thawing, the left ovary was intended for histological and immunohistochemical characterization by histochemical marker for MVH and right ovary was used for the tests with cell viability by trypan blue exclusion. **Results:** In HE slides was counting primordial, primary, pre antral and antral follicles. No significant difference was found in the proportion of high-quality primordial, primary and pre antral follicles after thawing/warming in the slow-freezing and vitrification group, respectively. The antral follicle counting was significant higher in vitrification group (p=0,004). In immunohistochemistry assay for MVH Antibody , MVH+ and MVH- follicles were counted and compared with the total number of follicles and slow freeze group had a higher number of not marked cells (p=0,012). **Conclusion:** Although both protocols showed similar results in the histological analysis for follicular counts, the vitrification protocol was significantly better for preserve the ovarian stem cell population.

Keywords: *Cryopreservation, infertility, ovarian tissue, primordial germ cell.*

3. LISTA DE ABREVIATURAS

C	Grupo Controle
°C	Graus Celsius
CEUA	Comissão de Ética no uso de Animais
CPE	Centro de Pesquisa Experimental
DMEM	*Dulbecco's Modified Eagle Medium*
DMSO	Dimetilsulfóxido
DPBS	*Dulbecco's Phosphate-Buffered Saline*
FBS	*Fetal Bovine Serum*
EG	Etileno Glicol
FGSC	*Female germline stem cell*
FOP	Falência Ovariana Precoce
g	Grama
GLY	Glicerol
Gy	Gray
HCPA	Hospital de Clínicas de Porto Alegre
HE	Hematoxilina e eosina
M	Molar

MVH *Mouse vasa homologue*

PROH Propanodiol

SF Grupo congelamento lento (Slow Freeze)

TRA Técnica de Reprodução Assistida

UEA Unidade de Experimentação Animal

UPE Unidade de Patologia Experimental

VIT Grupo Vitrificação

4. LISTA DE TABELAS

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6. INTRODUÇÃO

O câncer é diagnosticado em mais de 600.000 pessoas nos Estados Unidos a cada ano, 269.800 dos acometidos são mulheres e 8% apresentam idade abaixo de 40 anos [1]. Estima-se que em 2030 serão 21, 4 milhões de novos casos e 13,2 milhões de mortes por câncer no mundo [2]. Com o advento de novas terapias contra as doenças crônico degenerativas, especialmente as neoplasias, a expectativa de vida de muitas pacientes jovens foi aumentada e, aproximadamente, 80% dos doentes irão sobreviver.

Um dos principais problemas, especialmente para as jovens pacientes, é que a terapia destrói uma proporção significativa da população folicular, o que pode resultar em infertilidade permanente [3, 4, 5]. Embora o tratamento e a sobrevivência sejam o foco principal dos profissionais de saúde e também dos pacientes com câncer, é conveniente considerar a sua qualidade de vida após o tratamento, incluindo a possibilidade de ter filhos [6, 7].

A ação citotóxica da radiação ionizante e dos quimioterápicos, com grande frequência, leva à ocorrência de falência ovariana precoce (FOP), que implica sérias consequências em longo prazo, como redução da massa óssea levando a osteoporose, aumento na incidência de doenças cardiovasculares, ocorrência precoce de sintomas climatéricos e infertilidade [8]. A ausência temporária ou permanente das funções endócrina e reprodutiva dos ovários depende, principalmente, da idade da paciente no momento do tratamento gonadotóxico e das doses administradas [9, 10]. O risco de infertilidade varia de acordo com o tratamento, a quimioterapia induzida por esquemas de tratamento individual baseada em agentes alquilantes e platina possui o maior potencial de dano ao sistema reprodutivo [7, 11, 12].

Alguns protocolos de quimioterapia podem causar insuficiência prematura do ovário ou insuficiência ovariana aguda que ocorre durante ou logo após o tratamento do câncer, ou menopausa prematura seguida de uma janela de função normal após a terapia. Já para mulheres submetidas à radioterapia com doses maiores que 300 Gy, a incidência de FOP pode ser superior a 60%, sendo que a exposição a 4 Gy de irradiação pélvica é a dose letal para oócitos, e 97% das mulheres que recebem de 5 a 10,5 Gy vão posteriormente apresentar falência ovariana. A combinação de quimioterapia e radioterapia é particularmente devastadora.

No entanto, estudos em mulheres que conceberam anos após o seu tratamento quimioterápico não encontraram aumento significativo em malformações congênitas ou neoplasias malignas na prole [5, 13, 14, 15]. A cada dia mais pacientes estão sobrevivendo ao câncer, graças ao diagnóstico precoce e tratamento adequado. Embora adultos sobreviventes tendam a ter maior interesse na preservação e fertilidade reprodutiva, é fundamental ter em mente que outras opções devem ser disponibilizadas aos jovens pacientes com câncer. Os profissionais de saúde devem estar preparados para discutir sobre fertilidade com os pacientes, independentemente da sua idade ou estado civil no momento do início da terapia, para que eles compreendam as possíveis conseqüências do tratamento do câncer e conheçam as alternativas disponíveis para preservação da fertilidade [16].

Além das mulheres que receberam tratamento para o câncer outros grupos de paciente podem ser candidatos a preservação de fertilidade, incluindo mulheres com endometriose, e os pacientes tratados com agentes quimioterápicos para doenças não malignas como as auto-imunes e colágeno-

vasculares. Podem incluir ainda a incompatibilidade de idade da maternidade e do exercício dos planos de carreira, ou ainda não ter encontrado um parceiro adequado para iniciar uma família [17,18, 19].

A evolução contínua das técnicas de reprodução assistida (TRA) permite que se considerem várias estratégias de tentativa de manutenção da função reprodutiva em pacientes após tratamentos de doenças neoplásicas, dentre as quais se incluem manipulação hormonal, transposição dos ovários, criopreservação de embriões, gametas e de tecido cortical ovariano. O método rotineiramente utilizado em clínicas de reprodução assistida é a criopreservação de embriões, mas essa opção requer estimulação ovariana e coleta de oócitos, o que pode postergar o tratamento entre 2 a 5 semanas e o posterior emprego das TRA.

Assim, o atraso no início do tratamento quimioterápico para o armazenamento de embriões não é factível para algumas pacientes e pode ser, até mesmo, um fator de piora no prognóstico em alguns tipos de câncer. O congelamento de embriões também não se aplica a pacientes sem um parceiro fixo, com quem deseje constituir prole futura, como é o caso das adolescentes. Uma alternativa seria a utilização de amostras provenientes de banco de sêmen, entretanto este tipo de abordagem esbarra em questões religiosas, morais e éticas [3, 11, 20, 21].

Além disso, a estimulação não deve ser feita em mulheres que apresentam tumores hormônio dependentes, como o câncer de mama, apesar de terem surgido novos protocolos de estimulação com o uso do Tamoxifeno e de inibidores da aromatase (Letrozole), mostrando resultados animadores [22].

Embora a captação de oócitos possa ser realizada sem estimulação ovariana (ciclo natural), o rendimento final de produção embrionária em ciclos não estimulados é extremamente baixo [23] e, portanto, não recomendado para as pacientes com câncer [22].

Diante de todas estas questões, seguidas da difusão das técnicas de congelamento ovariano para preservação de fertilidade, tornou-se imprescindível avaliar a qualidade do tecido recuperado após a criopreservação.

A criopreservação de tecido ovariano fundamenta-se no princípio de que folículos inativos (primordiais) resistem melhor a criotoxicidade do que folículos em processo de maturação [24, 25], pois apresentam metabolismo reduzido, ausência de zona pelúcida e maior facilidade de penetração dos crioprotetores devido ao menor tamanho folicular [26]. Além disso, folículos primordiais têm maior potencial de reparo a danos nas organelas e outras estruturas sofridos durante a fase prolongada de congelamento na criopreservação.

Os danos celulares funcionais causados pela criopreservação podem, entretanto, ser irreversíveis se as temperaturas caírem abaixo dos níveis ideais. Variações térmicas muito abruptas podem interferir no transporte de água através da membrana celular e propiciar a formação de cristais de gelo e depósitos de sais no interior da célula [27]. Também as diferenças de pressão osmótica entre os meios intra e o extracelular podem levar a mudança de volume no oócito com consequente dano à membrana plasmática e às organelas [28, 29].

Outro fator que pode propiciar dano celular é a presença de fusos meióticos nas células foliculares; foi demonstrado que pequenas variações de temperatura podem danificar os microtúbulos que compõem o fuso, podendo cursar com perdas cromossômicas e aneuploidias durante o término da primeira divisão meiótica no processo de maturação subsequente [30]. Além disso, também tem sido demonstrado que o congelamento pode danificar o citoesqueleto das células ou oócitos, com prejuízo no tráfego de moléculas e organelas no processo de divisão celular [31]. A fase de reexpansão (descongelamento) do tecido também pode ser deletéria se o meio externo não for adequado [32].

O congelamento de tecido ovariano é atualmente proposto com o intuito principal de manter a função ovariana preservada, tanto do ponto de vista de fertilidade como de produção hormonal, o que não se alcança com os demais métodos. Além disso, essa opção surge no contexto atual para servir a grupos específicos de pacientes para as quais as demais técnicas não são recomendáveis, como: (1) pacientes pré-púberes, cujas gônadas ainda não estão sob controle do eixo hipotálamo-hipófise; (2) mulheres que não tenham parceiro e não desejam embriões oriundos da fertilização com sêmen de doador; (3) pacientes portadoras de neoplasias estrogênio-dependentes, como o câncer de mama; e (4) mulheres portadoras de neoplasias malignas que necessitam de abordagem imediata, para as quais o retardo no início do tratamento pelo tempo necessário para indução da ovulação poderia levar a mudanças no prognóstico da paciente.

Particularmente sob esse aspecto, a coleta do tecido cortical ovariano para criopreservação oferece a vantagem de poder ser realizada em qualquer

momento do ciclo menstrual da paciente, e possibilitar a aquisição de centenas de milhares de folículos primordiais [33, 34]. É relevante comentar sobre as desvantagens encontradas quanto à criopreservação de tecido cortical ovariano, sendo estas, (1) submeter à paciente a procedimentos cirúrgicos para colheita de tecido ovariano; e (2) possibilidade de reintroduzir células malignas no caso de posterior reimplante do tecido [34].

Nas duas últimas décadas, a criopreservação de oócitos e tecido ovariano ganhou popularidade e aumentou o seu sucesso devido ao método de vitrificação. Adicionalmente, há 18 casos reportados de mulheres que fizeram criopreservação e transplante de tecido ovariano, se curaram do câncer e tiveram filhos vários anos após a quimioterapia. Porém, não se pode afirmar que essas técnicas tenham sucesso inequívoco e que sejam seguras [35].

A vitrificação (formação de estado vítreo) foi idealizada por Luyet em 1937 [36]. Depois de quase 50 anos, Rall e Fahy [36] descreveram a vitrificação como uma alternativa ao processo de congelamento lento. A técnica de vitrificação envolve a exposição do material biológico a altas concentrações de agente crioprotetor (geralmente entre 4 e 6 mol/L) por um curto período de tempo (25 segundos a 5 minutos), geralmente à temperatura ambiente, seguido de um resfriamento ultra-rápido em nitrogênio líquido, não sendo necessária a utilização de equipamentos sofisticados e de alto custo.

De acordo com Stachecki e Cohen [37], a vitrificação possui dois aspectos básicos a serem levados em consideração; o primeiro consiste no fato de que as altas concentrações de agentes crioprotetores utilizadas na exposição aumentam os efeitos tóxicos e, em segundo lugar, apesar desse efeito durante

o período de equilíbrio, a vitrificação, por ser uma congelação altamente rápida, aumenta as taxas de sobrevivência. A vitrificação tem sido utilizada com êxito em tecido ovariano com o mínimo de mudanças na morfologia tecidual [15, 38, 39].

Os resultados obtidos com esta técnica têm maior número de recém-nascidos vivos quando comparado com o congelamento lento para a criopreservação de tecido ovariano. Norteando a sua incorporação na clínica como alternativa, entretanto a técnica de escolha para o procedimento não é consensual entre os autores e existem vários serviços que apresentam resultados satisfatórios com o congelamento lento. Neste sentido, recentemente alguns experimentos comparando a vitrificação e o congelamento lento das amostras do tecido do ovário de mamíferos mostrou que a vitrificação também pode garantir o armazenamento de folículos viáveis após o aquecimento, porém o congelamento lento é mais eficaz [37].

Estudos postulam a existência de células-tronco nos testículos e ovários, e a utilização destas tem efeito na restauração da gametogênese e também na geração de prole em modelos animais [40, 41]. Algumas décadas atrás se acreditava que a produção de oócitos nos ovários cessava antes do nascimento na maior parte das espécies de mamíferos [42]. Em 2004, estudos com ratas indicaram que a gônada feminina pode ter uma atividade regenerativa em animais jovens e adultos *in vivo* [43].

Outros estudos relataram o isolamento, cultura e proliferação das células-tronco germinativas femininas (FGSC) *in vitro,* de forma estável durante meses. Estas células produzem *mouse vasa homologue* (MVH) [44] proteína expressa

exclusivamente em células germinativas e tem capacidade de gerar espontaneamente oócito imaturos em cultura. [40, 45]. Estudos *in vivo* mostraram que as FGSC injetadas em ovários de camundongas, pós – quimioterapia foram capazes de produzir prole viável [40]. Estas descobertas abriram a possibilidade de utilizar as células tronco ovarianas para transplante em terapias destinadas a recuperar a função ovariana e fertilidade em fêmeas [46,47].

Os esforços vertem agora na tentativa de elucidar o potencial do tecido ovariano congelado e descongelado na manutenção da capacidade reprodutiva, seja através da maturação in vitro de oócitos isolados, autotransplante do tecido criopreservado ou isolamento de células-tronco ovarianas para transplante.

7. REVISÃO SISTEMÁTICA DA LITERATURA

ESQUEMA DE BUSCA NA LITERATURA

A revisão da literatura centrou-se nas seguintes palavras-chave: *Cryopreservation, infertility, ovarian tissue, Primordial germ cells.* A estratégia de busca envolveu as seguintes bases de dados: MEDLINE (site PubMed), LILACS e Scientific Eletronic Library On Line (SciELO). A tabela 1 sumariza a estratégia de busca das referências bibliográficas sobre as bases que fundamentam os objetivos do estudo.

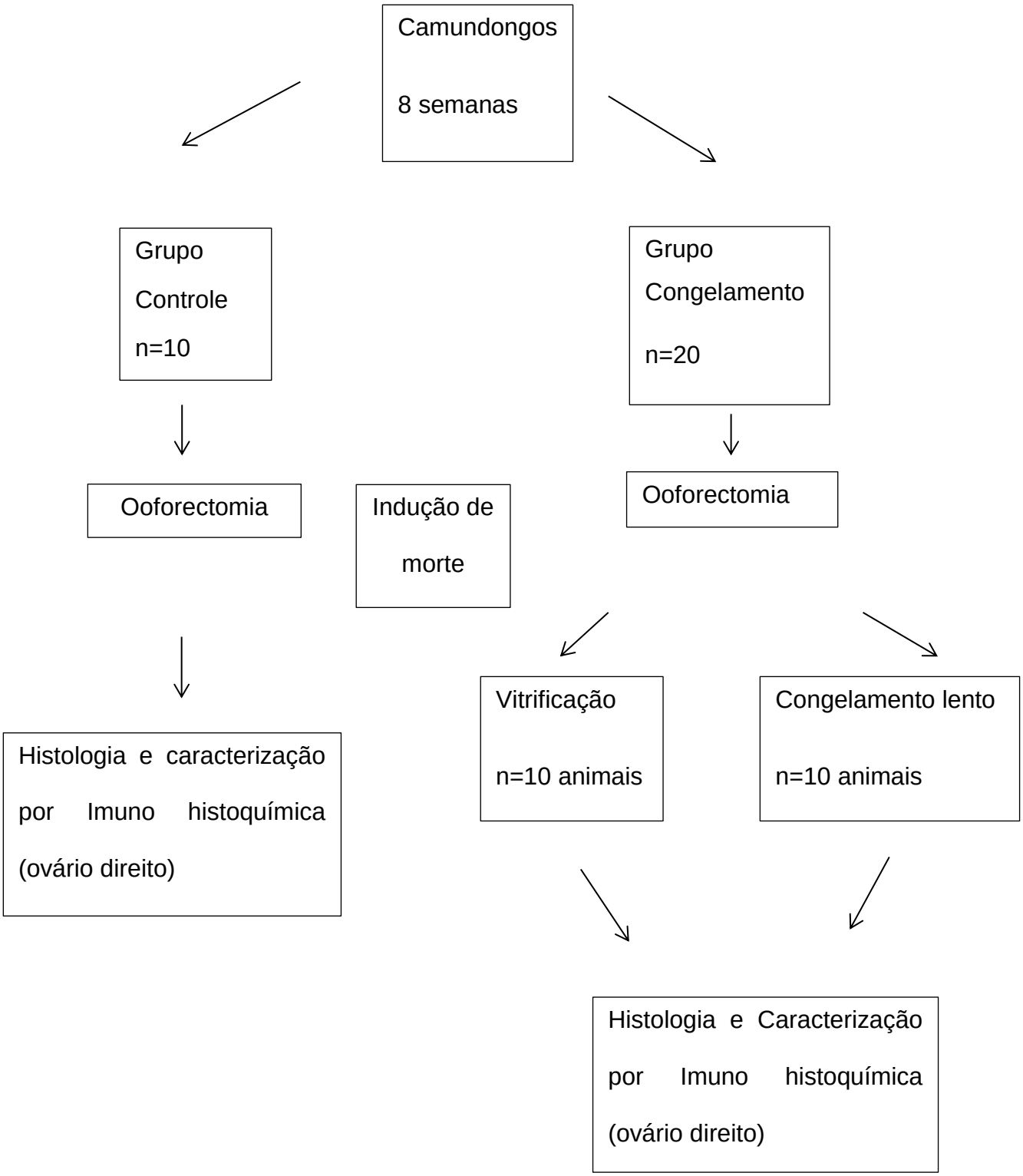
Tabela 01. Resultado de busca de referências bibliográficas nas bases de dados PubMed, LILACS e Scielo .

Palavras-chave	**PubMed**	**Lilacs**	**Scielo**
Cryopreservation	35.806	427	108
Infertility	80.634	2.056	359
Ovarian Tissue	28.468	151	2
Primordial germ cells	3.504	2.808	16

Tabela 02. Resultado do cruzamento de busca de referências bibliográficas nas bases de dados pesquisadas.

Palavras-chave	**PubMed**	**Lilacs**	**Scielo**
Cryopreservation e *Infertility*	2419	37	2
Cryopreservation e *Ovarian Tissue*	1252	10	8
Cryopreservation e Primordial germ cells	103	0	0
Infertility e *Ovarian tissue*	1502	8	4
Infertility e Primordial germ cells	213	168	0
Ovarian tissue e *Primordial germ cells*	280	0	0

8. MARCO CONCEITUAL ESQUEMÁTICO



9. JUSTIFICATIVA

A criopreservação de tecido ovariano, embora seja um procedimento em fase experimental, é uma alternativa promissora para a preservação da fertilidade em mulheres jovens.

Desta forma, as pesquisas visando à reversão da infertilidade feminina, com o transplante de tecido ovariano criopreservado, buscam oferecer alternativas terapêuticas, para pacientes submetidas a tratamentos com agentes quimioterápicos, radioterápicos ou cirurgias.

10. HIPÓTESES

HIPÓTESE NULA

O tecido ovariano fresco não terá a mesma viabilidade dos tecidos vitrificados ou congelados.

HIPÓTESE ALTERNATIVA

O tecido ovariano fresco terá a mesma viabilidade dos tecidos vitrificados ou congelados.

11. OBJETIVOS

Objetivo principal

Comparar a eficácia de dois protocolos diferentes de criopreservação de tecido ovariano, com curva lenta não automatizada ou vitrificação (curva ultra rápida).

Objetivos Específicos

- Obter ovários vitrificados ovários de camundongos CF1 adultos jovens (8 semanas).
- Obter ovários congelados, em curva lenta não automatizada, ovários de camundongos CF1 adultos jovens (8 semanas).
- Avaliar presença do marcador de células primordiais germinativas de ovário nos ovários controle ou congelados, através da técnica de imuno histoquímica.
- Avaliar o perfil de oócitos presentes nas amostras de ovários criopreservados com diferentes protocolos em comparação com o grupo controle à fresco.
- Comparar a viabilidade do tecido ovariano fresco, vitrificado ou congelado através da técnica de exclusão por azul de trypan.

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13. ARTIGO EM INGLÊS

OVARIAN TISSUE VITRIFICATION IS MORE EFFICIENT THAN SLOW FREEZING TO PRESERVE OVARIAN STEM CELLS IN CF-1 MICE

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OVARIAN TISSUE VITRIFICATION IS MORE EFFICIENT THAN SLOW FREEZING TO PRESERVE OVARIAN STEM CELLS IN CF-1 MICE

Abstract

The aim of this study was to investigate the efficacy of different protocols for cryopreservation of the whole mice ovary to compare the differences in MVH expression and viability of the ovary after vitrification or slow freezing. Female CF1 aged 40-45 days and weighing 29,29g±2,9 was randomly divided in three groups: control, vitrification or slow freezing. Ovaries were surgically removed, rinsed in saline solution and cryopreserved following. For vitrification protocol were used the VitKit Freeze (Irvine Scientific) and for slow freeze were used Ethilene Glycol (EG) 1.5M as cryoprotectant. Result(s): In HE slides was counting primordial, primary, pre antral and antral follicles. No significant difference was found in the proportion of high-quality primordial, primary and pre antral follicles after thawing/warming in the slow-freezing and vitrification group, respectively. The antral follicle counting was significant higher in vitrification group (p=0,004). Vitrification group shows lower number of viable single cell in comparison with control and slow freeze groups (p=0.043). In immunohistochemistry assay for MVH Antibody , MVH+ and MVH- follicles were counted and compared with the total number of follicles and slow freeze group had a higher number of not marked cells (p=0,012). Conclusion(s): Although both protocols showed similar results in the histological analysis for follicular

counts, the vitrification protocol was significantly better for preserve the ovarian stem cell population.

INTRODUCTION

Ovarian transplantation has been used for many years in animal model for studies of ovarian endocrine function (1), and later was adapted for studies of ovarian function after cryopreservation (2). Ovarian cryopreservation research is performed for the purpose of strain rescue (3) and to optimize the procedure for use in programs for human female fertility preservation (4). Fertility preservation in the human female aims at reserving/restoring fertility in girls and young adult women who are planned to undergo potentially gonadotoxic cancer treatment (5, 6, 7). However, in the light of that only a very limited number of live births have been reported and that most likely a large number of transplantation attempts have been performed, the procedure does most likely need further development to increase its effectiveness. Factors that should be improved are cryopreservation protocols and surgical transplantation procedures (8,9,10).

Most of the follicular loss in cryopreserved tissue does not occur during the cryopreservation/thawing process itself but during the warm ischemic time after retransplantation (11, 12). Interventions such as transplantation to granulation tissue (13) or pretransplantation tissue incubation with growth factors (14), vitamin E (15), or other antioxidants (16, 17, 18) have shown moderate or no effect to increase follicular survival. Whole ovary transplantation has been suggested as an approach to overcome the deleterious effect of the prolonged ischemic time after the tissue reintroduction (19–22). Thus, ongoing to develop

alternative techniques for whole ovary cryopreservation and transplantation with vascular anastomosis should be estimulate (23–25). Indeed, live births have been demonstrated after whole ovary cryopreservation and vascular retransplantation both in sheep (24) and in rat models (23), although the procedure as a whole is not effective low with low live-birth rates.

MATERIALS AND METHODS

Animals

Female CF-1 mice, aged 28 to 30 days and average weight 29.29g±2.9, were used. The animals were kept in group cages under controlled conditions (23°C) under a 12-hour light/dark cycle. They were fed pelleted food and tap water *ad libitum.* After an acclimatization period mice were randomly divided into three groups and were submitted to vaginal cytology, for confirmation of the estrous cycle before euthanize by isoflurane overdoses to remove the ovaries for experiments. These experiments were approved by the Animal Ethics Commission of HCPA (CEUA-HCPA).

Experimental Groups

The experiment was designed to compare the viability and MVH molecules expression by ovarian cells after different cryopreservation process. Each group consisted of ten animals (n= 20 ovaries) randomly allocated to the following groups: fresh control ovaries (C) or ovaries cryopreserved by either vitrification (VIT) or slow-freezing (SF) process.

Ovaries

Ovaries were collected after mice euthanasia by anesthetic overdose with isoflurane (5-10% at 100% O2) and were dissected to remove adipose and mesenteric tissue. Immediately after the ovaries were submitted to cryopreservation process according with their experimental group. The right and left ovary of the same animal were separately cryopreserved for different analysis.

Cryopreservation

Whole ovaries subjected to slow freezing (SF) were exposed for 15 minutes through a solution of DMEM supplemented with 1.5M EG and sucrose 0.5M at 4°C, positioned into a 1.5 mL Cryovial with 0.5 mL of 1.5M EG solution and placed into a container at room temperature (Cryo Freezing Container; Nalgene) with isopropanol. The larger container was placed in a -80°C freezer for 24 hours to allow freezing at a rate of approximately 1°C/minute and then was placed into liquid nitrogen and stored until thawing. The whole ovaries of the vitrification group (VIT) were immersed in the equilibrium solution with 7.5% ethylene glycol (EG) and 7.5% dimethyl sulfoxide (DMSO) in DPBS with 20% FBS for 10 minutes at room temperature and transferred to a vitrification solution (15% EG, 15% DMSO and 0.5 M sucrose) for 2 min. The ovaries were placed on a piece of sterile gauze to remove excess medium, placed in 1.5ml cryovial and plunged into liquid nitrogen.

Thawing

Cryovials were removed from the cryotank, after 30 days of storage, and positioned into a water bath at 37°C for 2 to 3 minutes to allow for complete thawing. Slow-Freeze ovaries was rinsed in DMEM medium supplemented with decreasing concentrations of sucrose (0.5, 0.25, 0.1M) for approximately 5 minutes in each washing step. After these steps to wash out the cryoprotectant, the ovary samples were processed for further viability tests. Ovaries submitted to vitrification process were quickly removed from LN_2 and fully immersed in the 37°C waterbath for 3 seconds. The ovary was dispensed directly to Thawing Solution (1M sucrose, 20% DSS, Gentamicin in M-199 Medium) for 1 minute,

after that was transferred to Dilution Solution (0.5 M sucrose, 20% DSS, Gentamicin in M-199 Medium) for 4 minutes and transferred to washing solution (20% DSS, Gentamicin, in M-199 Medium) for 4 minutes.

Histological Analysis

Ovary tissues were removed, fixed in formalin, embedded in paraffin and stained with hematoxylin and eosin.

Immunohistochemical Analysis

In order to evaluate the presence of germline stem cell markers MVH/DDX4 samples from groups were analyzed by immunohistochemistry technique. Briefly, the sections were incubated overnight at 4ºC with the primary antibody anti-DDX4, Rabbit polyclonal (Abcam), 1: 200. After the incubation interval, sections were washed and incubated with the detection system Goat anti-rabbit IgG (H + L) HRP (Millipore), 1: 200 and the reaction visualization was obtained with Liquid DAB (Dako, K3468), according to manufacturer's recommendations.

Estrous cycle detection

Females were submitted to vaginal cytology analysis before euthanasia procedure as described by Ceschin and collaborators in 2004, in order to confirm estrous cycle stage. Vaginal suspensions were collected with 0.25 ml saline 0.5% (27). The smears were evaluated according to Cooper et al. (27).

Follicular Classification

Follicles were classified according to the modified criteria of Oktay, et al; 1995 (28) as follows: follicles were analyzed and categorized as primordial, primary,

pre antral and antral. Primordial Follicles were identified as normal, even when they had citoplasmatic and/or irregular contour vacuolization, as these characteristics were considered reversible.

Statistics

Data were expressed as medians, percentages, quartiles, and ranges. Statistical comparisons were performed with One way ANOVA for parametric variables, p<.05 was considered statistically significant. Statistical evaluations were performed PASW 18.0 software.

RESULTS

Estrus cycle and body weight analysis

Estrus cycle phase and body weight (29.29g±2. 9)were evaluated and present similar results (Figure 1).

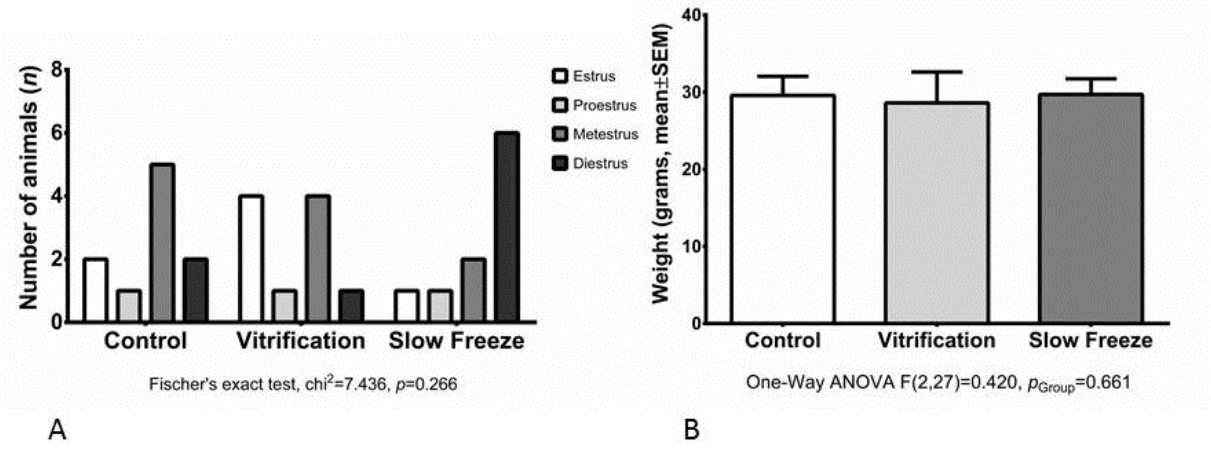

Fig1: Similar results were observed when Estrus Cycle phase (p=0.266) and body weight (p=0.661) analysis compare mice from different groups.

Histological evaluation

Hematoxylin and eosin slides were prepared to evaluate primordial, primary, pre antral and antral follicles presence. After analysis similar primordial, primary and pre antral follicles were detected after thawing/warming process. When samples submmited to slow-freezing and vitrification process. On the other hand when antral follicles samples were evaluated was possible to detect a statistical significant higher number of antral follicles on ovary samples submitted to vitrification process (p=0,004) (Figure 2).

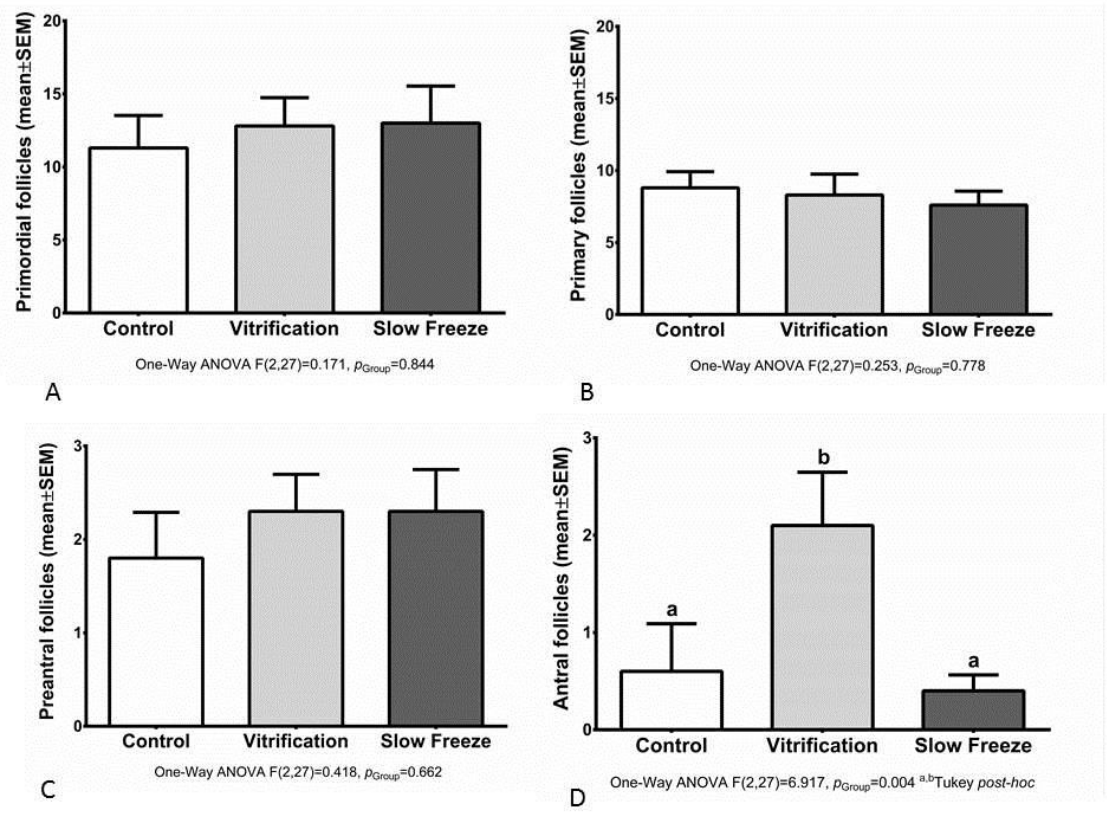

Fig2: Similar numbers of: A: primordial, B: primary and C: pre antral follicles counting were detected among treatments. D: Statistical difference in antral follicles count in vitrification group (p=0.004).

Immunohistochemistry for MVH cells

In order to assess the MVH expression in frozen/thawed ovaries immunohistochemistry was performed. The MVH+ and MVH- follicles were counted and compared with the total number of follicles observed in each ovary sample from different groups (Figure 3) (Figure 4). Total cells and total MVH positive cells were similar in different groups. Otherwise, when negative and positive MVH follicles where evaluated was possible to observe a higher proportion of negative MVH cells on the ovaries submitted to slow freeze process

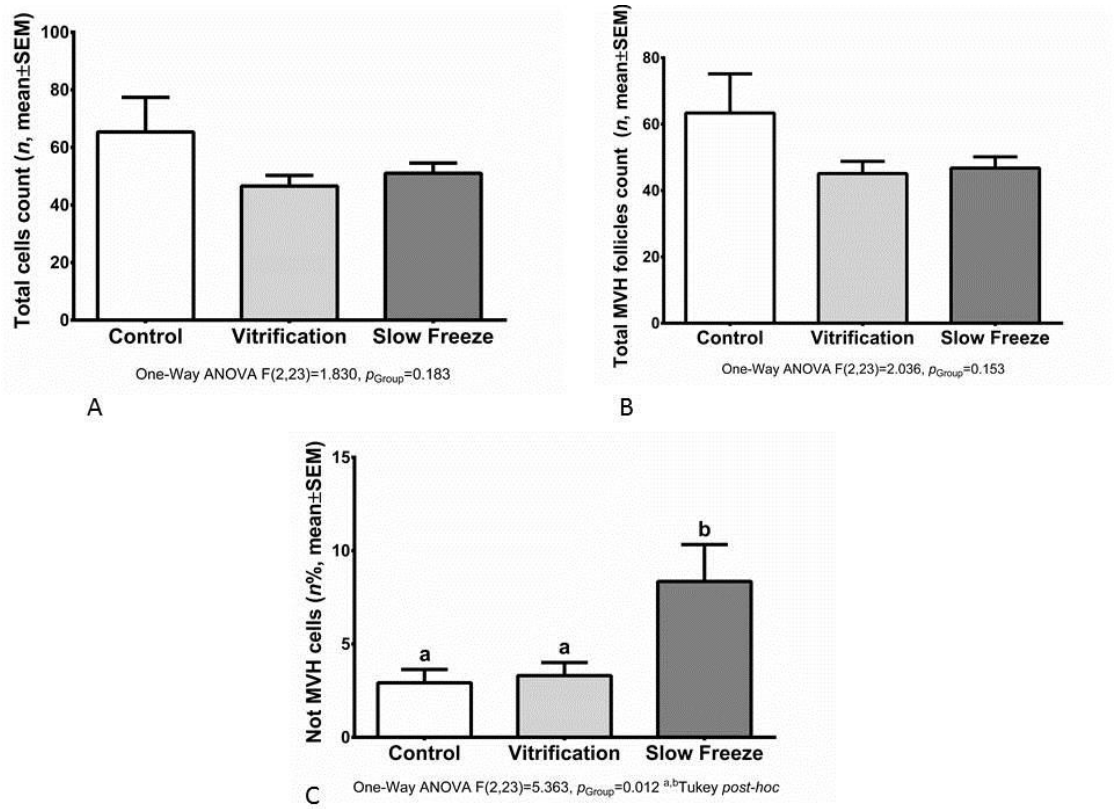

Fig3: A: No statistical difference among groups in total cell count number (p=0.183). B: No statistical difference among total follicles count (p=0.153). C: Statistical difference among groups in percentage number or MVH+. Slow freeze group had a higher number of not marked cells (p=0,012).

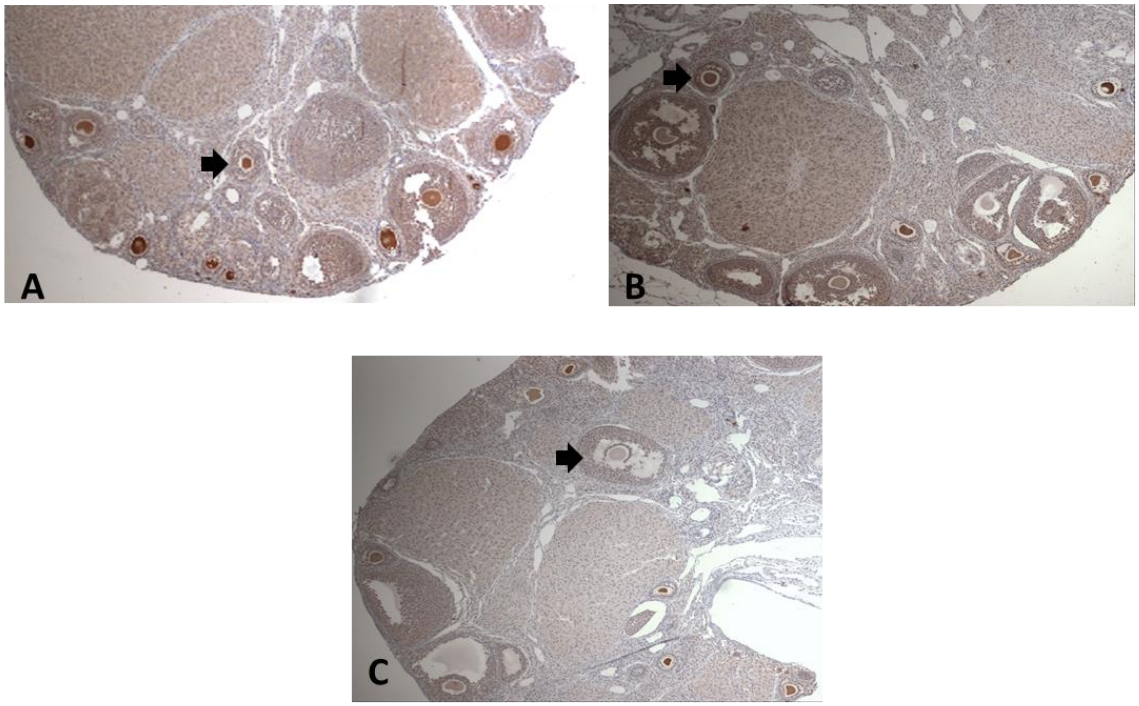

Fig.4: Photomicrograph from histological ovaries section showing viable follicles. A: Control Group IHC for MVH+ Cells. B: Vitrification Group IHC for MVH+ Cells and C: Slow Freeze Group IHC for MVH+ Cells. Magnification 40x.

DISCUSSION

Ovarian tissue cryopreservation is the primary treatment modality currently available to women at risk of losing their ovarian function due to cytotoxic therapy or radiotherapy. His effectiveness is still fairly low; around 15 live births have been reported (6) since the introduction of this method as an experimental procedure along more than 10 years (29), and the first healthy baby was reported in 2004 (30). Cells damage induced by ice crystals formation and how to optimize ovarian transplantation procedure are major questions to address in order to increase fertility rate after transplantation with cryopreserved ovaries.

Several animal models could be used to optimize cryopreservation protocols for human profit. Sheep is the animal model that has been extensively used in ovarian cryopreservation research (32, 33) considering ovine ovary size and because animals presents mono/diovulatory cicle. However, this experimental model is expensive compared to rodent models and inconvenient to work with. A murine model has many advantages as an experimental model for research given that it is a small and inexpensive animal with high reproductive efficiency. Moreover, the knowledge of ovarian function in the mouse, especially folliculogenesis, is vast (34). Rat ovary is nearly thirty-eight times smaller than human ovary and primordial follicle pool distribution within ovary structure is somewhat different and these characteristics must be consider (34). DMSO has been widely cryoprotectant used to ovarian tissue cryopreservation despite several others such as ethylene glycol were adopted demonstrating effectiveness in ovarian fertility preservation procedures both for the research or clinical applications (35).

The aim of this study was to compare two different cryopreservation protocols of the whole mice ovaryorgan in order to assess follicles viability and MVH molecules expression after vitrification or non-automated slow freezing processes. As is known ovarian tissue cryopreservation efficiency was defined as the amount of viable primordial and primary ovarian follicles detected in the processed ovarian tissue, whereas these structures were potentially able to generate mature oocytes in adequate conditions.

The present study compared the efficacy of two cryopreservation methods and were applied a specific cryoprotectant solution to each method. Vitrification method was compound by EG and DMSO (v/v) and non-authomated slow freezing process was performed with a EG containing cryoprotectant solution.

Regarding the histological evaluation no significant difference was found in the proportion of viable primordial, primary and pre antral follicles was detected in control group (92,7%) after thawing/warming in the slow-freezing and vitrification group, with 89,7% and 91,5% of viable follicles respectively. On the other hand antral follicle presence was significant higher when ovary tissues were submitted to vitrification process group (p=0.004) (Figure 2). This important result was probably due to the lower embryonic cell toxicity and higher cell membrane permeability of EG (36). Comparing four different cryoprotectants (PROH, GLY, DMSO, EG) Lucci et al. (2004) (37) demonstrate more effective outcomes with DMSO- and PROH-based cryoprotectants, which preserved the structural integrity of somatic and germ cells. This difference can be explained due to interspecies peculiarities in ovarian tissue; probably bovine follicles are more sensitive EG toxic effects than other species. Furthermore, EG cryopreservation seems to be more effective (38) considering was possible

to preserve 88% morphologically normal follicles after thaw murine ovarian tissues; however, the authors warned that prolonged exposure to EG might decrease follicular viability. In another study with human ovarian tissue, 84% of follicles survived after cryopreservation in EG (38). These findings are in accordance with our results: EG demonstrated to be less toxic as a freezing solution, as shown by superior preservation rates of ovarian tissues structural integrity of. Besides that a significant presence of antral follicles encountered when ovary samples were cryopreserved by vitrification process ($p = 0.04$). In parallel the group of experimental females were analyzed and 80% of them were in the estrous phase, wich corresponds to ovulatory phase with accented follicular growth, or in metaestrus phase, wich corresponds to the period right after ovulation, although there is no statistical difference among different analyzed cryopreservation ovary tissue processes when oestrus phases were compared.

In the last decade severall studies have compared conventional freezing process with vitrification with controversial results (39, 40, 41). Isachenko et al. (2009) tested vitrification versus conventional freezing process of human ovarian tissue and concluded that conventional freezing is a better technique, as tissue thus preserved conserves a higher development potential.

Based on the study of Zou et al. 2009 (42) which transplanted ovarian stem cells into mice and obtained offspring and Terraciano et al. 2014 (43), which compare the ADSC or MVH cells transplant to restore fertility in mice with significant positive results, our initial purpose was to compare the expression of MVH in different types of freezing processes for subsequent cell transplantation. In order to assess the MVH expression in frozen/thawed

ovaries immunohistochemistry was performed. MVH is the homolog of the Drosophila vasa gene, which is specifically expressed in all germ cell lineages and is known as a specific marker of reproductive cells (44, 45). Therefore, in general, MVH are taken as the marker of germ stem cells. In our study the MVH+ and MVH- follicles were measured and compared with the total number of follicles and we found a higher number of not marked cells percentage in slow freeze group (p=0,012).Our data showed that vitrification of ovarian tissue using combination of cryoprotectants (EG with DMSO) had no harmful effect on the morphology and MVH expression of ovarian tissue, where the cell structure is complex.

The major difficulty of vitrification protocols is high toxicity due to the high concentration of cryoprotectants used, which can cause severe osmotic shock and compromise tissue survival after thawing (46,47,48). Reduction of toxicity can be achieved by using a combination of two cryoprotectants and a gradual exposure of the cells to the concentrated solutions prior to cooling. This technique was used in this study, similarly to other studies (49, 50). Although both protocols showed similar results in the histological analysis for follicular counts, the vitrification protocol was significantly better for preserve the ovarian stem cell population.

Cecshin et al. (2006) using immunohistochemical analysis with Ki-67 concluded that although both conventional freezing and vitrification were feasible methods for ovarian tissue cryopreservation, vitrification was associated with the recovery of a greater number of potentially viable primordial follicles in rats, similarly to ours conclusions.

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14. CONSIDERAÇÕES FINAIS

Os ensaios de viabilidade por exclusão de azul de trypan realizados neste projeto não foram inseridos nos resultados do artigo. Os dados de contagem da viabilidade da suspensão celular do grupo vitrificação foram significativamente menores quando comparados aos grupos controle ou congelamento lento. Como os ovários foram congelados inteiros e desagregados com tripsina e colagenase para esta análise, obtivemos uma suspensão heterogênea de células do ovário. O fato da contagem de viabilidade do grupo vitrificação ter sido menor, não interfere no nosso resultado final, que foi a mensuração da expressão de MVH, marcador de células tronco ovarianas.

Cell viability by trypan blue exclusion

The control or frozen/thawed ovaries were disaggregated with collagenase/trypsin method and the single cell suspension was staining with Trypan Blue and counting to estimate cell viability.

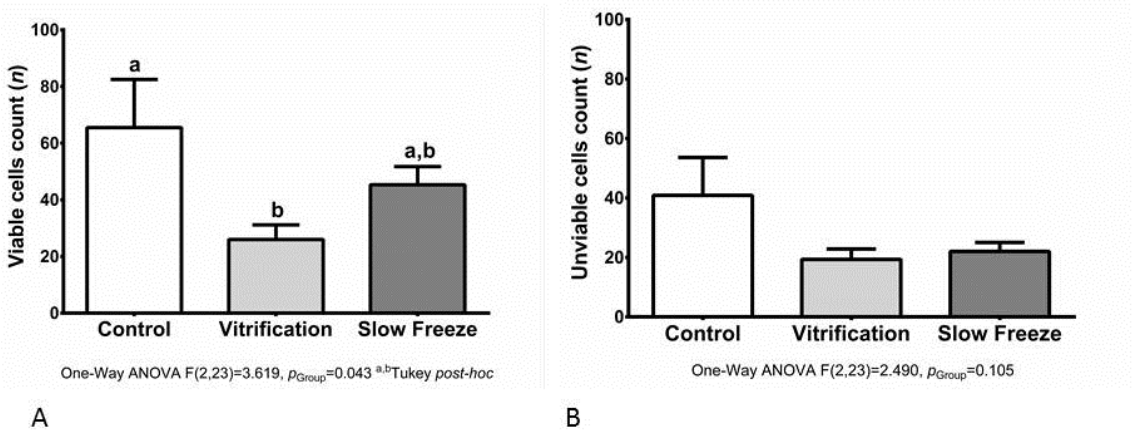

Fig: Statistical difference among groups. A: Vitrification group shows lower number of viable single cell counting in comparison with control and slow freeze

groups, p=0.043. B: No statistical difference among groups in unviable single cells counting.

15. PERSPECTIVAS

Com a finalidade de ampliarmos os resultados obtidos, para corroborar nossa afirmação, iremos realizar, nos blocos de parafina provenientes do experimento, outro ensaio de imunohistoquimica. Para tanto o marcador Oct 4, expresso em embriões e células-tronco embrionárias e considerado um importante marcador da pluripotência será avaliado, nos mesmos moldes do marcador MVH.