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Nanoembriologia: nanotecnologia, biotecnologia e a produção *in vitro* de embriões

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Pelotas, 2017

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“ Sem o respaldo do desempenho e do trabalho duro, as palavras não significam nada. ”

Michael Jordan

Resumo

REMIÃO, Mariana Härter. **Nanoembriologia: nanotecnologia, biotecnologia e a produção *in vitro* de embriões.** 2017. 100f. Tese (Doutorado) - Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas, Pelotas.

A nanobiotecnologia é uma área da nanotecnologia que tem muito a contribuir para as ciências reprodutivas. Além das diversas possibilidades para auxiliar em tratamentos e diagnósticos na medicina reprodutiva, ela traz alternativas para a produção *in vitro* de embriões (PIVE), passando assim a ser denominada nanoembriologia. A nanoembriologia tem potencial para auxiliar problemas que ainda permanecem em torno das tecnologias de reprodução assistida na espécie humana, como a estimulação ovariana, a gestação múltipla e as alternativas para casais com problemas genéticos. No entanto, sua principal abordagem tem sido a busca por uma alternativa à otimização dos protocolos de suplementação dos meios utilizados durante a PIVE a fim de otimizar o processo em todas as espécies que utilizam desta biotécnica da reprodução. Neste documento, além da proposta da utilização da nanotecnologia para solucionar problemas relacionados à medicina reprodutiva, é descrita uma nova abordagem da nanoembriologia, onde nanocápsulas de núcleo lipídico (LNC) carreadas com resveratrol (Res-LNC) são adicionadas ao meio de maturação *in vitro* de oócitos bovinos em diferentes concentrações (0,5; 1 e 2 µM da molécula). Essa estratégia tem por finalidade potencializar o efeito antioxidante que o resveratrol possui, aumentando sua biodisponibilidade, sua solubilidade e sua proteção da luz em um sistema de entrega controlada. Análises de expansão de células do cumulus, maturação oocitária e viabilidade celular demonstram que o tratamento proposto não causa citotoxicidade aos oócitos. A quantificação de glutationa endógena (GSH) e espécies reativas de oxigênio (EROs) demonstram efeito benéfico na utilização de Res-LNC, onde houve um aumento de síntese de GSH em relação aos grupos controle, e uma diminuição na produção de EROs quando utilizada a 1 µM em relação à nanocápsula não carreada. Além destes resultados, o presente documento apresenta o desenvolvimento de um dispositivo que visa auxiliar nos procedimentos de vitrificação de oócitos e embriões. A vitrificação é um método de criopreservação que vem demonstrando ótimos resultados, e esse recipiente se torna útil durante este processo, principalmente em se tratando de animais de produção que possuem grandes quantidades de estruturas a serem criopreservadas de forma fracionada. Por fim, conclui-se que as ciências reprodutivas ainda têm muitos problemas a serem solucionados e a busca por novas alternativas e tecnologias pode auxiliar no desenvolvimento de processos em diversas espécies. A nanotecnologia é uma das principais áreas a serem exploradas e, portanto, novos estudos devem surgir para validarem sua contribuição aos processos relacionados à PIVE.

Palavras-chave: Antioxidante, bovino, maturação *in vitro*, nanocáspulas de núcleo lipídico, oócito, resveratrol.

Abstract

REMIÃO, Mariana Härter. **Nanoembryology: nanotechnology, biotechnology and the *in vitro* embryo production**. 2017. 100p. Thesis (Doctorate) - Graduate Program in Biotechnology. Federal University of Pelotas, Pelotas.

Nanobiotechnology is an area of nanotechnology that has much to contribute to the reproductive sciences. In addition to the diverse possibilities to assist in treatments and diagnostics in reproductive medicine, it provides alternatives for the *in vitro* embryo production (IVEP), and in this case, it is now known as nanoembryology. Nanoembryology has the potential to support problems that still remain around technologies of assisted reproduction in the human species, such as ovarian stimulation, multiple gestation and alternatives for couples with genetic problems. However, until nowadays, its main approach has been the search for an alternative to the optimization of the protocols through the supplementation of the media used during the IVEP in order to optimize the process in all the species that use of this biotechnique of the reproduction. In this paper, in addition to the proposal to use nanotechnology to solve problems related to reproductive medicine, a new approach to nanoembryology is described, in which resveratrol-loaded lipid core nanocapsules (LNC) are added to the *in vitro* maturation medium of bovine oocytes at different concentrations (0.5, 1 and 2 µM of the molecule). This strategy aims to potentiate the antioxidant effect that resveratrol has, increasing its bioavailability, solubility and light protection in a controlled delivery system. Cumulus cell expansion, oocyte maturation, and cell viability analyses demonstrate that the proposed treatments do not cause cytotoxicity to oocytes. The quantification of endogenous glutathione (GSH) and reactive oxygen species (ROS) demonstrated a beneficial effect on the use of Res-LNC, which there was an increase in GSH synthesis in relation to the control groups, and a decrease in ROS production when 1 µM in relation to the unloaded nanocapsule. In addition to these results, this document presents the development of a device that aims to assist in the procedures of vitrification of oocytes and embryos. Vitrification is a method of cryopreservation that has shown excellent results, and this container becomes useful during this process, especially in the case of farm animals that have large amounts of structures to be cryopreserved in a fractional manner. Finally, it is concluded that the reproductive sciences still have many problems to be solved and the search for new alternatives and technologies can help in the development of processes in several species. Nanotechnology is one of the main areas to be explored and, therefore, new studies must emerge to validate their contribution to the processes related to IVEP.

Keywords: Antioxidant, bovine, *in vitro* maturation, lipid core nanocapsules, oocyte, resveratrol.

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Lista de Abreviaturas

BSA – *Bovine serum albumine* (albumina sérica bovina)

Cas9 – *CRISPR associated protein 9* (proteína 9 associada a CRISPR)

CRISPR – C*lustered regularly interspaced short palindromic repeats* (repetições palindrômicas curtas agrupadas e regularmente interespaçadas)

CIV – Cultivo *in vitro*

DNA - *Deoxyribonucleic acid* (ácido desoxirribonucleico)

EDTA – *Ethylenediamine tetraacetic acid* (ácido etilenodiamino tetra-acético)

EROs- Espécies reativas de oxigênio

FIV – Fertilização *in vitro*

FSH – *Follicle stimulating hormone* (hormônio folículo estimulante)

ICSI – *Intracytoplasmic sperm injection* (injeção intracitoplasmática de espermatozoides)

LNC – *Lipid core nanocapsules* (nanocápsulas de núcleo lipídico)

LH – *Luteinizing hormone* (hormônio luteinizante)

MIV – Maturação *in vitro*

OHSS – *Ovarian hyperstimulation syndrome* (Síndrome de Hiperestimulação Ovariana)

PCOS – *Polycystic ovary syndrome* (Síndrome dos ovários policísticos)

PIVE – Produção *in vitro* de embriões

RNA – *Ribonucleic acid* (ácido ribonucleico)

SFB – Soro fetal bovino

UV – ultravioleta

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1 INTRODUÇÃO GERAL

Desde o nascimento do primeiro bebê produzido por fertilização *in vitro* (FIV) e cultivo *in vitro* (CIV) de embriões (Steptoe & Edwards, 1978) e do primeiro bovino nascido resultado das técnicas de maturação *in vitro* de oócitos (MIV), FIV e CIV (Brackett et al., 1982), muito já se avançou em relação à produção *in vitro* de embriões (PIVE). A técnica de PIVE está compreendida dentro das biotécnicas da reprodução e é altamente utilizada para finalidades comerciais, em especial às espécies humana e bovina, e para finalidades científicas. Em pesquisas científicas, a PIVE auxilia na produção de animais transgênicos (Eyestone, 1999; Bevacqua et al., 2017), nos estudos de toxicologia (Beker van Woudenberg et al., 2012; Santos et al., 2014; Ferris et al., 2016) e na produção de células-tronco embrionárias (Aikawa et al., 2014; Saadeldin et al., 2017), por exemplo. No entanto, a ciência também busca formas de auxiliar o melhor desenvolvimento da PIVE, a fim de contornar dificuldades que ainda são encontrados durante a aplicação desta técnica.

Alguns problemas são encontrados principalmente na PIVE voltada à espécie humana. Dentre eles, podemos citar as complicações relacionadas à estimulação ovariana (Chang et al., 2014), a alta incidência de gestações múltiplas (Vahratian et al., 2003) e a necessidade de busca por doadores de gametas para casais que possuem anomalias genéticas (Verlinsky & Kuliev, 2003). Já um problema que se dá principalmente para as espécies animais é a necessidade de criopreservação de diversas estruturas (oócitos ou embriões) em uma mesma rotina, em que um dispositivo de bancada seguro para o armazenamento de nitrogênio líquido se torna necessário. No entanto, um problema que ocorre tanto na PIVE aplicada à humanos quanto a animais é a produção exacerbada de espécies reativas de oxigênio (EROs) durante o processo por parte dos gametas e embriões (Agarwal et al., 2006).

A produção exacerbada de EROs durante a PIVE acontece devido à própria manipulação *in vitro* das estruturas, à exposição à luz, às altas tensões de oxigênio encontradas no ambiente e à composição artificial dos meios de cultivo (Agarwal et al., 2006; Gupta et al., 2010). Esse descontrole na produção de EROs faz com que a incidência de estresse oxidativo seja muito maior nos embriões produzidos *in vitro*, resultando em embriões com uma qualidade muito inferior aos que são produzidos *in vivo* (Gupta et al., 2010; Dang-Nguyen et al., 2011). Para contornar este problema,

grupos de pesquisa ao redor do mundo vêm testando diversas alternativas a fim de tentar controlar essa produção exacerbada de EROs durante a PIVE. Dentre elas, uma das opções mais promissoras é a adição de moléculas antioxidantes nos meios de maturação e cultivo (Guérin et al., 2001; Hosseini et al., 2009; Truong et al., 2016).

O resveratrol é um dos antioxidantes que tem demonstrado resultados interessantes como a diminuição da produção de EROs (Kwak et al., 2012; Wang et al., 2014; Lee et al., 2015), o aumento da síntese de glutationa (Kwak et al., 2012; Wang et al., 2014; Lee et al., 2015), o aumento das taxas de produção de blastocisto (Wang et al., 2014; Sovernigo et al., 2017) e o aumento da qualidade de blastocisto (Takeo et al., 2014; Wang et al., 2014; Itami et al., 2015; Sovernigo et al., 2017). Essa molécula, no entanto, possui propriedades instáveis, como a sua fotossensibilidade, e por isso ela pode não estar exercendo sua atividade com total capacidade, quando utilizada em sistemas *in vitro* (Trela & Waterhouse, 1996; López-Hernández et al., 2006; Koga et al., 2016).

Nesse sentido, a nanotecnologia surge como uma alternativa interessante para proteção e aumento da estabilidade do resveratrol através da nanoencapsulação (Frozza et al., 2010), seguido de suplementação dos meios utilizados em PIVE. Trabalhos prévios já demonstraram que a suplementação de meios de MIV (Lucas et al., 2015; Remião et al., 2016) e CIV (Komninou et al., 2016) com moléculas nanoencapsuladas faz com que os efeitos benéficos proporcionados pelos fármacos sejam potencializados, já que as taxas de produção e a qualidade embrionária das estruturas tratadas com o fármaco nanoencapsulado são maiores em relação ao fármaco livre.

Neste documento serão apresentados três documentos: um manuscrito de perspectiva, um manuscrito de pesquisa e um pedido de patente. O manuscrito de perspectiva sugere a utilização da nanotecnologia para a busca de novas soluções para problemas relacionados à PIVE de humanos; o manuscrito de pesquisa apresenta uma alternativa de suplementação do meio de MIV de oócitos bovinos utilizando nanocápsulas de núcleo lipídico carregadas com resveratrol, e pedido de patente é relativo a um dispositivo de bancada que auxilia na vitrificação de grandes quantidades de oócitos e embriões.

2 REVISÃO BIBLIOGRÁFICA

2.1 A produção *in vitro* de embriões (PIVE)

A produção *in vitro* de embriões é uma biotécnica da reprodução relativamente recente que visa produzir embriões fora do trato reprodutor feminino. Atualmente a PIVE é uma técnica amplamente utilizada, tendo sua aplicação consolidada devido a diversos estudos realizados com a finalidade de aprimorá-la. Estes estudos já ocorrem desde o século passado, e dentre os primeiros relatos está o do pesquisador Dérbes, em 1847, que usou o ouriço do mar como seu modelo para melhor compreender os eventos que ocorriam durante a fertilização (Parrington et al., 2007). No entanto, somente mais de um século depois é que ocorreram os principais avanços na área de PIVE de mamíferos; quando houve o reconhecimento do processo de capacitação espermática (Austin, 1951; Chang, 1951).

Cada caso e cada espécie possuem suas particularidades, mas, no geral, a PIVE consiste de três principais etapas. As três etapas da PIVE são: a maturação dos oócitos, a fecundação do oócito pelo espermatozoide e o cultivo em laboratório do zigoto que logo se tornará embrião, até o momento da transferência deste para o trato reprodutor feminino.

A etapa de maturação dos oócitos pode acontecer *in vivo* ou *in vitro*. Esse evento é extremamente importante pois os oócitos que se encontram nos folículos primordiais necessitam sofrer uma série de modificações moleculares, citoplasmáticas e nucleares para se tornarem aptos a receberem os espermatozoides e darem origem a um embrião (Campos Júnior et al., 2009; Chaves & Figueiredo, 2010). No organismo, este evento ocorre após o pico pré-ovulatório de hormônio luteinizante (LH) que acontece no início da puberdade em humanos, ou no estro em animais (McGee & Hsueh, 2000; Smitz & Cortvrindt, 2002).

Quando é necessária a maturação *in vivo* dos oócitos, a quantidade hormonal natural não é o suficiente para que seja aplicado o processo de PIVE. Isso ocorre porque ao final, salvo raros casos, a maturação oocitária natural resulta em apenas um folículo que atinge o estágio de folículo de Graaf, ou seja, um folículo que contém o oócito maturado (Gougeon & Introduction, 1996; Smitz & Cortvrindt, 2002).

Portanto é necessário que seja administrado um protocolo hormonal que estimule a fêmea a produzir mais oócitos maduros. Esse processo é comumente utilizado para humanos, mesmo trazendo, muitas vezes, efeitos colaterais, riscos e complicações (Kumar et al., 2011; Raju et al., 2013). O ideal nestes casos seria a realização da maturação *in vitro,* que acaba por não ser amplamente utilizada para a espécie humana devido ao fato de o protocolo não estar bem estabelecido e não apresentar os mesmos resultados de taxas de produção que a maturação *in vivo* (Combelles et al., 2002; Chang et al., 2014) . Na maturação *in vitro* (MIV), os oócitos são coletados dos ovários não estimulados e incubados em um meio de cultivo que contém, além de fontes protéicas como soro fetal bovino (SFB) e albumina sérica bovina (BSA), gonadotrofinas (FSH e LH) que são as principais responsáveis pelo início dos eventos de maturação (Hwu et al., 1998; Galli et al., 2003). No entanto, sabe-se também que a própria retirada do oócito de dentro do folículo já desencadeia o início do processo de maturação já que, nesse ato, é interrompido o mecanismo de bloqueio meiótico por parte das células foliculares (Sirard & Coenen, 1993).

Com os oócitos maturados, é possível realizar a fertilização. Na PIVE, esse evento pode se dar de duas principais formas: pelo processo de fertilização *in vitro* (FIV) convencional, onde os oócitos são mantidos em estufa junto dos espermatozoides em uma concentração adequada; ou por injeção intracitoplasmática (ICSI), na qual o manipulador seleciona um único espermatozoide a ser microinjetado no ooplasma do oócito (Figura 1) (Landim-Alvarenga et al., 2008).

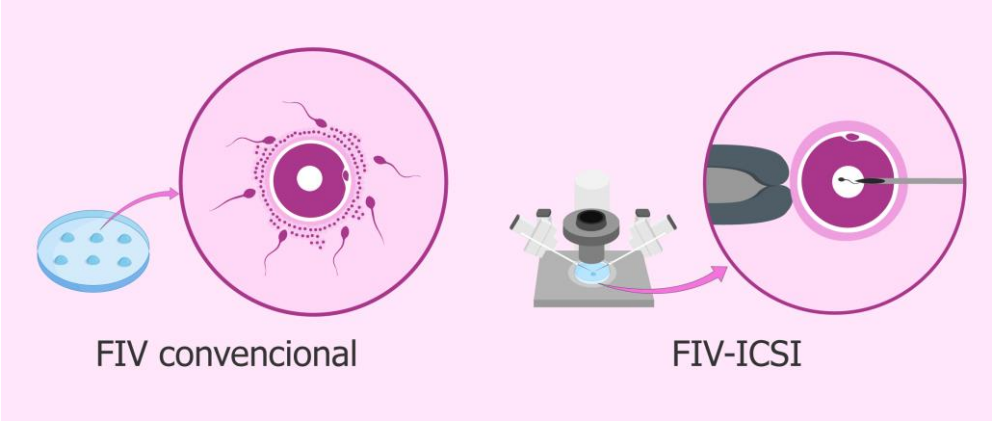

Figura 1. Esquema que demonstra a diferença entre FIV convencional e ICSI. Na FIV os espermatozoides são incubados juntos dos oócitos e, na ICSI, com ajuda de um micromanipulador, o espermatozoide é inserido no ooplasma. (Fonte:

Reproduccion Asistida ORG, disponível em: https://www.reproduccionasistida.org/fiv-icsi/fiv_vs_icsi/)

Já na etapa de CIV, após a fertilização, os presumíveis zigotos são mantidos em meio de cultivo, em atmosfera controlada, para que ocorra o desenvolvimento embrionário. Os embriões passam a iniciar as suas clivagens e se desenvolvem *in vitro* até o momento em que são transferidos para o trato reprodutor da fêmea (Karaki et al., 2002; Hinrichs, 2010; Paramio & Izquierdo, 2014). É importante que a fêmea esteja sincronizada com o embrião para que haja compatibilidade entre o embrião e o endométrio e a prenhez seja estabelecida (Baruselli et al., 2010; Ming et al., 2012). Geralmente são transferidos os embriões nos estágios de 2 a 8 células, principalmente na espécie humana, ou no estágio de blastocisto (Sallam, 2005; Papanikolaou et al., 2008).

Atualmente a PIVE é utilizada para finalidades comerciais e científicas para diversas espécies como humanos (Dyer et al., 2016; Ma et al., 2017; Zegers-Hochschild et al., 2017), murinos (Aikawa et al., 2014; Nakagawa et al., 2015), suínos (Gil et al., 2010; Romar et al., 2016), equinos (Landim-Alvarenga et al., 2008; Hinrichs, 2010), caprinos (Magalhães et al., 2011; de Souza-Fabjan et al., 2014), bubalinos (Manjunatha et al., 2009; Aquino et al., 2013; Selokar et al., 2015) e bovinos (Lonergan & Fair, 2008; Machaty et al., 2012). Dentre as principais contribuições científicas que a PIVE proporciona está a transgênese (Eyestone, 1999; Bevacqua et al., 2017), os estudos de toxicologia (Beker van Woudenberg et al., 2012; Santos et al., 2014; Ferris et al., 2016) e a produção de células-tronco embrionárias (Aikawa et al., 2014; Saadeldin et al., 2017). Em relação às aplicações comerciais, as espécies bovina e humana são as que mais se utilizam desta biotécnica: para humanos a principal aplicação é no auxílio a casais que possuem algum tipo de distúrbio que os leva à infertilidade (Dyer et al., 2016) e para bovinos a busca é por melhoria genética e aumento de produção (Verma et al., 2012). Ainda, é importante destacar que muito dos avanços na medicina reprodutiva só foram alcançáveis devida os estudos em animais, dentre eles, na espécie bovina.

2.2 Criopreservação de gametas e embriões

Com certeza, dentro das biotécnicas da reprodução, uma das mais revolucionárias é a criopreservação de embriões e gametas. Esta tecnologia tem

proporcionado um grande avanço na área reprodutiva pois permite a preservação de células e tecidos por um longo período de tempo e garante sua viabilidade no momento em que se deseja utilizá-los (Aflatoonian et al., 2013; Zegers-Hochschild et al., 2017).

Para a espécie humana, os benefícios trazidos pela criopreservação são inúmeros. A partir dela é possível o armazenamento de gametas de pacientes que passarão por algum procedimento que possa prejudicar sua fertilidade, como a radioterapia e a quimioterapia (Seli & Tangir, 2005; Vermeulen et al., 2017); a preservação de gametas e embriões que serão doados a pacientes que não podem utilizar de seus próprios gametas para estabelecimento de gestação (Fosas et al., 2003); ou a conservação de embriões excedentes de um procedimento de PIVE que não puderam ser transferidos para o útero naquele ciclo (Schnorr et al., 2001).

Em animais, a criopreservação de gametas e embriões propicia a manutenção da genética, tanto de animais de mérito genético superior quanto de espécies ameaçadas de extinção (Boettcher et al., 2005; Strand et al., 2016). Além disso, com esta técnica, é possível a geração de uma maior diversidade genética já que, a partir dela, o comércio e o deslocamento de gametas e embriões torna mais viável o cruzamento entre animais que se encontram distantes. Também, a utilização de gametas e embriões criopreservados traz uma maior segurança em relação ao controle sanitário de doenças transmissíveis, pois o contato dos animais na monta natural não se torna mais necessário (Bailey et al., 2000).

Para que a manutenção das células seja possível por um longo período, a amostra criopreservada é mantida a uma temperatura de – 196 ºC em nitrogênio líquido. Esta baixa temperatura leva a uma redução drástica do metabolismo celular, onde as células se encontrarão em um estado quiescente (Kami, 2012; Wakchaure et al., 2016). Para que as células não sofram com as injúrias que esta redução de temperatura pode causar, componentes crioprotetores são utilizados antes do congelamento (Pegg, 2007). As soluções crioprotetoras protegem a célula de possíveis danos, como a ruptura de membranas celulares e danos no citoplasma causados principalmente pela formação de cristais de gelo (Wakchaure et al., 2016). Os crioprotetores são portanto muito importantes para a viabilidade da técnica e podem atuar extracelularmente ou intracelularmente (Pegg, 2007; Wakchaure et al., 2016).

Em se tratando de congelamento de oócitos e embriões, existem dois principais métodos: o congelamento lento e a vitrificação (Wang et al., 2016). As principais diferenças entre as duas é a velocidade em que a amostra atinge a temperatura de – 196 ºC e a concentração de crioprotetores (Rienzi et al., 2016). A vitrificação é uma técnica que vem sendo cada vez mais empregada para oócitos e embriões devido à sua praticidade e ótimos resultados (Sanfilippo et al., 2015; Rienzi et al., 2016). Ela é executada de forma muito mais rápida do que o congelamento lento e emprega uma maior concentração de crioprotetores, tornando a solução altamente viscosa e evitando a formação de cristais de gelo a partir da água existente nos espaços extra e intracelulares (Mapletoft & Hasler, 2005). No entanto, devido esta alta concentração de crioprotetores, que são altamente tóxicos às células em temperatura ambiente por um longo período (Greer, 2015), este método de criopreservação deve ser realizado com agilidade pelo manipulador. Nesta técnica, a amostra contendo o oócito ou o embrião junto da solução de vitrificação, é colocada em contato com o vapor do nitrogênio líquido e, em seguida, imersos neste líquido, passando rapidamente da temperatura ambiente para – 196 ºC (Rienzi et al., 2016). Ao contrário do congelamento lento, essa técnica não requer aparelhagem específica, tornando-a mais acessível, rápida e barata.

Em se tratando principalmente de animais de produção, a quantidade de estruturas a serem vitrificadas geralmente é grande, fazendo-se necessária a manipulação fracionada. Por isso é importante a utilização de um recipiente auxiliar que matenha nitrogênio líquido durante o processo de criopreservação de todas as estruturas antes do armazenamento definitivo em botijões de nitrogênio líquido. A atual demanda é suprida por caixas comuns de isopor com tampa que acabam sendo de dificil higienização, ou por caixas compostas de aço inoxidável ou polipropileno, materiais que não são eficientes na manutenção da temperatura e acabam fazendo com que o líquido criogênico evapore mais rápido. Por isso, torna-se necessária a utilização de algum dispositivo de bancada que mantenha o nitrogênio líquido em segurança, de forma asséptica, na bancada de trabalho ou dentro do fluxo laminar, para auxiliar nas rotinas de vitrificação. Além disso, um recipiente que possibilite a organização das amostras durante o preparo seria uma alternativa muito interessante que ajudaria a evitar possíveis perdas que eventualmente ocorrem após a imersão das palhetas no nitrogênio líquido.

2.3 Problemas relacionados à PIVE

Desde o nascimento do primeiro bebê gerado por FIV em 1978 (Steptoe & Edwards, 1978) e do primeiro bovino nascido após procedimento de MIV e FIV em 1981 (Brackett et al., 1982) muito já se avançou em relação à PIVE. Como já foi mencionado, este procedimento é altamente utilizado para diversas finalidades, porém, apesar das altas taxas de sucesso, muitos problemas ainda são encontrados. Serão aqui mencionadas diversas questões que ainda necessitam de atenção de grupos de pesquisa focados em buscar novas soluções.

Especificamente na medicina reprodutiva, alguns pontos, que apesar de já terem evoluído bastante, ainda geram alguns transtornos são: a estimulação ovariana (Chang et al., 2014), a gestação múltipla em pacientes que recorreram à reprodução assistida (Vahratian et al., 2003), os pacientes que recorrem à PIVE por portarem anomalias genéticas (Verlinsky & Kuliev, 2003), além da diferença de qualidade inferior de embriões produzidos *in vitro* em relação aos produzidos *in vivo* (Agarwal et al., 2006). Em relação à estimulação ovariana, o problema que se enfrenta é das pacientes que possuem sensibilidade ao protocolo hormonal como mulheres com risco de síndrome de hiperestimulação ovárica (OHSS); com síndrome dos ovários policísticos (PCOS); com tumores sensíveis ao estrogênio; e as que necessitam da preservação da fertilidade com urgência, antes de iniciar um tratamento potencialmente gonadotóxico (Chang et al., 2014). Nestes casos a maturação *in vivo* dos oócitos se torna arriscada devido às altas doses hormonais que devem ser administradas. A maturação *in vitro* nesses casos seria uma boa alternativa, no entanto, os protocolos para a espécie humana ainda não estão bem consolidados (Combelles et al., 2002; Chang et al., 2014).

Outra questão é a gestação múltipla, recorrente em casos de reprodução assistida. Esta condição acaba por ser preocupante já que esse tipo de gestação está relacionado a nascimentos prematuros ou com baixo peso, além de outras complicações e riscos tanto aos bebês quanto às mães (Fauser et al., 2005; Sinclair, 2008). Ela ocorre devido a transferência de mais de um embrião para o trato reprodutor feminino a fim de garantir uma gestação. Por isso, novas formas de seleção para que um único embrião seja transferido se fazem necessárias.

A reprodução assitida também é uma alternativa para casais que possuem anomalias genéticas; casais que já possuem filhos com anomalias genéticas; ou casais que sofrem de abortos espontâneos recorrentes (que estão frequentemente relacionados à distúrbios genéticos) (Scriven et al., 2011; Chen et al., 2017). Nesses casos é possível produzir embriões *in vitro* seguido de biópsia embrionária para seleção genética (Fiorentino et al., 2003). Porém, algumas vezes os gametas dos pais são afetados e a única opção que se tem é a de optar pela doação de gametas e embriões, já que uma correção genética do embrião ainda não é possível de ser realizada.

Outro problema que acontece, e é uma das questões mais discutidas acerca da PIVE, é a diferença de qualidade dos embriões que são produzidos *in vivo* em relação aos produzidos *in vitro* (Pomar et al., 2005; Corcoran et al., 2006). Os primeiros ainda apresentam melhor qualidade (Pomar et al., 2005; Corcoran et al., 2006). Diversos motivos são sugeridos a fim de explicar por quê isto ocorre, mas dentre os principais estariam a produção exacerbada de espécies reativas de oxigênio (EROs) pelos gametas e embriões manipulados *in vitro* (Agarwal et al., 2006; Gupta et al., 2010). O excesso de EROs ocorre não só pela manipulação dos gametas, mas também pela sua exposição à luz, pelas altas tensões de oxigênio do ambiente em relação ao trato reprodutor feminino e pela sua manutenção em meios de cultivo artificiais, por exemplo (Agarwal et al., 2006). A fim de tentar diminuir esses efeitos, pesquisadores têm buscado diferentes estratégias, como a adição de agentes quelantes e de moléculas antioxidantes aos meios utilizados para cultivo de gametas e embriões (Hosseini et al., 2009; Combelles & Hennet, 2012; Sovernigo et al., 2017; Khazaei et al., 2017).

2.4 Suplementação de meios como alternativa no controle da produção de EROs em PIVE : o antioxidante resveratrol

Os meios de cultivo utilizados para PIVE tornam possível a produção *in vitro* de embriões. Eles podem variar na sua composição de acordo com a etapa de produção, mas basicamente são compostos de carboidratos, sais minerais, metabólitos, tampão e proteínas (Biggers & Summers, 2008; Morbeck et al., 2014). A composição dos meios, bem como suas propriedades físicas e químicas, são muito importantes no cultivo de gametas e embriões. Sabe-se que o ambiente onde as

estruturas se encontram influencia nos padrões de expressão gênica das células, e um dos grandes responsáveis por essa alteração e também responsável por outros tipos de danos celulares, é o excesso de EROs intracitoplasmático (Takahashi, 2012).

Por ser extremamente nociva à PIVE, algumas estratégias já foram desenvolvidas para tentar controlar a produção de EROs quando as células são expostas ao ambiente *in vitro*. Dentre as principais formas de controlar essa produção estão: a redução das tensões de oxigênio da incubadora (Nagao et al., 2008), o cultivo dos embriões junto de células somáticas (Bavister, 1995), a redução da exposição à luz (Goto et al., 1993) ou também a adição de antioxidantes como metais quelantes (EDTA) (Combelles & Hennet, 2012), vitaminas (E e C) (Feugang et al., 2004; Gajda et al., 2008; Branco et al., 2010) e outras moléculas como β-mercaptoetanol (Takahashi et al., 2002; Feugang et al., 2004; Nikseresht et al., 2017), cisteamina (Merton et al., 2013; Nikseresht et al., 2017), polifenóis (Wahyudi & Sargowo, 2007; Wang et al., 2013), melatonina (Cebrian-Serrano et al., 2013; Tian et al., 2014; Remião et al., 2016) e resveratrol (Wang et al., 2014; Mukherjee et al., 2014; Sprícigo et al., 2017).

O resveratrol (trans-resveratrol; 3,5,4'-trihidroxiestilbeno) é um dos suplementos que vem sendo adicionados em meios de PIVE, apresentando resultados interessantes. Esta molécula é um polifenol natural presente em abundância na casca de uvas (Jang, 1997), no vinho tinto (Baur & Sinclair, 2006) e em menor quantidade em amendoins (Wang et al., 2005), cacau (Hurst et al., 2008) dentre outros alimentos. Algumas propriedades biológicas interessantes já foram relacionadas a essa molécula como efeito de cardioproteção (Hung et al., 2004), propriedades anticâncer (Jang, 1997), antidiabetes (Palsamy & Subramanian, 2008), proliferação e diferenciação celular (Olson et al., 2005; Dai et al., 2007) e prolongamento do tempo de vida em diferentes espécies (Howitz et al., 2003; Valenzano et al., 2006). Meios de cultivo utilizados em PIVE suplementados com resveratrol demonstram um aumento de produção e qualidade embrionária (Takeo et al., 2014; Wang et al., 2014; Sovernigo et al., 2017). Esse efeito já foi observado em diversas espécies como bovinos (Takeo et al., 2013; Takeo et al., 2014; Wang et al., 2014; Sovernigo et al., 2017; Sprícigo et al., 2017) , suínos (Kwak et al., 2012; Lee et al., 2015; Ma et al., 2015) e caprinos (Mukherjee et al., 2014). O resveratrol

adicionado ao meio de maturação de oócitos bovinos tem sido descrito por diminuir a produção de espécies reativas de oxigênio (Kwak et al., 2012; Wang et al., 2014; Lee et al., 2015), aumentar a síntese de glutationa (Kwak et al., 2012; Wang et al., 2014; Lee et al., 2015), aumentar as taxas de produção de blastocisto (Wang et al., 2014; Sovernigo et al., 2017), aumentar a qualidade dos blastocistos (Takeo et al., 2014; Wang et al., 2014; Itami et al., 2015; Sovernigo et al., 2017) e regular a expressão gênica (Lin et al., 2012; Itami et al., 2015; Sprícigo et al., 2017).

No entanto, o resveratrol é uma molécula que possui instabilidades em sua estrutura, principalmente em relação à sua fotossensibilidade (Koga et al., 2016). Essa instabilidade ocorre quando o isômero trans-resveratrol, o composto comercial que possui mais atividades biológicas de interesse farmacêutico, é exposto à luz UV (Vian et al., 2005; Koga et al., 2016). Quando isto ocorre, ele fotoisomeriza para a forma cis-resveratrol de forma irreversível, perdendo grande parte de suas propriedades (Figura 2) (Vian et al., 2005). Por este motivo, além da baixa solubilidade do resveratrol em água (Amri et al., 2012), é provável que esta molécula, quando utilizada em sistemas de PIVE, possa não conseguir atuar em sua totalidade. Por isso, uma alternativa para esse problema é a nanoencapsulação do resveratrol que, além de proteção à luz, pode oferecer aumento da sua solubilidade e a sua liberação controlada (Frozza et al., 2013; Jeon et al., 2016).

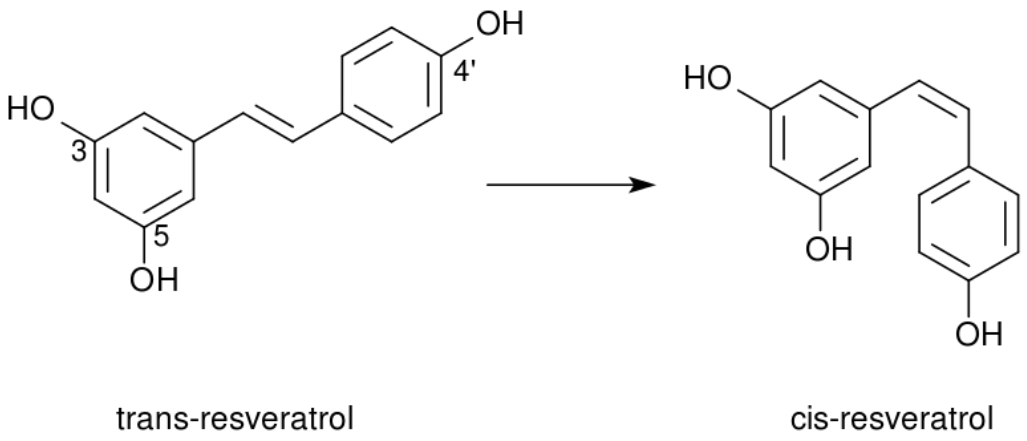

Figura 2. Isômeros do resveratrol. O trans-reveratrol quando exposto à luz UV fotoisomeriza para cis-resveratrol. (Fonte: Wikimedia Commons, disponível em: https://commons.wikimedia.org/wiki/File:Trans-cis-resveratrol.svg)

2.5 Nanotecnologia

A manipulação da matéria em escala atômica e nuclear é uma possibilidade recente que surge com o desenvolvimento da nanotecnologia. Essa área da ciência que estuda e desenvolve produtos na escala nanométrica vem se destacando por trazer possibilidades multidisciplinares (Ebbesen & Jensen, 2006; Jain et al., 2013). Na área médica, sua contribuição tem sido extremamente importante a partir do desenvolvimento de novas alternativas para diagnósticos e terapias. A utilização de materiais em escala manométrica permite o aumento da especificidade, sensibilidade e seletividade, características extremamente importantes para uma eficaz detecção de patologias e para um tratamento de doenças de forma não invasiva, ou minimamente invasiva (Barkalina, Charalambous, et al., 2014; Riley & Vermerris, 2017).

No caso dos métodos diagnósticos, a nanotecnologia está ajudando a torná-los mais rápidos, baratos e reprodutíveis. Um exemplo são os nanobiosensores dispostos em chips, utilizados para detecção de diversos tipos de materiais biológicos como antígenos, proteínas, ácidos nucléicos, dentre outros (Figura 3) (Barkalina, Charalambous, et al., 2014; Zhu et al., 2015). Estes testes que, além de todas as vantagens já citadas, requerem volumes mínimos de reagente e analito, têm sido nomeados como ‘*lab-on-a-chip’* (Craighead, 2006; Hill & Li, 2017). Em se tratando de medicina reprodutiva, esses dispositivos podem vir a contribuir muito em diversas vertentes, pois facilitariam as análises em laboratório, dispensando a terceirização de serviços. Um caso que pode ser explorado, por exemplo, seria nos diagnósticos pré-implantacionais de embriões em que um resultado rápido dispensaria a necessidade de congelamento do embrião e a transferência deste em um novo ciclo, o que ocorre atualmente devido à espera dos resultados das análises das biópsias.

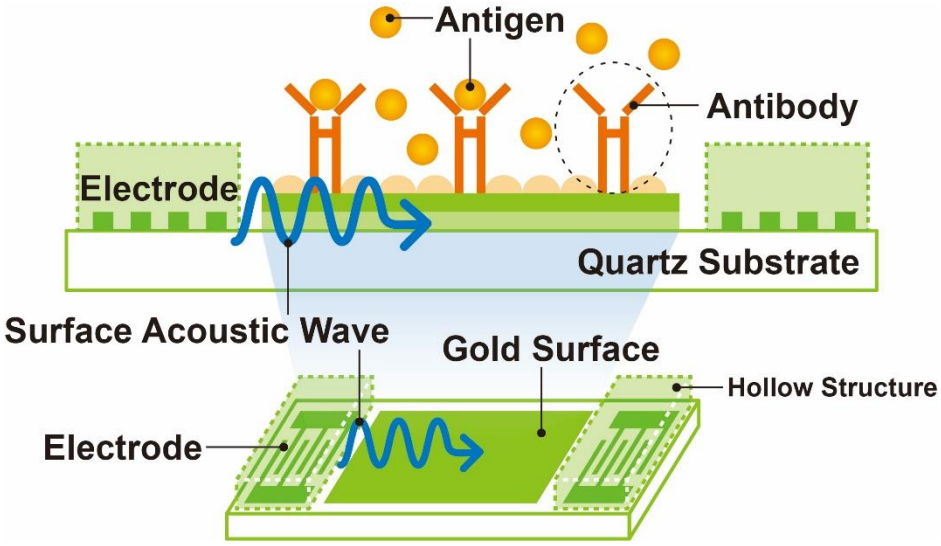

Figura 3. Exemplo de utilização de biossensor. O exemplo apresenta uma superfície de quartzo sensibilizada com anticorpos que quando detectam a ligação do antígeno geram um sinal elétrico no nanochip. (Fonte: Wireless Life-Sciences Alliance, disponível em: http://wirelesslifesciences.org/2015/03/oj-bio-at-wlsa-convergence-summit-2015)

Um ramo dentro da nanobiotecnologia que pode em breve beneficiar a medicina reprodutiva é a nanoembriologia. Trata-se da utilização de materiais em nanoescala durante qualquer etapa da PIVE, tendo sido descrito pela primeira vez por Lucas e colaboradores (2015). A estratégia mais explorada até o presente momento é o de suplementação dos meios utilizados na maturação e no cultivo *in vitro*, com nanocápsulas carregadas com moléculas de interesse (Lucas et al., 2015; Remião et al., 2016; Komninou et al., 2016). O primeiro trabalho publicado relata a utilização da molécula tretinoína associada a nanocápsulas de núcleo lipídico (LNC) em meio MIV, onde foi demonstrado que essa suplementação foi benéfica para o aumento das taxas de produção de blastocisto, diminuindo a produção de EROs e da proteína S36-p66Shc. A associação da tretinoína com a LNC fez com que uma menor concentração de molécula encapsulada tivesse mesmo efeito do que uma concentração maior da sua forma livre (Lucas et al., 2015). Utilizando o neuro-hormônio melatonina associado às LNCs, tanto durante a MIV (Remião et al., 2016) quanto durante o CIV (Komninou et al., 2016), é possível observar diminuição da produção de EROs, aumento nas taxas de produção de blastocistos e aumento da qualidade embrionária quando comparada à molécula livre e, mais ainda, ao controle sem tratamento. Recentemente foi relatado que as LNCs, mesmo em altas

concentrações durante a MIV, não possuem efeito tóxico e não migram para o óleo mineral utilizado para cobertura dos meios durante a PIVE (Lucas et al., 2017). Essa vertente da nanobiotecnologia ainda pode ser muito explorada com a associação de outras moléculas que vem se destacando na suplementação de meios, como o resveratrol, já aqui mencionado.

Outra questão em que a nanotecnologia vem contribuindo é na edição de genomas. A manipulação de nanoestruturas traz benefícios através do desenvolvimento de métodos de transformação gênica não-viral (Das et al., 2016). Essa estratégia seria interessante não somente para produção de animais transgênicos como também para uma aplicação futura de correção de anomalias genéticas em embriões humanos.

Em relação à transgênese, a modificação gênica mediada por espermatozoides é uma das estratégias mais exploradas por ser mais simples e barata do que outras técnicas como a microinjeção pronuclear (Tasic et al., 2011), a transferência nuclear de célula somática (Ren et al., 2014) ou a transformação mediada por lentivirus (Chen et al., 2016), por exemplo. Nesse sentido, diferentes materiais nanoestruturados já foram testados para entrega de ácidos nucléicos ou proteínas a espermatozoides. Dentre estes materiais estão os nanotubos de haloisita (Campos et al., 2011), as nanopartículas de sílica (Barkalina, Jones, et al., 2014), as nanopartículas magnéticas de ferro (Kim et al., 2010) e as nanopartículas de óxido de ferro cobertas por álcool polivinílico (Makhluf et al., 2008).

Quanto à correção de anomalias genéticas em embriões humanos, dois trabalhos recentes utilizando a metodologia de direcionamento gênico CRISPR-Cas9, demonstraram que essa estratégia é possível de ser realizada (Liang et al., 2015; Ma et al., 2017). Apesar de ter sido feita em embriões inviáveis que não prosseguiram para a transferência intrauterina por razões de bioética e pela técnica ainda não ter comprovada eficiência e segurança, esses estudos foram importantes para trazerem uma alternativa futura para tratar doenças genéticas que são atualmente intratáveis, antes mesmo do estabelecimento da gestação (Ishii, 2017). A nanotecnologia pode contribuir na entrega da molécula Cas9 junto do RNA guia, necessários para a realização da técnica. Um estudo relata esta entrega eficiente em células humanas de osteosarcoma que foram depositadas em camundongo, utilizando ‘nanoclews’, que seriam arranjos de moléculas de DNA, cobertos por

polietilenamida (Sun et al., 2015).

Por fim, é possível observar que a produção *in vitro* de embriões, bem como a medicina reprodutiva, mesmo sendo amplamente exploradas e utilizadas, possuem diversos pontos a serem investigados, a fim de buscar sua excelência. A nanotecnologia ainda é uma ciência pouco aplicada nestas áreas, no entanto, ela tem muito a oferecer para o desenvolvimento de métodos e protocolos mais eficientes, trazendo maior produção na área animal e maior satisfação na área da medicina humana. Esta tese tem como objetivo promover perspectivas em relação à utilização da nanotecnologia na medicina reprodutiva, propor um novo protocolo de maturação *in vitro* de oócitos bovinos utilizando a suplementação de nanocápsulas de núcleo lipídico carreadas com resveratrol, e apresentar um novo dispositivo utilizado para facilitar a técnica de vitrificação de oócitos e embriões em larga escala.

3 OBJETIVOS

3.1 Objetivo Geral

Propor o uso da nanotecnologia nas ciências reprodutivas através da apresentação de perspectivas e de testes experimentais de suplementação de meio de maturação *in vitro* de oócitos bovinos com nanocápsulas de núcleo lipídico carregadas com resveratrol.

3.3 Objetivos Específicos

- Avaliar o efeito dose-dependente das concentrações do resveratrol livre e nanoencapsulado na produção *in vitro* de embriões;

- Avaliar a citotoxicidade do resveratrol nanoencapsulado frente aos oócitos maturados;

- Avaliar o efeito antioxidante do resveratrol nanoencapsulado em oócitos bovinos;

- Avaliar as taxas de maturação após oócitos serem maturados *in vitro* na presença do resveratrol nanoencapsulado;

- Propor de forma teórica a maior utilização da nanotecnologia na medicina reprodutiva;

- Desenvolver uma tecnologia para auxiliar na criopreservação de oócitos e embriões de animais de produção.

4 CAPÍTULOS

4.1 Manuscrito 1 – The potential of nanotechnology in medically assisted reproduction

Manuscrito aceito para publicação na revista *Frontiers in Pharmacology* (Fator de impacto: 4,400 – Qualis Biotecnologia: A1)

PERSPECTIVE

Abstract 196 words

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Table or Figure (1)

The potential of nanotechnology in medically assisted reproduction

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Conflict of Interest Statement: The authors declare that the work was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Abstract

Reproductive medicine is a field of science which searches for new alternatives not only to help couples achieve pregnancy and preserve fertility, but also to diagnose and treat diseases which can impair the normal operation of the reproductive tract. Assisted reproductive technology (ART) is a set of methodologies applied to cases related to infertility. Despite being highly practiced worldwide, ART presents some challenges, which still require special attention. Nanotechnology, as a tool for reproductive medicine, has been considered to help overcome some of those impairments. Over recent years, nanotechnology approaches applied to reproductive medicine have provided strategies to improve diagnosis and increase specificity and sensitivity. For *in vitro* embryo production, studies in non-human models have been used to deliver molecules to gametes and embryos. The exploration of nanotechnology for ART would bring great advances. In this way, experiments in non-human models to test the development and safety of new protocols using nanomaterials are very important for informing potential future employment in humans. This paper presents recent developments in nanotechnology regarding impairments still faced by ART: ovary stimulation, multiple pregnancy, and genetic disorders. New perspectives for further use of nanotechnology in reproductive medicine studies are also discussed.

Keywords: **assisted reproductive technologies, nanotechnology, nanobiotechnology, multiple pregnancy, in vitro maturation, gene therapy, and embryology.**

Reproductive medicine and nanotechnology

Infertility and subfertility defined as the difficulty to conceive are conditions affecting people worldwide. The World Health Organization considers infertile couples those who fail to achieve a clinical pregnancy after, at least, one year of regular unprotected sexual intercourse (Zegers-Hochschild et al., 2017). Couples who experience these difficulties can turn to reproductive medicine technologies to help solve the problem. One of the most revolutionary treatments in this area is assisted reproductive technology (ART) comprising of *in vitro* embryo production (IVEP).

Regarding reproductive medicine, nanotechnology can be very useful in the development of non-invasive detection, diagnosis, and minimally invasive treatment of infertility-related disorders (oncological or non-oncological) (Barkalina et al., 2014a). To improve diagnostics, nanotechnology is applied mainly to the development and improvement of nanobiosensors and imaging techniques. Nanobiosensors are devices capable of identifying antigens, proteins, nucleic acids, and reactive oxygen and nitrogen species with quickness and sensitivity (Shi et al., 2007; Zhu et al., 2015). These technologies are underlying the development of interesting 'lab-on-a-chip' tools. Besides advantages of nanotechnology, this tool requires small volumes of analyte and reagents (Craighead, 2006; Hill and Li, 2017). The functionalization of zinc oxide nanoroads – gold nanoparticles (Gasparotto et al., 2017), iron oxide nanoparticles (Pal et al., 2015), and silica coated gold nanoparticles with cadmiun selenide quantum dots (Johari-Ahar et al., 2015) with anti-CA125 antibodies represent successful strategies to develop higher sensitivity tools for ovarian cancer detection. In addition, anti –HE4 antibody attached to silver nanoparticles was also used to develop a fast, specific, and stable ovarian cancer detection system (Yuan et al., 2012).

Biosensors using anti-PSA antibodies to detect PSA antigen represent one of the most used strategies for detection of prostate cancer. Gold nanoparticles functionalized with anti-PSA antibodies have been used in a bio-barcode assay showing ultrasensitivity (Thaxton et al., 2009) and in silicon nanowire field effect

transistors providing real-time prostate cancer detection (Presnova et al., 2017). Gold nanoparticles and anti-PSA antibodies supported in graphene oxide (Pal and Khan, 2017) or bound in cuprous oxide@ceric dioxide core-shell nanocomposites (Li et al., 2017) were also used to develop novel, accurate, and sensitive electrochemical immunosensors.

Diagnostic imaging has been improved by metallic and nanostructured particles, as these nanomaterials have great benefits compared to contrast agents. Iron oxide is one of the main contrast agents used for magnetic resonance imaging (MRI), and when nanostructured, it can be functionalized for additional benefits. Iron oxide nanoparticles can be formed in poly(vinyl alcohol), rendering them degradable over time (Bannerman et al., 2017) or, as showed in tumor xenograft animal models, can be associated to diatoms to improve tumour retention when a magnetic field is applied (Todd et al., 2014). In addition, iron oxide nanoparticles can be directed to a tumour site. For example, the functionalization of superparamagnetic iron oxide nanoparticles (SPIONs) with anti-prostate specific membrane antigen (PSMA) increased the detection limit and the sensitivity of MRI in prostate tumour cell culture (Sillerud, 2016). In addition to iron oxide, other nanomaterials already tested in cell culture and/or animal models can be used as contrast agents to enhance imaging diagnostics, including gold nanoparticles (Cole et al., 2015; Indrasekara et al., 2013), carbon nanotubes (Liu et al., 2007; Vittorio et al., 2011), liposomes (Martina et al., 2005; Mukundan et al., 2006), dendrimers (Miyake et al., 2015), and quantum dots (Guo et al., 2014; Yao et al., 2016).

In the treatment of oncological diseases of the reproductive system, recent drug delivery and cell-target strategies have been developed. For example, one of the main anticancer drugs used, doxorubicin, has been associated to nanoformulations to increase its efficacy. These include mesoporous silica nanoparticles (Guo et al., 2017) and lipid coated mesoporous iron oxide –based magnetic nanoassemblies (Pradhan et al., 2016) tested in human cell culture and xenograft mouse models, respectively. PEGylated liposomes have also been tested for cervical and ovarian cancer using human cells (Sriraman et al., 2016). Magnetic nanoparticles (Hua et al., 2017) have been used to treat cervical cancer in human cell cultures and xenograft mice. Other strategies include the delivering of siRNA in cationic dendritic starch

(Engelberth et al., 2017), layer-by-layer engineering of upconversion nanoparticles (Lin et al., 2017), and mesoporous silica nanoparticles (Roberts et al., 2017) resulting in improved cell death in human ovarian cancer cells.

For non-oncological diseases of the reproductive system, some alternatives were tested in human cell culture. To treat uterine leiomyoma, strategies included the use of magnetic nanoparticles complexed to adenovirus (Shalaby et al., 2016) and nanoparticles loaded with 2-methoxyestradiol (Ali et al., 2013). In animal models, carbosilane dendrimer (Chonco et al., 2012) and nanoparticles-in-film (Cunha-Reis et al., 2016) were tested for the treatment of HIV infections. Another condition that could impair fertility is endometriosis, and the strategies already generated using nanomaterials are listed in Table 1.

In the field of fertility preservation, nanotechnology was shown to improve the potential of cryopreserved human immature testicular tissue to restore fertility. Dextran-chitosan nanoparticles loaded with vascular endothelial growth factor (VEGF) were tested for tissue engraftment after cryopreservation of the tissue in mice, resulting in higher vascular density and spermatogonia recovery in transplanted tissues (Poels et al., 2016). For female gametes, an interesting strategy for swine oocyte cryopreservation was developed. The addition of low concentrations of hydroxy apatite nanoparticles (less than 0.5%) in cryoprotectant agents increased the developmental rate of vitrified/devitrified germinal vesicles oocytes (Li et al, 2016). These are a few of the different contributions that nanotechnology has been giving to medically assisted reproduction.

Potential contributions of nanotechnology to assisted reproductive technology

Although ART is successfully applied as a clinical treatment worldwide, some challenges remain. Because of this, strategies developed in animal models are highly important for identifying new alternatives to overcome these problems. When it comes to ART, embryo development in mammalian models is highly similar to humans (Barkalina et al., 2016; Niemann and Wrenzycki, 2000). Lagomorph, murine, swine, bovine, and non-human primates are the main species used to study IVEP techniques to be applied to humans.

Similarly, the implementation of nanotechnology, which has already been developed for non-human animals, could be applied to assisted reproduction in humans (Barkalina et al., 2016; Langbeen et al., 2015). As mentioned previously, this technology has already been tested and used in sectors adjacent to reproductive medicine. Therefore, the main challenges of ART nowadays are how nanotechnology can intervene in order to boost the techniques already used today.

Ovarian stimulation and *in vitro* maturation

To perform ART procedures, ovarian stimulation is routinelly required in order to obtain a higher number of oocytes and increase the chances of embryo production to enable the selection of the best quality embryos for transfer (Fauser et al., 2005). Despite the increased number of oocytes that can be obtained using this procedure, some impairment has been observed. In addition to the high costs and the modest success rates, there are also potential health risks for the patients such as ovarian hyperstimulation syndrome in case of hypersresponse to ovarian stimulation (Huang et al., 2010).

In vitro maturation (IVM) is one of the most promising strategies for overcoming problems related to ovarian stimulation. Oocyte maturation consists of modification of genomic structures, organelle restructurations, and molecular production to allow the gamete to receive spermatozoa for fertilization (Fulka Jr. et al., 1998; Mao et al., 2014). Using the IVM technique, immature oocytes are collected from ovaries of non-stimulated patients, followed by selection and exposure to IVM medium consisting of a base medium for cell culture supplemented with hormones, including FSH, LH and estradiol. However, despite its clinical utility and successfull application in farm animals (Goto et al., 1988; Hwu et al., 1998), IVM of human oocytes remains an experimental approach not widely accepted in fertility clinics worldwide (Chang et al., 2014; Tannus et al., 2017). This is likely due to the lower pregnancy and live birth rates using *in vitro* compared to *in vivo* maturated oocytes, likely due to inadequacies of the culture media (Combelles et al., 2002; Ortega-Hrepich et al., 2013).

It is well established that embryo quality is dependent on oocyte quality (Ferris et al., 2016; Lonergan et al., 2003). In addition, correct and complete oocyte maturation is essential to efficient embryo production. Regarding IVM, the process can be disrupted by excess production of ROS, which is one of the major causes of oocyte depletion (Karuputhula et al., 2013; Tamura et al., 2008). For IVM, the addition of antioxidants is helpful, but these molecules may not exert their function with high efficiency due to their instability in *in vitro* enviroment, making utilization of nanomaterials an interesting strategy for molecule protection (Duarah et al., 2017; Komninou et al., 2016; Lucas et al., 2015; Manconi et al., 2017; Remião et al., 2016). One study from our group has show increased cleavage and blastocyst production rates, decreased ROS levels, and decreased the number of apoptotic cells/blastocyst when bovine oocytes were supplemented with nanoencapsulated melatonin in a IVM medium (Remião et al., 2016).

In another study, tretinoin was nanoencapsulated in lipid-core nanocapsules (LNC) and supplementation with the minor tested concentration (0,25 µM) in IVM medium was benneficial for bovine oocytes, resulting in higher cleavage and blastocyst rates, decreased P66Shc protein levels (the 66-kDa isoform of the growth-factor adapter Shc), and decreased ROS production. These benefits were not observed using the same concentration of non-encapsulated tretinoin (Lucas et al., 2015). Therefore, this represents a potential strategy for increasing the effectivness of human IVM and IVEP.

Multiple pregnancy

Multiple pregnancies are a current problem in ART. The incidence of multiple pregnancies is related to pre-term birth, birth of babies with low weight and other complications, and risks to mothers and babies (Fauser et al., 2005; Vulliemoz et al., 2012). The high incidence of multiple pregnancy when using ART is related to the fact that sometimes more than one embryo is transferred into the female reproductive tract (Friedman et al., 2011; Mersereau et al., 2017).

In order to overcome the multiple pregnancy problems in ART, one alternative is the transfer of single-embryos performed at a higher frequency (Mancuso et al.,

2016). The methodologies assisting this condition are IVM, *in vitro* blastocyst culture, and embryo cryopreservation, techniques that have been highly studied in small and large animals and have been utilized commercially for many years (Sinclair, 2008).

Preimplantational genetic screening (PGS) and preimplantational genetic diagnosis (PGD) can also be useful to avoid multiple pregnancies, by discarding embryos with genetic disorders. To perform PGS and PGD, embryos are biopsied and evaluated using techniques such as karyotyping, fluorescent in situ hybridization (FISH), quantitative polymerase chain reaction (qPCR), array comparative genomic hybridization (aCGH), and next generation sequencing (NGS) (Chen et al., 2017).

Nanotechnology can help researches improve the application of PGS and PGD. Although highly employed, the current detection methods could be more sensitive and specific, more affordable and accessible to patients, faster, and easier to use to facilitate use in human reproduction clinics. Gold, silver, carbon, and magnetic nanomaterials are the main materials used to develop new methods of genetic diagnostics (Zhu et al., 2015). Nanotechnology combined with colorimetric (Stoeva et al., 2006) and electrochemical (Ozsoz et al., 2003) methods for nucleic acid analysis and detection, has brought more sensitivity, lower cost, and increased simplicity and portability to diagnostics. This and other strategies recently developed for DNA analysis can be applied in the future to simplify PGD and PGS diagnostic procedures.

Another strategy for embryo selection is the culture of human embryos until day 5/6, when they reach the blastocyst stage. It has been previously shown that blastocyst transfer (day 5/6) presents better results than cleavage embryos (day 2/3) (Abuzeid et al., 2014; Yin et al., 2017). However, some clinics transfer embryos at the cleavage stage because most embryos fail to reach day 5/6 due to difficulties in mimicking the complexities of the *in vivo* environment (Alper et al., 2001; Tsirigotis, 1998). *In vitro* culture and manipulation of gametes and embryos stimulates production of exogenous ROS and leads to oxidative stress, reducing embryo quality (Agarwal et al., 2006; Truong et al., 2016). To overcome the challenge of embryo culture leading up to the blastocyst stage, research groups have looked for

alternative approaches to improve *in vitro* embryo culture, including the addition of antioxidant molecules to the medium.

Studies on *in vitro* embryo production in animal models indicate antioxidant supplementation in medium is beneficial for blastocyst production. Antioxidants presenting beneficial effects in animal model *in vitro* embryo cultures include L-carnitine (Abdelrazik et al., 2009), hyaluronan (Romek et al., 2017), resveratrol (Salzano et al., 2014), and melatonin (Wang et al., 2013, 2014). However, in the case of bovine IVM, nanotechnology provides interesting alternatives for protecting of these molecules in *in vitro* environments (Komninou et al., 2016; Lucas et al., 2015; Remião et al., 2016). A recent publication confirmed this approach may represent a relevant alternative: supplementation of IVC medium with melatonin-loaded LNC increased embryo quality and blastocyst hatching in a bovine model (Komninou et al., 2016). This strategy is beneficial since the nanocapsules are biodegradable and do not result in toxicity when exposed to bovine oocytes (Lucas et al., 2017) or administered intradermally in rats (Bulcão et al., 2014).

Genetic disorders

The development and improvement of genome editing technology in the last few years has introduced gene therapy as a pre-emptive solution for correction of genetic anomalies. Monogenic diseases may be easily corrected using gene therapy, as they are caused by a single defective gene (Ma et al., 2017). Some monogenic diseases have already been targeted by gene therapy techniques, including lipoprotein lipase deficiency (Gaudet et al., 2016), haemophilia B (Nathwani et al., 2017), β-hemoglobinopathies (Negre et al., 2016), Wiskott-Aldrich syndrome (Aiuti et al., 2013; Morris et al., 2017), and inherited retinal degenerations (Gupta and Huckefeldt, 2017), although these diseases have not been treated in embryos.

Two recent reports have already shown the possibility of gene editing human embryos to correct genetic disorders. The studies used the CRISPR-Cas9 method to fix the human β-globin gene (Liang et al., 2015) and heterozygous *MYBPC3* mutation (Ma et al., 2017), mutations responsible for β-thalassemia and hypertrophic cardiomyopathy, respectively. Although these studies have raised ethical concerns

and the technology is still experimental, without proven efficacy and safety, both publications bring an important alternative to reproductive medicine through the treatment of diseases that until now were considered incurable (Ishii, 2017).

Nanotechnology development has resulted in some interesting non-viral strategies for molecule delivery in cells (Barkalina et al., 2015) contributing to the optimization of gene editing. One of them is the study of Sun et al., 2015, that delivered the Cas9 protein and a guide RNA through a DNA nanoclew to human osteosarcoma tumors in mice. Diverse studies have shown efficient gene delivery in mammalian cells via nanomaterials (Guan and Rosenecker, 2017; Riley and Vermerris, 2017). To produce genetically modified embryos, nanomaterials can be used to increase the efficiency of gene transfer via sperm mediated gene transfer. Silica nanoparticles (Barkalina et al., 2014b), magnetic iron nanoparticles (Kim et al., 2010), halloysite clay nanotubes (Campos et al., 2011), and poly(vinyl alcohol)-coated iron oxide nanoparticles (Makhluf et al., 2008) have already shown promise for delivery of nucleic acids and/or proteins to bovine spermatozoa.

Single-cell embryos can also be directly modified using nanomaterials. Das and collaborators (2016) hypothesized that if single-cell stage zona-free bubaline embryos are transfected with commercial transfecting agents and developed until the blastocyst stage (Selokar et al., 2015), nanomaterials could also be used to introduce genes into embryos at this stage and condition. This could be an alternative to not only viral vectors, but also other expensive methods such as pronuclear microinjection (Das et al., 2016). However, more studies are needed before introducing this technology into practice due to the possible toxic effects.

Challenges for the use of nanotechnology in reproductive science

Nanotechnology has already and can continue to provide advantages for reproductive medicine. Despite the great solutions it can offer, some challenges still faces the use of this technology in medicine. As it is an emerging science, few studies have been performed to validate all the possibilities for treatments or diagnostics. One of the main questions that still needs to be addressed regarding the use of nanotechnology is the toxicity it could cause. Despite new diagnostic

methodologies being closer to being applied commercially, the ways in which nanomaterials are administered to organisms, embryos, or gametes needs to be further studied.

Some nanomaterials are toxic to organism, mainly when exposure occurs during pregnancy and embryo development. For example, when pregnant mice are exposed to titanium dioxide nanomaterials, these materials can cross the placental barrier and cause anatomical defects in the fetuses (Melnik et al., 2013; Naserzadeh et al., 2017). In addition, silver nanoparticles decrease oestrogen plasma levels, increasing the number of resorbed foetuses (Campagnolo et al., 2017) and affecting embryonic growth (Austin et al., 2016). Carbon nanotubes also decrease the number of live foetuses per dam (Fujitani et al., 2015), the number of blood vessels on placenta, and increase the number of abortions (Qi et al., 2014).

Because of this, the utilization of these nanomaterials for reproductive proposes should be done carefully. One alternative is to search for additional nanomaterials that do not present toxicity, with biodegradable structures as the LNC and the dextran/chitosan nanoparticles, representing the most promising nanostructures for use in health applications.

Final considerations

Despite the recent advances in assisted reproductive technologies, some challenges remain, mainly related to pregnancy rates, multiple births, and genetic disorders. To overcome these problems, new alternatives must be identified. Nanotechnology represents a valuable tool that must be explored further to help researchers identify solutions for reproductive medicine. Nanomaterials can bring specificity, practice, and sensibility to next-generation diagnostic and treatment modalities.

It is expected that, as in other areas of medicine, the employment of nanotechnology could be helpful and beneficial to patients. In addition, researchers must be encouraged to develop more *in vitro* and *in vivo* tests using animal models to test safety and efficiency of these new methodologies. In addition, human clinical

reproductive trials may also help accelerate commercial availability of new alternatives for ART.

Author contributions

M. H. Remião, N. V. Segatto, A. R. Pohlmann, S. S. Guterres, F. K. Seixas and T. Collares had an equal participation in writing and approving the present manuscript.

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Table 1. Recent studies developing strategies for treating endometriosis using materials in nanoscale.

Nanomaterial	Strategy	Animal model (cell type)	Main results	Bibliographic reference
Poly(lactic-co-glycolic acid) (PLGA) nanoparticles	PLGA nanoparticle to carry an anti-CD4 antibody	Female C57 endometriosis mouse model	The proposed treatment inhibited IL-10 and TGF-beta secreted by CD4+CD25+Treg cells.	(Liu et al., 2017)
Polyethylenimine-grafted chitosan oligosaccharide (CSO-PEI) with hyaluronic acid (HA)	Gene delivery of aquaporin 2 - small interfering RNA by polymeric nanoparticles	Ishikawa (IK) cells and female Sprague-Dawley rats with induced endometrial lesions	The tested strategy decreased the endometriotic lesion sizes with atrophy and degeneration of the ectopic endometrium. Also, the epithelial cells of ectopic endometrium showed a significant decrease of CD44 expression.	(Zhao et al., 2016)
Polyvinylpyrrolidone (PVP K-30)	Nanoencapsulation of copaiba oil-resin	Primary cell cultures of endometrial stromal cells (ESCs) obtained from ectopic endometrium of patients with endometriosis (EuESCs), ESCs obtained from ectopic endometrium of patients without endometriosis (CESCs) and ESCs from endometriotic lesions (EctESCs)	The proposed method reduced viability and proliferation of endometriotic cell cultures upon COPA nanocomposite treatment.	(De Almeida Borges et al., 2016)
Poly(lactic-co-glycolic) (PLGA) nanoparticles	Nanoencapsulation of epigallocatechin gallate and doxycycline	Human skin keratinocyte (HACAT) cell line and Swiss albino female mice	The proposed treatment decreased oxidative stress, matrix metalloproteinase activity, angiogenesis, endometrial gland presence and microvessel density, and improved oocyte quality.	(Singh et al., 2015)
Unmodified silica nanoparticles (UMNPs) and modified by aminopropyl groups silica	Nanoencapsulation of glucosaminyl muramyldipeptide (N-acetylglucosaminyl- N-	Peritoneal mononuclear cells (MNC) derived from peritoneal fluid of women with endometriosis	The proposed strategy improved immunomodulatory effect of GMDP by the nanoencapsulation in silica nanoparticles.	(Antsiferova et al., 2013)

nanoparticles (AMNPs)	acetylmuramyl-L-alanyl-D-isoglutamine) (GMDP)			
Cerium oxide nanoparticles (nanoceria)	Mitigation of endometrial lesions by nanoceria	CD-1 strain Swiss Albino female mice endometriosis induced	The nanoceria decreased oxidative stress, inhibited angiogenesis and protected oocytes from endometriosis-related adverse effects.	(Chaudhury et al., 2013)
Chitosan derived - polymeric micelles with glycolipid-like structure	Gene delivery of pigment epithelium derived factor gene by micelles	Female Sprague-Dawley rats with induced endometrial lesions	The proposed gene therapy caused a decrease in the sizes of the endometriotic lesions, an atrophy and degeneration of ectopic endometrium, a significantly decrease in microvessel density and increased index of apoptotic in endometriotic lesions.	(Zhao et al., 2012)

4.2 Manuscrito 2 – Effect of nanoencapsulated resveratrol in *in vitro* maturation of bovine oocytes

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Effect of nanoencapsulated resveratrol in *in vitro* maturation of bovine oocytes

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ABSTRACT

Nanoembryology is an area of nanobiotechnology comprising the use of nanomaterials during *in vitro* embryo production (IVEP). The supplementation of mediums used during IVEP with nanoencapsulated antioxidants is one of the great advances emerging from this area of science and bringing great results. In this current work, it was investigated the effects of the supplementation of lipid-core nanocapsules loaded with resveratrol (Res-LNC) at 0.5, 1 and 2 µM of the molecule, during *in vitro* maturation of bovine oocytes. In addition, unloaded nanocapsule (LNC), non-encapsulated resveratrol (Res), and a control without treatment were tested for cumulus cells expansion, oocyte maturation, cytotoxicity and viability of oocytes, and reactive oxygen species (ROS) and glutathione (GSH) levels. Res-LNC increased GSH synthesis during IVM in comparison to LNC and control. There was no difference of Res and Res-LNC in the observed parameters. It was also possible to state that the proposed treatments did not present toxicity to oocytes, as well as LNC and Res-LNC did not impair cumulus cells expansion, oocyte maturation and viability.

Keywords: antioxidant; lipid-core nanocapsules; nanoembryology; nanotechnology; oocyte maturation; resveratrol.

HIGHLIGHTS

- Lipid-core nanocapsules loaded with resveratrol (Res-LNC) is not cytotoxic to bovine oocytes;

- Res- LNC increase glutathione levels in comparison to control and unloaded LNC.
- Res- LNC present similar effects than non-encapsulated resveratrol.

1. INTRODUCTION

The search for new alternatives to overcome exacerbated reactive oxygen (ROS) production by *in vitro* cultured (IVC) embryo and gametes has emerged a new field of research: nanoembryology. Nanoembryology is an area of nanobiotechnology comprising the use of materials in a nanometric scale during *in vitro* embryo production (IVEP). It arise from the search of new strategies to protect molecules from degradation during IVEP medium supplementation (Lucas et al., 2015). The nanoencapsulation of molecules added during *in vitro* maturation (IVM) (Lucas et al., 2017, 2015; Remião et al., 2016) and IVC (Komninou et al., 2016) provided an increase of embryo quality, problably due to the molecule stabilization (Frozza et al., 2013; Koyani and Vazquez-Duhalt, 2016; Pan et al., 2018) and controlled release (Ji et al., 2017; Khan et al., 2015) in a non-toxic and biodegradable way (Bulcão et al., 2014; Lucas et al., 2017; Remião et al., 2016).

The supplementation of mediums for IVEP with antioxidants is one of the main strategies to overcome oxidative stress (Agarwal et al., 2006; Sovernigo et al., 2017). Generally, beneficial effects, as the increase of blastocyst rate and quality are observed when a controlled delivery of antioxidants is applied to IVEP (Komninou et al., 2016; Remião et al., 2016). Probably one of the most benefitted steps of IVEP with controlled release of antioxidants is IVM, as oocytes are one of the most sensitive structures to oxidative stress (Wale and Gardner, 2015). Oxidative stress is

one of the main causes of oocyte depletion, and an inappropriate *in vitro* maturation of oocytes results in an inefficient IVEP (Kwak and Hyun, 2012). Among the different antioxidants that have already been tested during IVM, resveratrol is one of the most promising.

Resveratrol (3,4′,-5-trihydroxystilebene) is a natural polyphenol which has been related to antioxidant action and other desirable biological properties such as cardioprotection (Hung et al., 2004), anticancer (Jang, 1997) and antidiabetic properties (Palsamy and Subramanian, 2008), and prolongation of life span (Valenzano et al., 2006). When added to IVM medium, resveratrol has brought beneficial effects to bovine (Sovernigo et al., 2017; Sprícigo et al., 2017; Takeo et al., 2014, 2013; Wang et al., 2014), swine (Kwak et al., 2012; Lee et al., 2015; Ma et al., 2015) and caprine species (Mukherjee et al., 2014). However, due to the instability of this molecule, it is likely that all antioxidant effects aforementioned could be potentialized if it the molecule was not so exposed to *in vitro* handling. The resveratrol protection using nanocapsules may increase its solubility and time of release (Baur and Sinclair, 2006; Frozza et al., 2013; Wenzel and Somoza, 2005). This strategy also seems to be interesting due to a light sensibility of resveratrol. It has two isomers, cis-resveratrol and trans-resveratrol, in which the trans-isomer is related to more biological effects in comparison to cis. Trans-resveratrol, when exposed to UV light undergoes photoisomerization to cis-resveratrol losing the majority of its biological activities (Vian et al., 2005).

Therefore, nanoencapsulation could be a recommended approach to protect the molecule from UV exposure, contributing to the increase in resveratrol antioxidant effects *in vitro* when compared to non-encapsulated molecules. However, the effects of IVM medium supplementation with resveratrol lipid-core nanocapsules are not

known, thus, this study has focused on the evaluation of some effects in bovine oocytes after the supplementation with different concentrations.

2. MATERIAL AND METHODS

2.1Materials

Formulations were prepared using poly(ε-caprolactone) (PCL, Mw 65 kg mol^{-1}, Aldrich, Strasbourg, France), *trans*-resveratrol (Nigbo Vitax Biotech Co. Ltd., China), capric/caprylic triglyceride (Delaware, Porto Alegre, Brazil), sorbitan monostearate (Sigma Chemical Co.), polysorbate 80 (Vetec, Rio de Janeiro, Brazil), ethanol and acetone (Neon, São Paulo, Brazil). All reagents and solvents used were of analytical or pharmaceutical grades. The other chemicals used in experiments were purchased from Sigma Chemical Co. (St Louis, MO, USA) unless otherwise indicated.

2.2 Methods

2.2.1 Preparation of Lipid-Core Nanocapsules

Trans-Resveratrol-loaded lipid-core nanocapsules dispersed in water were prepared by self-assembling method.. At 40 °C, *trans*-resveratrol (0.010 g), poly(□-caprolactone) (0.100 g), capric/caprylic triglyceride (0.165 mL), and sorbitan monostearate (0.0385 g) were dissolved in acetone (24 mL) and ethanol (3 mL). This organic phase was injected into the 53 mL aqueous phase containing polysorbate 80 (0.0770 g) under magnetic stirring at room temperature. After 10 min, the organic solvents were eliminated and the formulation concentrated under reduced pressure at 40 °C (9 mL). Since resveratrol is very sensitive to light, the resveratrol-loaded

lipid-core nanocapsule formulation was produced in amber flasks protected from light. A control formulation was prepared without trans-resveratrol (lipid-core nanocapsules, LNC).

2.2.2 Physicochemical Characterization of the Formulations

The pH values of the suspensions were determined using a potentiometer DM-22 (Digimed, Brazil). Mean diameters (z-average), polydispersity index, and zeta potentials were measured at 25 °C by using a Zetasizer® nano-ZS ZEN 3600 model (Nanoseries, Malvern, UK), after diluting the samples with MilliQ® water or with 0.01 mol/L NaCl aqueous solution, respectively. To ensure that the particle size of the formulations was submicrometric, the suspensions were also analyzed by laser diffraction (Mastersizer® 2000, Malvern Instruments, Malvern, UK).

2.2.3 Analytical Procedure

Trans-Resveratrol was analyzed by high-performance liquid chromatography (HPLC) at 305 nm. The content of resveratrol (total concentration, C_t) in the formulation (105 µL) was determined after dissolving the drug-loaded lipid-core nanocapsules into acetonitrile (10 mL) (Tedia Company Inc., Ohio, USA) and filtering (Millipore 0.45 µL) for analysis. Chromatography analysis was conducted on a Shimadzu® HPLC model Shimadzu LC-20A system (LC-20AT pump, SPD-M20A photodiode-array (PDA) detector, CBM-20A system controller, SIL-20A auto-sampler (Tokyo, Japan) and a Phenomenex RP-Gemini C18 (150 x 4.6 mm, 5 µm) with a

guard-column. The mobile phase was prepared by using Milli-Q® water and HPLC grade acetonitrile, and consisted of acetonitrile/water (1:1 v/v) containing 0.5 mg of TBA per milliliter of mobile phase and adjusted to an apparent pH of 3.0 ± 0.5 corrected with 10% (v/v) acetic acid. The flow rate used was 0.8 mL/min and injection volume of 20µL. The HPLC method was validated presenting linearity between 3.5 and 17.5 µg/mL^{-1}, (y= 190381x + 21596, r > 0,9996), inter- and intraday variability lower than 2.0%, detection and quantification limits of 0.2 µg/mL^{-1} and 0.9 µg/mL^{-1}, respectively. The encapsulation efficiency (EE%) was calculated (Eq. 1) determining the total drug (C_t) in the nanocapsules and the drug concentration in the continuous phase (C_w) by ultrafiltration-centrifugation technique (Ultrafree Microcon 10,000 MW, Merck Millipore, Darmstadt, Germany), at 5000 rpm for 10 min.

$$EE\% = \frac{C_t - C_w}{C_t} \times 100 \quad (1)$$

2.2.4 Oocyte collection and in vitro maturation

Ovaries were collected from a local slaughterhouse (Pelotas, Rio Grande do Sul, Brazil) and transported to the laboratory in thermal cases to maintain temperature. The cumulus-oocyte complexes (COCs) were aspirated from follicles between 2 and 8 mm using a sterile 18-gauge needle attached to a vacuum pump. Under a stereomicroscope, COCs were classified and only the ones with homogeneous cytoplasm and a minimum of three layers of compact cumulus cells were selected for further analysis. COCs were washed in TCM-199 HEPES supplemented with 10% fetal calf serum, 0.2 mM sodium pyruvate and 83.4 mg/ml amikacin (In Vitro Brasil®, Mogi Mirim, Brazil). Groups of around 15 COCs were transferred to drops of 100 µl of

IVM medium provided by In Vitro Brasil® company (TCM-199, 10% FCS, 1 µg/ml, 50 µg/ml hCG, 1 µg/ml 17 β-estradiol, 0.2 mM sodium pyruvate, and 83.4 µg/ml amikacin) under mineral oil. The COCs were kept at 38.5 ºC, 5% CO^2 and 90% humidity for 23–25 h.

2.2.5 Assessment of the cumulus cells expansion and nuclear status

After 24 h in the IVM medium, COCs were evaluated by cumulus cells expansion degree under a stereomicroscope. The cumulus cells expansion was classified as expanded, partially expanded, and not expanded as described by Marei et al. (2010) (Marei et al., 2010).

The COCs were then denudated from cumulus cells using hyaluronidase (160 IU/ml) to access cytoplasm. Denuded oocytes were washed in three drops of 70 µl of PBS-PVP solution (1 µg/ml polyvinylpyrrolidone in PBS) and incubated with Hoechst 33342 (10 µg /mL) for 30 min at 37 °C in the dark. The oocytes were washed again in three drops of PBS-PBS and evaluated in an inverted fluorescent microscope (IX 71; Olympus, Tokyo, Japan) with UV filters (330–385 nm) for oocyte classification. The oocytes were classified as matured, immature and degenerated according to previous literature (Chankitisakul et al., 2013; Del Collado et al., 2016). The degenerated oocytes were discarded of evaluations, and for each experimental group the percentage of matured oocytes was calculated. The experiments were performed in triplicate.

2.2.6 Viability and cytotoxicity assay

After COCs maturation, live/dead assay was performed to evaluate if the proposed supplementation of IVM medium is toxic to gametes. To perform this staining, COCs were denuded from cumulus cells using hyaluronidase (160 IU/ml), followed by three washes in 70 µl drops of PBS-PVP and incubation in LIVE/ DEAD ® Viability/ Cytotoxicity kit (Molecular Probes Inc.), following manufacturer's instructions with minimal modifications. The oocytes were analysed using an inverted fluorescent microscope IX 71 (Olympus Co.) equipped with an UV filter (excitation of 450–490 nm and emission of 515–565 nm). The gametes were classified between damaged membrane (red fluorescence) and not damaged membrane (green fluorescence). The experiment was replicated three times, and the percentage of not damaged membrane was determined for each experimental group.

2.2.7 Measurement of reactive oxygen species (ROS) and endogenous glutathione (GSH) levels

After COCs maturation, the gametes were stripped from cumulus cells with hyaluronidase (160 IU/ml) and washed three times in 70 µl drops of PBS-PVP solution. The denuded oocytes were put in 100 µl of a solution containing 10 µM of 2',7'-diclorodihydrofluorescein (DCHFDA; Sigma-Aldrich Co.) and 10 µM of 4-chloromethyl-6,8-difluoro-7-hydroxycoumarin (Cell Tracker Blue, CMF2HC; Molecular Probes; Beyotime Institute of Biotechnology) in PBS-PVP. Also, propidium iodide (PI; Molecular Probes) at 72 µM was added to exclude the inviable oocytes in the measurement. They were kept in the dark at 38.5 °C for 30 min and then washed for three times in 70 µl drops of PBS-PVP solution. Then, oocytes were evaluated in a fluorescence microscope IX71 (Olympus Co.) with UV filters (450–490 nm for DCHFDA and 330–395 nm for CMF2HC), attached to a digital camera DP72

(Olympus Co.) for image capturing. Images were analysed using Cell^F software (Olympus SIS) for pixel intensity measurement. The experiment was made in triplicate.

2.2.8 Experimental design

Bovine COCs were randomly distributed in IVM medium containing: non-encapsulated resveratrol (Res) at 0.5, 1 and 2 µM; resveratrol-loaded lipid-core nanocapsules (Res-LNC) at 0.5, 1 and 2 µM of resveratrol; unloaded lipid-core nanocapsules (LNC) in a volume correspondent to the highest of Res-LNC (Res-LNC 2 µM); and a control without supplementation.

Due to the low solubility of non-encapsulated resveratrol, the molecule was dissolved in dimethyl sulfoxide in a concentration of 1 mg/ml. Res-LNC was prepared to have a final solution of 1 mg/ml of resveratrol, and LNC was prepared using the correspondent volumes. To achieve the final concentration in the IVM drop, serial dilution was made using IVM media, resulting in a solution that when 1.8 µl was added in 98.2 µl of IVM media and oocytes, the desired concentration would be reached.

2.2.9 Statistical analysis

Chi-square analysis was performed to compare cumulus cell expansion and oocyte maturation. Cytotoxicity test was analysed by one-way analysis of variance (ANOVA) and ROS and GSH levels were compared among experimental groups using Kruskal Wallis test followed by Tukey test for multiple comparison. The results

were reported as the mean values for each set of data ± SEM, and the degree of statistical significance in all analyses was defined at a probability level of $P < 0.05$.

3. RESULTS

3.1. Production and characterization of LNCs

In a macroscopic evaluation, the lipid-core nanocapsules formulation loaded with either resveratrol (Res-LNC) or not (LNC), resulted in a homogeneous white milky liquid. The mean pH value was 6.1 and 5.7 and the Zeta potential was – 13.3 mV and – 13.2 mV for LNC and Res-LNC, respectively. Laser diffraction analysis showed volume-weighted mean diameter, D [4,3], of 222 for LNC and 156 nm for Res-LNC. The dynamic light scattering analysis showed a mean z-average diameter of 198.1 for LNC and 181.2 for Res-LNC. The resveratrol content was 1 $mg.ml^{-1}$ in the Res-LNC formulation.

3.2 Effect on cumulus cells expansion and nuclear maturation of oocytes

The different treatments during IVM (control, LNC, Res at 0.5, 1 and 2 µM, and Res-LNC at 0.5, 1 and 2 µM) did not affect the cumulus cell expansion, showing that Res-LNC does not influence this parameter of oocyte maturation (Table 1). Only control groups and Res-LNC presented not expanded cumulus cells but it was not statistically significant. In relation to nuclear maturation, the percentage of matured oocytes varied between 72.0% and 90.9%, but statistical differences between groups and concentrations in relation to the control group were not detected (Table 2).

Table 1. Cumulus cells expansion classification in cumulus-oocyte-complexes (COC) submitted to different treatments during IVM.

Experimental groups	Cumulus cells expansion		
	Totally expanded	Partially expanded	Not expanded
Control	2 (5.3%)	33 (86.8%)	3 (7.9%)
LNC 2 µM	6 (20.7%)	23 (79.3%)	0
Res 0.5 µM	5 (17.2%)	24 (82.8%)	0
Res 1 µM	4 (13.3%)	26 (86.7%)	0
Res 2 µM	8 (25.8%)	23 (74.2%)	0
Res-LNC 0.5 µM	5 (12.8%)	32 (82.1%)	2 (5.1%)
Res-LNC 1 µM	6 (18.8%)	26 (81.2%)	0
Res-LNC 2 µM	4 (17.4%)	19 (82.6%)	0

LNC: lipid-core nanocapsule; Res: resveratrol; Res-LNC: Lipid-core nanocapsule loaded with resveratrol. It was not observed differences in cumulus cells expansion between treatments, $P>0.05$. Data represents three replicates.

Table 2. Nuclear maturation classification in cumulus-oocyte complexes (COC) submitted to different treatments during IVM.

Experimental groups	Immature oocytes	Matured oocytes
Control	10 (25.0%)	30 (75.0%)
LNC 2 µM	6 (18.8%)	26 (81.2%)
Res 0.5 µM	7 (28.0%)	18 (72.0%)
Res 1 µM	3 (11.5%)	23 (88.5%)
Res 2 µM	3 (9.1%)	30 (90.9%)
Res-LNC 0.5 µM	8 (19.5%)	33 (80.5%)
Res-LNC 1 µM	7 (24.1%)	22 (75.9%)
Res-LNC 2 µM	6 (23.1%)	20 (76.9%)

LNC: lipid-core nanocapsule; Res: resveratrol; Res-LNC: Lipid-core nanocapsule loaded with resveratrol. It was not observed differences in nuclear maturation between treatments, $P>0.05$. Data represents three replicates.

3.3 Cytotoxicity and viability assay

To evaluate if the treatments have influence on membrane integrity of oocytes, Live/Dead assay was performed demonstrating that there were no statistical differences between the groups in relation to control (Fig. 1). The experiment was performed in triplicate with around 15 oocytes per group in each independent repetition.

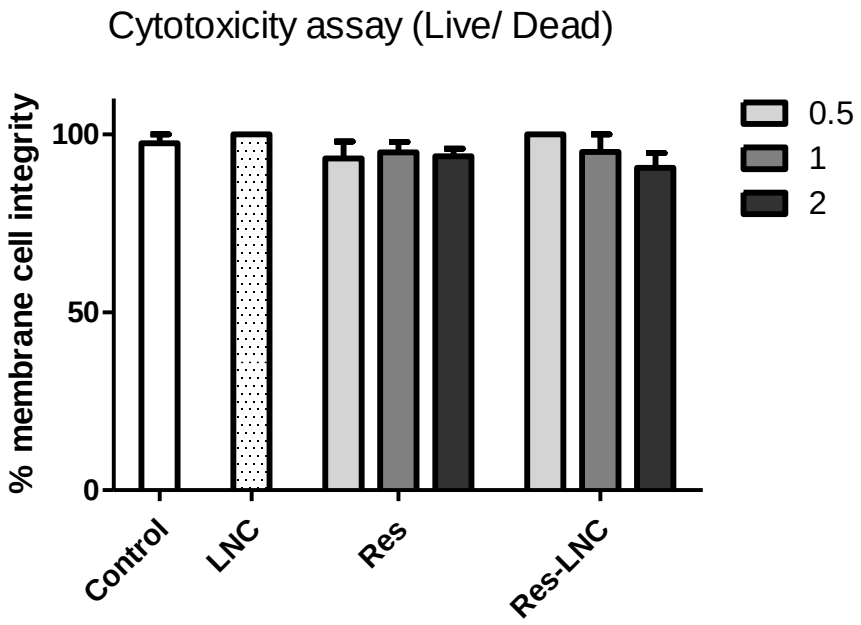

Figure 1. Citotoxicity and viability assay (Live/ Dead assay) for oocytes after IVM treatment. It is observed that there was a small percentage of oocytes classified as inviable in this assay in all experimental groups: Control, LNC (unloaded lipid-core nanocapsule), Res (non-encapsulated resveratrol) at 0.5, 1 and 2 µM, and Res-LNC (resveratrol-loaded lipid-core nanocapsules) at 0.5, 1 and 2 µM of resveratrol. It was not observed differences between treatments, $P>0.05$. Data represents three replicates.

3.4 Effect on levels of ROS and GSH of oocytes

Oocytes were stained for mensuration of its fluorescence intensity to measure ROS and GSH levels in the different treatments during IVM (Fig. 2 C). Non-encapsulated resveratrol, independently from its concentration, and Res-LNC 1 presented a lower production of ROS in relation to LNC. On the other hand, the Res –LNC (0.5 and 2) and control groups did not statistically differ from the other groups, (Fig 2 A).

In relation to GSH, resveratrol, nanoencapsulated in LNC or not, independently of the concentration, demonstrated an effect of increasing the levels, when oocytes were exposed to the treatments during IVM in relation to control and LNC (Fig. 2 B).

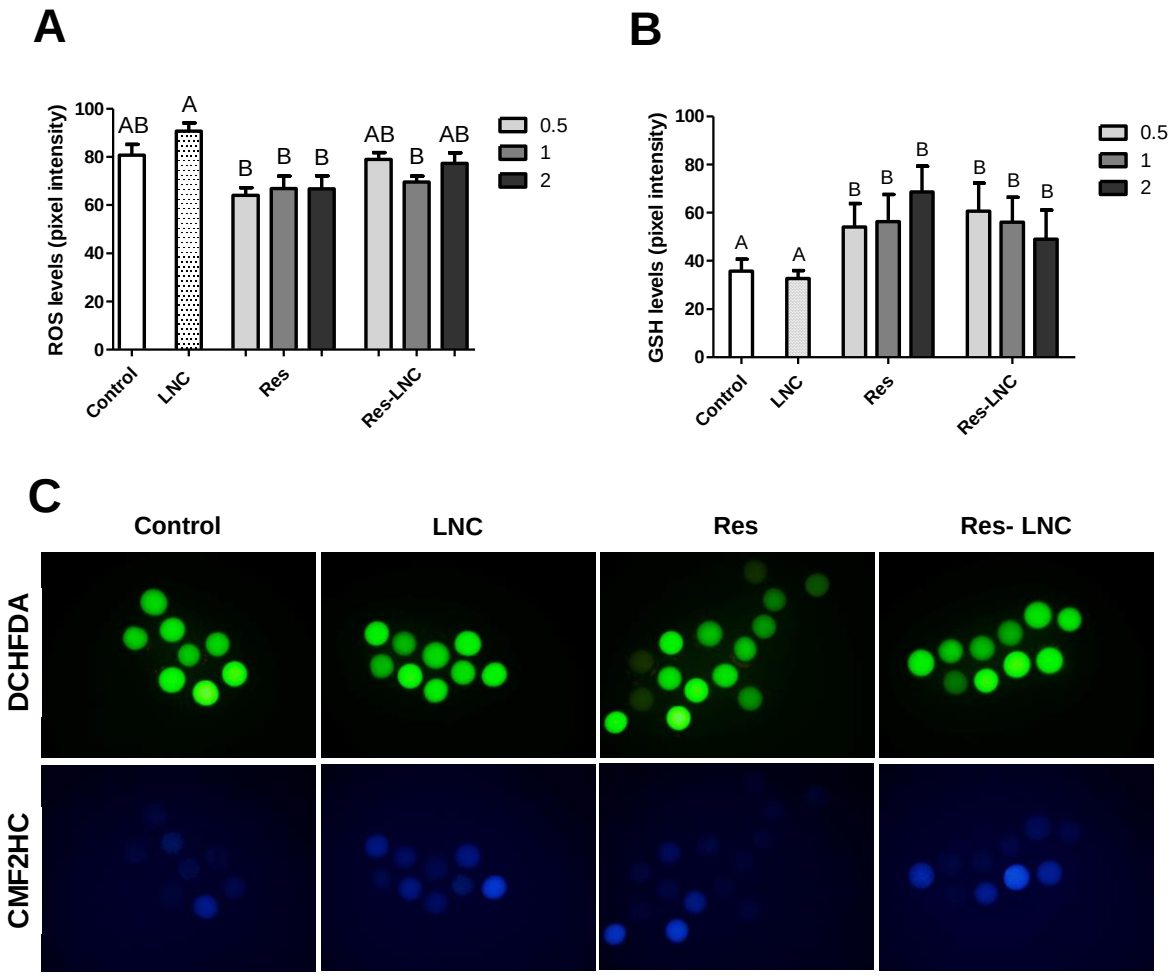

Figure 2**.** Evaluation of ROS and GSH levels in oocytes treated during IVM. A) ROS levels in oocytes from control, LNC (unloaded lipid-core nanocapsule), Res (non-encapsulated resveratrol) at 0.5, 1 and 2 µM, and Res-LNC (resveratrol-loaded lipid-core nanocapsule) at 0.5, 1 and 2 µM of resveratrol, measured by pixel intensity. B) ROS levels in oocytes from control, LNC, Res at 0.5, 1 and 2 µM, and Res-LNC at 0.5, 1 and 2 µM of resveratrol, measured by pixel intensity. C) Representative images of oocytes coloured with the fluorescent probes DCHFDA and CMF2HC in control, LNC, Res at 2 µM and Res-LNC 2 µM. Different letters above the bars represent significant differences in mean fluorescent intensities, P>0.05. Data represents three replicates.

4. DISCUSSION

The nanoencapsulation of resveratrol seems to be an interesting alternative to protect this molecule and increase its properties during the IVEP. In the current study, using the analysis performed in oocytes after IVM supplemented with LNC and Res-LNC, it is possible to conclude that the Res-LNC at 0.5, 1 and 2 µM did not present toxicity to the female gametes.

The evaluations of viability, cell expansion and nuclear maturation demonstrated no difference between all the treatments and the control group. These results agree with previous results which did not detect toxicity of LNC in COCs (Lucas et al., 2017, 2015; Remião et al., 2016) as in *in vivo* tests of intradermal administration (Bulcão et al., 2014). The effect of LNC in cumulus cells expansion was also previously evaluated in different concentrations, some of them much higher than the ones tested in this study (Lucas et al., 2017), while in our study, the different treatments did not alter this parameter after IVM. In relation to unloaded resveratrol, Wang and collaborators (2014) have reported that when bovine COCs were matured in the presence of 1 and 10 µM of this molecule, there was an increase in the gene expression of pentraxin 3 gene (PTX3) in cumulus cells, a gene that is more expressed after the cumulus cells expansion (Wang et al., 2014). Morphologically, our study could not detect a difference in the cumulus cells expansion in the group of unloaded resveratrol at 1 µM.

Our study demonstrated that there was not difference in extrusion of polar body in COCs treated with resveratrol in relation to other groups. Other studies that supplemented IVM medium with unloaded resveratrol diverge in this parameter: some of them corroborate with the current study finding no difference between the

supplementation and the control, and others found an increase in polar body extrusion when resveratrol is added. The latter ones did not find any differences using unloaded resveratrol at 2 µM of concentration in bovine (Sovernigo et al., 2017) and swine (Itami et al., 2015; Ma et al., 2015) IVM. However, Kwak and collaborators, also using unloaded resveratrol at 2 µM, reported an increase in nuclear maturation in swine oocytes (Kwak et al., 2012). For bovine oocytes, Wang and collaborators also found an increase in polar body extrusion when unloaded resveratrol supplements IVM medium, but at 0.1 and 1 µM (Wang et al., 2014). In relation to LNC, in the parameter of extrusion of polar body, it demonstrates no detrimental effect as it does not diverge from the control group, corroborating with previous studies (Lucas et al., 2017, 2015; Remião et al., 2016).

The antioxidant effect of resveratrol seems to be very beneficial for oocytes. The control of oxidative stress of resveratrol in IVEP procedures is suggested by some studies to be due to the increase of GSH synthesis (Kwak et al., 2012; Mukherjee et al., 2014). The GSH synthesis during IVM is very beneficial for oocytes: it protects the meiotic spindle against the damages caused by oxidative stress (Bai et al., 2008; Ma et al., 2017; Roushandeh and Roudkenar, 2009) and improves the subsequent embryo development (De Matos et al., 1996; Khazaei et al., 2017). In the current study, the GSH levels were higher in the groups treated with resveratrol, either nanoencapsulated or not. The data of unloaded resveratrol is consistent with the literature (Kwak et al., 2012; Lee et al., 2015; Wang et al., 2014) which reports a promotion of GSH levels in treated COCs.

Besides increasing GSH levels, resveratrol has already been described for decreasing ROS production when added in an unloaded form in IVM medium in oocytes (Kwak et al., 2012; Wang et al., 2014) and embryos (Lee et al., 2015). In our

study, a slight decrease in ROS is observed in Res in all concentrations and Res-LNC 1 µM, but it was not sufficient to diverge from the control group. In the study of Takeo and collaborators (2013), a difference in ROS production of oocytes that were incubated in IVM medium with 2 µM of unloaded resveratrol (Takeo et al., 2013) was not observed. In the current study, LNC group presented higher ROS production, but it was not different from the control group, corroborating with other published studies (Lucas et al., 2017, 2015; Remião et al., 2016) and demonstrating no damaging effects for this parameter.

The Res-LNC demonstrated not to be cytotoxic for oocytes and it acts similarly to Res for the evaluated parameters. However, a beneficial effect generated by Res-LNC supplementation of IVM may be observed in subsequent parameters such as cleavage and blastocyst rate or cryotolerance, for example. A previous study of nanoencapsulated melatonin in LNC has shown no statistical difference in nuclear maturation when this molecule was added in the IVM medium but later it was observed an increase in cleavage and blastocyst rate and a decrease in proportion of apoptotic cells/total number of cells per blastocyst (Remião et al., 2016). Probably this effect was observed due to the controlled release of LNCs. Due to this fact, new studies should also be performed using Res-LNC in other stages of IVEP.

In conclusion, this study shows that *in vitro* maturation medium of bovine oocytes can be supplemented with lipid-core nanocapsules loaded with resveratrol (Res-LNC) without detrimental effects, as it does not alter maturation and viability parameters of oocytes. Although an increase in GSH synthesis of oocytes was observed in nanoencapsulated resveratrol, which is relevant for the gamete, no statistical difference was found in relation to the non-encapsulated resveratrol. More

studies are recommended to evaluate the effect of this supplementation in *in vitro* embryo development.

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4.3 Pedido de patente – Recipiente auxiliar de bancada para estoque de nitrogênio líquido durante o procedimento de vitrificação e desvitrificação de oócitos ou embriões

Pedido de patente de invenção depositada no Instituto Nacional de Propriedade Industrial sob o número de processo BR 10 2017 009395 6

INSTITUTO NACIONAL DA PROPRIEDADE INDUSTRIAL

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Pedido nacional de Invenção, Modelo de Utilidade, Certificado de Adição de Invenção e entrada na fase nacional do PCT

Número do Processo: BR 10 2017 009395 6

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PETICIONAMENTO ELETRÔNICO

Esta solicitação foi enviada pelo sistema Peticionamento Eletrônico em 04/05/2017 às 10:07, Petição 870170029411

Dados do Pedido

Natureza Patente: 10 - Patente de Invenção (PI)

Título da Invenção ou Modelo de Utilidade (54): Recipiente auxiliar de bancada para estoque de nitrogênio líquido durante o procedimento de vitrificação e desvitrificação de oócitos ou embriões

Resumo: A presente invenção refere-se aum recipiente térmico utilizado para armazenamento em nitrogênio líquido deamostras de oócitos e embriões vitrificados, antes da sua estocagem definitiva em botijões que contenham esse líquido criogênico. Este recipiente surge como uma alternativa à métodos utilizados atualmente, os quais são de difícil higienização, ocupam espaço ou acabam desperdiçando o líquido criogênico por sua rápida evaporação. O recipiente térmico proposto é composto internamente de isopor, material que mantém por mais tempo o líquido criogênico sem evaporar, e externamente de aço inoxidável ou plástico resistente a baixas temperaturas, o que facilita a sua assepsia. A caixa é compacta, e por isso não ocupa muito espaço em capelas de fluxo laminar e bancadas em geral. A caixa possui um sistema de encaixe onde um suporte composto de aço inoxidável ou plástico resistente a baixas temperaturas será acoplado. Este suporte é utilizado paramanter tubos de 15 e 50 mililitros inclinados. Os tubos possuem orifícios que permitem a entrada de líquido criogênico, o que mantém as amostras devidamente criopreservadas e faz com que o tubo não bóie no canister do botijão de nitrogênio líquido. Nesse sentido, o presente invento é uma alternativa prática para a realização de vitrificação de oócitos e embriões.

Figura a publicar: 1

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Documentos anexados

Tipo Anexo	Nome
Comprovante de pagamento de GRU 200	Comprovante pgmto GRU 00.000.2.2.17.0008733.3.pdf
Desenho	Desenhos.pdf
Termo de Posse	Termo de Posse MEC.pdf
Procuração	PROCURAÇÃO_2017.pdf
Reivindicação	Revindicações.pdf
Relatório Descritivo	Relatório descritivo.pdf
Resumo	Resumo.pdf

Acesso ao Patrimônio Genético

☑ Declaração Negativa de Acesso - Declaro que o objeto do presente pedido de patente de invenção não foi obtido em decorrência de acesso à amostra de componente do Patrimônio Genético Brasileiro, o acesso foi realizado antes de 30 de junho de 2000, ou não se aplica.

Declaração de veracidade

☑ Declaro, sob as penas da lei, que todas as informações acima prestadas são completas e verdadeiras.

5 CONCLUSÃO GERAL

Conclui-se a partir deste trabalho que a nanotecnologia pode ser uma grande aliada no desenvolvimento das ciências reprodutivas. Apesar de não ser ainda tão difundida nesse campo, diversas estratégias podem ser utilizadas a fim de otimizar tratamentos e protocolos e é na produção *in vitro* de embriões que estratégias associadas à nanotecnologia vêm sendo testadas com mais frequência. Seguindo esta linha, o presente documento apresenta uma proposta de suplementação do meio de maturação *in vitro* de oócitos com nanocápsulas de núcleo lipídico carregadas com resveratrol.

Testando esta proposta, os resultados aqui obtidos demonstram que a estratégia de nanoencapsulamento do antioxidante resveratrol não traz toxicidade aos oócitos. Pelo contrário, esse tratamento, nas concentrações testadas, é capaz de aumentar a síntese de glutationa endógena quando comparado ao controle sem tratamento ou à nanocápsula não carregada. No entanto, também foi observado que a suplementação do meio com o resveratrol nanoencapsulado é similar ao resveratrol não nanoencapsulado. Esse resultado foi encontrado para os parâmetros testados, mas é possível que o efeito de liberação controlada da molécula pela nanocápsula faça com que outros benefícios sejam alcançados quando observado o desenvolvimento embrionário destes oócitos tratados em baixas concentrações.

Por fim, conclui-se que é de extrema importância a busca por avanços nas áreas das ciências reprodutivas, e que áreas de destaque científico como a nanotecnologia podem vir a contribuir na resolução de diversos problemas que ainda permanecem acerca da produção *in vitro* de embriões.

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