Casa abierta al tiempo

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VITRIFICACIÓN DE OVOCITOS PORCINOS Y SU EFECTO A NIVEL ESTRUCTURAL EN EMBRIONES Y EN EL ADN DE LAS CÉLULAS DEL CÚMULO

TESIS

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PRESENTA

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"Me enseñaron que el camino del progreso

no es ni rápido ni fácil"

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RESUMEN

La vitrificación es una técnica que se utiliza principalmente para criopreservar embriones y gametos femeninos. Esta técnica permite mantener la viabilidad celular, la funcionalidad y el potencial de desarrollo a bajas temperaturas en nitrógeno líquido a -196 ºC. Para ello, se requiere de la adición de agentes crioprotectores (CPAs), que son sustancias que brindan protección celular durante el enfriamiento y el calentamiento. Sin embargo, se ha informado que son potencialmente tóxicos, reduciendo la viabilidad de los ovocitos, la maduración, la fertilización y el desarrollo embrionario (DE), posiblemente alterando la estructura del citoesqueleto celular y de la cromatina (CR). Estudios previos han evaluado los efectos de la vitrificación de manera directa en ovocitos en vesícula germinal (VG), metafase II (MII), cigotos y blastocistos, pero el conocimiento de su impacto en el desarrollo posterior de los embriones es limitado. Otros estudios han evaluado el papel de los microfilamentos (MF) de actina y de la CR, basados en las tasas obtenidas de la fertilización y del DE, pero no la evaluación directa de estas estructuras en embriones producidos a partir de ovocitos inmaduros vitrificados. Por lo tanto, este estudio fue diseñado para evaluar cómo la vitrificación de ovocitos inmaduros porcinos afecta al desarrollo del embrión temprano mediante la evaluación de la distribución de MF de actina y la integridad de la CR. Los resultados demuestran que el daño generado por la vitrificación de ovocitos inmaduros afecta la viabilidad, la maduración, el DE, la distribución de MF de actina y la integridad de la CR observada en embriones tempranos, pero no afectó la fertilización *in vitro*. Por lo tanto, se sugiere que la vitrificación podría afectan los mecanismos de reparación de los ovocitos en esas estructuras, siendo uno de los mecanismos que explicar las bajas tasas de DE después de la vitrificación.

Por otro lado, la evaluación del daño del ADN generado en las células del cúmulo (CC) después de la vitrificación de los complejos ovocitos-células del cúmulo (COCs) maduros puede

considerarse como indicador de la calidad de los ovocitos, ya que estas células juegan un papel importante en la competencia del desarrollo de los ovocitos. Por lo tanto, el segundo objetivo de este estudio fue determinar si la exposición de los COCs maduros a CPAs o a la vitrificación afectan la viabilidad de los ovocitos y de las CC, además si se genera daño al ADN en las CC, afectando la fertilización y el DE. El daño del ADN en las CC se midió utilizando el ensayo cometa alcalino y se expresó como longitud de la cola del cometa (LCC) y el tiempo momento (OTM, por sus siglas en inglés). Los resultados demuestran que la exposición de los ovocitos a los CPAs (grupo toxicidad) o la vitrificación redujo la viabilidad de los ovocitos (75.5 ± 3.69 %, toxicidad; 66.7 ± 4.57 %, vitrificación) y de las CC (32.7 ± 5.85 %, toxicidad; 7.7 ± 2.21 %, vitrificación) en comparación con el control (95.5 % ± 4.04 %, ovocitos; 89 ± 4.24 %, CC). Además, se generó daño en el ADN significativamente mayor expresado como OTM en las CC después de la exposición a CPAs y vitrificación (39 ± 17.41, 33.6 ± 16.69, respectivamente) en comparación con el control (7.4 ± 4.22). Además, las tasas de fertilización y de DE también disminuyeron después de la exposición a CPAs (35.3 ± 16.65 %, 22.6 ± 3.05 %, respectivamente) y a la vitrificación (32.3 ± 9.29 %, 20 ± 1 %, respectivamente). Este estudio demuestra que la exposición de los ovocitos a los CPAs o a la vitrificación redujo la viabilidad de los ovocitos y de las CC y generó daños en el ADN de las CC, lo que afectó las tasas de fertilización y de DE. Estos hallazgos permitirán comprender algunos de los mecanismos de daño de los ovocitos tras la vitrificación que comprometen su capacidad de desarrollo, así como la búsqueda de nuevas estrategias de vitrificación para incrementar las tasas de fecundación y de DE preservando la integridad de las CC.

ABSTRACT

Vitrification is mainly used to cryopreserve embryos and female gametes. This technique allows maintaining cell viability, functionality, and developmental potential at low temperatures into liquid nitrogen at -196 ºC. For this, the addition of cryoprotectant agents, which are substances that provide cell protection during cooling and warming, is required. However, they have been reported to be toxic, reducing oocyte viability, maturation, fertilization, and embryo development, possibly by altering cell cytoskeleton structure and chromatin. Previous studies have evaluated the effects of vitrification in the germinal vesicle, metaphase II oocytes, zygotes, and blastocysts, but the knowledge of its impact on their further embryo development is limited. Other studies have evaluated the role of actin microfilaments and chromatin, based on the fertilization and embryo development rates obtained, but not the direct evaluation of these structures in embryos produced from vitrified immature oocytes. Therefore, this study was designed to evaluate how the vitrification of porcine immature oocytes affects early embryo development by the evaluation of actin microfilament distribution and chromatin integrity. Results demonstrate that the damage generated by the vitrification of immature oocytes affects viability, maturation, and the distribution of actin microfilaments and chromatin integrity, observed in early embryos, but did not affect *in vitro* fertilization. Therefore, it is suggested that vitrification could affect oocyte repair mechanisms in those structures, being one of the mechanisms that explain the low embryo development rates after vitrification.

On the other hand, the evaluation of the DNA damage generated in cumulus cells after mature cumulus-oocyte complexes vitrification can be considered as an indicator of oocyte quality since these cells play important roles in oocyte developmental competence. Therefore, the aim of this study was to determine if matured cumulus-oocyte complexes exposure to cryoprotectants (CPAs) or vitrification affects oocytes and cumulus cells viability, but also if DNA damage is

generated in cumulus cells, affecting fertilization and embryo development. The DNA damage in cumulus cells was measured using the alkaline comet assay and expressed as Comet Tail Length and Olive Tail Moment (OTM). Results demonstrate that oocyte exposure to CPAs or vitrification reduced oocyte (75.5 ± 3.69 %, toxicity; 66.7 ± 4.57 %, vitrification) and cumulus cells viability (32.7 ± 5.85 %, toxicity; 7.7 ± 2.21 %, vitrification) compared to control (95.5 ± 4.04 %, oocytes; 89 ± 4.24 %, cumulus cells). Also, significantly higher DNA damage expressed as OTM was generated in the cumulus cells after exposure to CPAs and vitrification (39 ± 17.41, 33.6 ± 16.69, respectively) compared to control (7.4 ± 4.22). In addition, fertilization and embryo development rates also decreased after exposure to CPAs (35.3 ± 16.65 %, 22.6 ± 3.05 %, respectively) and vitrification (32.3 ± 9.29 %, 20 ± 1 %, respectively). This study demonstrates that oocyte exposure to CPAs or vitrification reduced viability in oocytes and cumulus cells, and generated DNA damage in the cumulus cells, affecting fertilization and embryo development rates. These findings will allow to understand some of the mechanisms of oocyte damage after vitrification that compromise their developmental capacity, as well as the search for new vitrification strategies to increase fertilization and embryo development rates by preserving the integrity of the cumulus cells.

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% ADN: integridad del ADN

AC: actina cortical

AC/D: actina cortical con daño en la CR

AC/SD: actina cortical sin daño en la CR

ACD: actina cortical dispersa

ACD/D: actina cortical disgregada con daño en la CR

ACD/SD: actina cortical disgregada sin daño en la CR

ACT: activado

AD: actina dispersa

AD/D: actina disgregada con daño en la CR

AD/SD: actina disgregada sin daño en la CR

ADN: ácido desoxirribonucléico

AMPc: monofosfato de adenosina cíclico

ARNm: ácido ribonucleico mensajero

ATP: trifosfato de adenosina

Ca^{2+}: calcio

CC: células del cúmulo

CO_2: dióxido de carbono

COC(s): complejo(s) ovocito-células del cúmulo

CPA(s): agente(s) crioprotector(es)

CR: cromatina

CRA: cromatina anormal

D: con daño/dañado

DE: desarrollo embrionario

DMSO: dimetilsulfóxido

ds-ADN: ADN de doble hebra *(doble stranded DNA)*

EG: etilenglicol

ERO: especies reactivas de oxígeno

FITC: isotiocianato de fluoresceína

FIV: fertilización *in vitro*

FSH: hormona folículo estimulante

GL: glicerol

GSH: glutatión

GTP: guanosín trifosfato

HM: *holding medium*

ICSI: inyección intracitoplasmática de espermatozoides *(Intracytoplasmic Sperm Injection)*

IGF-I: factor de crecimiento de insulina I

LCC: longitud de la cola del cometa

LH: hormona luteinizante

MAPs: proteínas asociadas a microtúbulos

MF: microfilamentos

MII: metafase II

MIV: maduración *in vitro*

MP: monospérmico

MTOCs: centro organizador de microtúbulos

MTT: metil tetrazolio

N: núcleo

n: número de células evaluadas

N_2L: nitrógeno líquido

nc: número de células evaluadas del control

NCSU-23: *North Carolina State University-23*

NF: no fertilizado

nv: número de células evaluadas de vitrificación

OPS: *Open Pulled Straw*

OTM: tiempo momento *(Olive Tail Moment*)

PBS: *phosphate buffered saline*

PICSI: inyección fisiológica intracitoplasmática de espermatozoides *(Physiological Intracytoplasmic Sperm Injection)*

PN: pronúcleo

PP: polispérmico

PROH: 1 , 2 – propanediol

SD: sin daño

ss-ADN: ADN de una sola hebra *(single stranded DNA)*

TBMm: *tris-buffer medium*

TCM-199: *tissue culture medium 199*

TL HEPES-PVA: *Tyrode's lactate (TL)-HEPES-polyvinyl alcohol (PVA) medium*

TRA: tecnologías de reproducción asistida

VG: vesícula germinal

I. INTRODUCCIÓN

1.1 Desarrollo de las tecnologías de reproducción asistida

La reproducción asistida es un campo de la medicina reproductiva que ha presentado desarrollo acelerado, debido a que el número de pacientes que padecen infertilidad va en constante aumento. Por lo que existe incremento en la necesidad de desarrollar y mejorar las tecnologías de reproducción asistida (TRA) (López *et al*, 2021 a).

Dentro de las TRA que se realizan en México se encuentran la fertilización *in vitro* (FIV) y la inyección intracitoplasmática de espermatozoides (ICSI, por sus siglas en inglés) como técnicas de fertilización, la crioconservación de gametos, la transferencia embrionaria y la donación de gametos, entre otras (López *et al*, 2021 a). En la crioconservación de gametos, se incluyen tecnologías como la congelación lenta y la vitrificación. Esta última, en estudio de meta-análisis en humanos, ha mostrado mejores resultados en el gameto femenino en comparación con la congelación lenta en parámetros como la supervivencia (82% vs 66%), tasa de embarazo en curso por ciclo (37.5% vs 13%) (Rienzi *et al*, 2017).

En humanos, las TRA son ampliamente utilizadas en centros de reproducción asistida para parejas o personas solteras con algún problema de infertilidad o condición reproductiva. En el caso de las mujeres, estas técnicas también son utilizadas para las que deciden postergar la maternidad o que serán sometidas a tratamientos de quimioterapia y que planean la maternidad a futuro (Luke *et al*, 2016; McCray *et al*, 2016). Así mismo, han tenido una puntual relevancia en animales domésticos y silvestres para el mejoramiento genético, en la producción de especies con importancia económica para el humano y en la preservación de aquellas que se encuentran en peligro de extinción (Mullen y Fahy, 2012).

La aplicación de las TRA ha tenido una especial relevancia en el área de la investigación

científica, ya que los avances tecnológicos en la reproducción asistida, la clonación de células somáticas, la edición del genoma y la transgénesis han permitido la creación de modelos animales para la investigación biomédica durante los últimos 40 a 45 años (Gutierrez *et al*, 2015; Walters *et al*, 2017). El porcino, se ha planteado como un modelo adecuado de estudio para la aplicación en los humanos debido a las similitudes que comparte con él, como lo es el tamaño, la fisiología, la anatomía y el perfil metabólico (Gutierrez *et al*, 2015), resultando en un modelo óptimo para estudios en enfermedades humanas, el xenotransplante, la toxicología y la generación de fármacos (Walters *et al*, 2017). Además, su implementación ha sido importante para el avance en la medicina reproductiva humana, en la que los modelos animales han permitido la experimentación en las TRA que han sido posteriormente aplicadas en los humanos, ya que en humanos se presentan limitaciones debido a la restricción de la manipulación de gametos y embriones condicionada al manejo ético y a consentimientos informados por parte de pacientes en clínicas para la donación de gametos y embriones para la investigación (*Ethics Committee of American Society for Reproductive Medicine,* 2014).

1.2 Sistemas para la criopreservación de gametos

La criopreservación es una técnica aplicada para la preservación de células, tejidos, gametos y embriones; cuya finalidad es mantener la viabilidad, funcionalidad y el potencial del desarrollo celular a bajas temperaturas (Ávila-Portillo *et al*, 2006; Chian, 2010; Casillas *et al*, 2014).

Son dos los sistemas para la criopreservación actualmente utilizados, la congelación lenta y la vitrificación, estos permiten la conservación de las células a -196 ºC deteniendo los procesos bioquímicos, estructurales y fisiológicos hasta su calentamiento. La congelación lenta tiene como principio la deshidratación progresiva de la célula como respuesta del cambio osmótico,

mediante la incorporación lenta de concentraciones relativamente bajas de los CPAs, sustancias que confieren protección a la célula durante la criopreservación, además, de requerir de equipo especializado como cámaras de congelación y almacenaje que permiten descender de forma gradual la temperatura de las células, pasando de manera gradual de -4 ºC, -30/-40 ºC, -80 ºC hasta -196 ºC (Somoskoi *et al*, 2015). En caso contrario, la vitrificación, es un método más rápido y menos costoso al no requerir equipo sofisticado para su realización, así mismo, alcanza tasas de enfriamiento y calentamiento óptimas que aumentan la supervivencia celular y reduce la deshidratación excesiva, común en la congelación lenta, mejorando los resultados (Fahy, 2016). Por lo anterior, se ha posicionado como el método de criopreservación más utilizado, reflejandose en el número de pacientes que han sido sometidos a esta tecnología comparado a la congelación lenta, según el registro Human Oocyte Preservation Experiences (HOPE, por sus siglas en inglés) del 2017, registro observacional prospectivo de clínicas de reproducción asistida en los EUA (Nagy *et al*, 2017). En la Tabla 1 se presentan algunas características comparativas entre ambas tecnologías de criopreservación.

Tabla 1. Características comparativas entre la congelación lenta y la vitrificación

CARACTERÍSTICA	**TECNOLOGÍA DE CRIOPRESERVACIÓN**	
	Congelación lenta	**Vitrificación**
Tiempo de trabajo	Lento. Más de 3 horas	Rápido. Menos de 10 minutos
Costo	Costoso. Se requiere equipo de congelación especializado	Económico. No se requiere de equipo especializado
Volumen de la muestra	100-250 µL	1-2 µL
Concentración de CPAs	Baja. ~ 1.5 M	Elevada. De 7.5 a 16%
Riesgo de lesiones por congelación (incluida la formación de cristales de hielo)	Elevado	Bajo

Viabilidad posterior al calentamiento	Elevada	Elevada
Riesgo de toxicidad por los CPAs	Bajo	Elevado
Tipos de sistemas de almacenamiento	Sistemas cerrados	Sistemas abiertos y cerrados
Contaminación potencial con agentes patógenos	Baja	Elevada
Habilidad requerida para la manipulación	Fácil	Difícil

Modificado de Jang *et al*, 2017.

Para la selección del método de criopreservación más adecuado es importante considerar algunos factores como la especie animal, el tipo celular y la naturaleza de los CPA a utilizar; los cuales son sustancias indispensables que conferirán protección a las células durante el enfriamiento y el calentamiento, etapas importantes de la criopreservación. El enfriamiento consta del descenso de la temperatura posterior a la exposición de los CPAs y el calentamiento implica la rehidratación y reactivación metabólica celular para el retorno a condiciones fisiológicas mediante la expocisión a CPAs impermeables (explicados en el tema 'agentes crioprotectores' de la sección 1.3) después del almacenamiento en nitrógeno líquido (N_2L) a -196 ºC (Fahy, 2016).

Durante la crioconservación pueden surgir algunos criodaños por el manejo inadecuado durante el proceso, como el daño por "efectos de solución" que causa la hinchazon de la célula por el choque osmótico durante el enfriamiento lento y la formación de hielo intracelular letal que provoca la fractura de estructuras celulares producido por el enfriamiento rápido. Estas fracturas pueden participar como sitios de nucleación que es la formación de una particula inicial que indica el comienzo del cambio de estado de líquido a sólido, que incrementan el problema de la formación de hielo durante el calentamiento (Jang et al, 2017; Fahy, 2021). Esta etapa es crítica

debido al riesgo de fractura por desvitrificación que implica la formación de hielo desde el estado vitrificado, así como por la recristalización que comprende el crecimiento del tamaño de los cristales de hielo (Fahy, 2021). En la Figura 1 se representan los posibles criodaños en las células durante el enfriamiento lento y rápido. En color rojo se indica la manera en que la vitrificación ayuda a impedir esos criodaños.

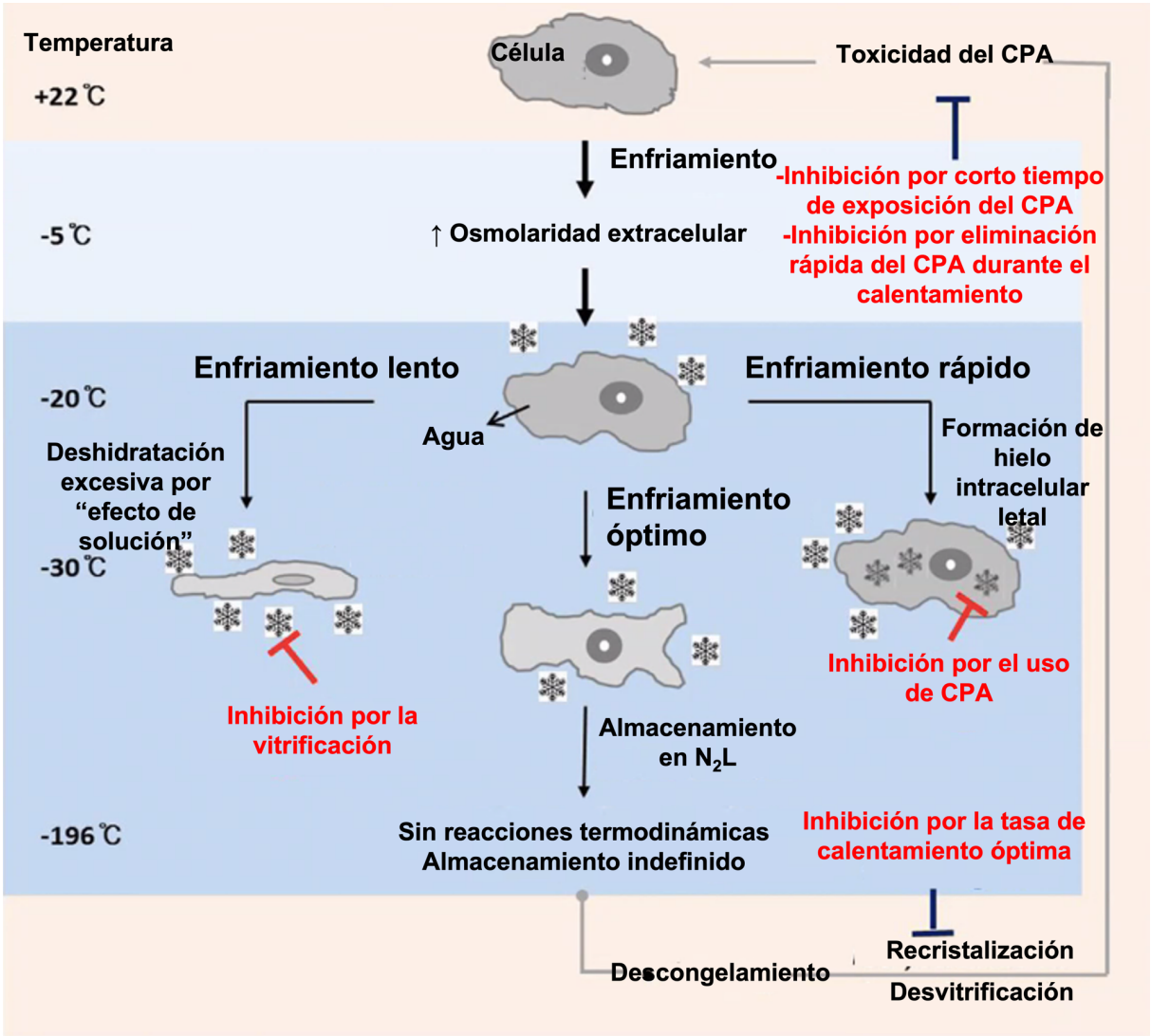

Figura 1. Criodaños en las células por la congelación durante el enfriamiento lento y rápido. Modificado de Jang *et al*, 2017.

1.3 Vitrificación

La vitrificación ha sido una herramienta útil en las TRA como recurso en el tratamiento de afecciones reproductivas y de infertilidad en humanos, así como para mejorar la capacidad y calidad reproductiva en especies con importancia económica en la producción animal y en especies en peligro de extinción (Mullen y Fahy, 2012; Tharasanit y Thuwanut, 2021). La vitrificación es utilizada en tejido ovárico, testicular y en ovocitos de especies como el porcino (López *et al*, 2021 b), el ovino (Fernández-Reyes *et al*, 2012; Barrera *et al*, 2018) y el humano (Lei *et al*, 2014; Khalili *et al*, 2017).

El fundamento de la vitrificación consiste en pasar del rango de temperatura crítica (-30 °C a -80 °C) hasta los -196 °C de forma ultra-rápida mediante la transición de una solución acuosa en estado líquido a un estado sólido vítreo evitando la formación de hielo intracelular para reducir el riesgo de daño en estructuras celulares ya que no requiere de una velocidad de enfriamiento óptima debido a la rapidez del descenso de la temperatura (Fahy, 2021). Sin embargo, presenta desventajas debido a la necesidad del uso de altas concentraciones de CPAs que implican riesgo de toxicidad y daño osmótico. Además, el enfriamiento a la temperatura del N_2L puede inducir fracturas en estructuras como la zona pelúcida de los ovocitos, que pueden favorecer la formación de sitios de nucleación durante el calentamiento (Jang *et al*, 2017; Fahy, 2021).

Dos principios físicos pueden ayudar a explicar lo que sucede en la célula durante el enfriamiento: el principio de ósmosis y el principio del descenso del punto de congelación. El primero se puede entender si consideramos que en una célula semipermeable al estar en una solución salina, el agua fluirá en sentido tal que busque igualar las concentraciones de ambos sistemas (medio interno de la célula y la solución salina externa). Por lo tanto, si exponemos a la célula a un medio con mayor concentración de sales (hipertónico) que su medio interno,

entonces el agua que se encuentra en el interior de la célula tenderá a salir, provocando que la célula se deshidrate y reduzca su volúmen. Por el contrario, si la célula es expuesta a un medio más diluido (hipotónico) que su medio interno, entonces el agua ingresará al interior de la célula provocando su hinchazón. El segundo principio se basa en la congelación del agua pura a los 0 ºC, sin embargo, si disolvemos sales en esa agua la temperatura de congelación entonces será por debajo de los 0 ºC, en correlación, cuanto más concentración de sales presente el agua, la temperatura de congelación de esta será más baja (Jang *et al, 2017).*

Agentes crioprotectores (CPAs)

En consideración a los dos principios físicos anteriormente mencionados, los CPAs son sustancias indispensables para la vitrificación que conferirán protección a las células durante este proceso (Fahy, 2016), estas sustancias ayudarán a promover la formación de estos fenómenos físicos. Durante la vitrificación la célula es expuesta de manera gradual a mayores concentraciones de CPAs permeables e impermeables, estos últimos CPAs aumentarán la osmolaridad (concentración de solutos) de las soluciones de vitrificación y como consecuencia promueven la deshidratación celular por la salida del agua interna hacia el medio externo que busca compensar las diferencias de concentraciones. La deshidratación celular provocará que los solutos del interior se encuentren altamente concentrados reduciendo el punto de congelación, esto implica que la temperatura a la que se realiza la nucleación del hielo sea más baja cuanto más concentración se presente, dificultando la formación de cristales de hielo en el interior de la célula que pueden ocasionar el daño de las estructuras celulares por fractura. Por otro lado, los CPAs permeables ocuparán el lugar que el agua tenía en el interior de la célula promoviendo la transición del estado líquido al sólido vítreo, además de evitar la deshidratación excesiva (Fahy, 2016; Son *et al*, 2019). La adición y eliminación de los CPAs crea un

desequilibrio osmótico a través de la membrana del ovocito que puede resultar en grandes cambios volumétricos por la hinchazón o deshidratación excesiva, que pueden causar la fractura de la membrana celular y las estructuras del citoesqueleto y con ello comprometer la viabilidad y desarrollo del ovocito (Chian *et al*, 2014), estos daños están relacionados con la generación de criolesiones. Durante todas las fases de la vitrificación de ovocitos se pueden presentar lesiones relacionadas a la temperatura, como el daño por frío (entre 15 ºC y −5 ºC), la formación de cristales de hielo (entre −5 ºC y −80 ºC) y el daño por fracturas (entre −50 ºC y −150 ºC) (Son *et al*, 2019); no obstante, se ha reportado que el mayor riesgo de daño durante la vitrificación es el empleo necesario de altas concentraciones de CPAs que aumentan el riesgo de daño osmótico y principalmente por la citotoxicidad ocasionada por su naturaleza química de estas sustancias (Fahy, 2016). Sin embargo, la administración de CPAs en concentraciones adecuadas ayudará a aumentar de forma considerable las tasas de supervivencia de la célula (Chian *et al*, 2014). Esto es debido a que los CPAs aumentan la osmolaridad de las soluciones de vitrificación, reduciendo el punto de congelación, y de esta forma previene o reduce la formación de cristales de hielo (Fahy *et al*, 2021, Son *et al*, 2019).

Los CPAs de tipo intracelulares más utilizados para la vitrificación son el dimetilsulfóxido (DMSO), etilenglicol (EG), propilenglicol (PROH) y el glicerol (GL), debido a que presentan bajo peso molecular y penetran rápidamente a la célula. Estos CPAs disminuyen el punto de congelación, evitan la unión de los electrolitos que son capaces de unirse a la membrana, además de interaccionar con la membrana celular manteniendo su estructura (Brambillasca *et al*, 2013; Chian *et al*, 2014; Tharasanit *et al*, 2021). En los ovocitos y mórulas de cerdo el DMSO, EG y GL se desplazan por difusión simple, en los blastocistos expandidos por difusión facilitada. Dado que los blastocistos expandidos de cerdo expresan de manera abundante ARNm de acuaporina 3, esta podría estar involucrada en la difusión facilitada. En el caso del PROH se

mueve principalmente por difusión simple, independientemente de la etapa de desarrollo celular (Edashige, 2016).

El PROH y el DMSO son tóxicos a concentraciones más bajas que las requeridas para desnaturalizar proteínas y su uso individual aumenta el riesgo de daño (Fahy, 2016), por lo que se ha sugerido emplear mezclas de CPAs, ya que el uso de dos o más CPAs en las soluciones de vitrificación, reducen la toxicidad en la célula que el uso individual de los CPAs a las mismas concentraciones (Cuello *et al*, 2008; Brambillasca *et al*, 2013; Fahy, 2016).

La sacarosa y trehalosa son utilizados como CPAs impermeables o extracelulares, ya que tienen un peso molecular alto que les impide atravesar la membrana celular y promueven la rápida deshidratación por el aumento del gradiente de osmolaridad, ayudando de forma indirecta a la difusión del CPA permeable (Brambillasca *et al*, 2013; Tharasanit *et al*, 2021). Se han utilizado varios mono y disacáridos, siendo la sacarosa el azúcar más común para el calentamiento como contrafuerza osmótica para impedir la penetración excesiva de agua en el ovocito y con ello prevenir una lesión por hinchazón que conduce al posible rompimiento de la membrana o a la modificación de la estructura celular que puede repercutir en alteraciones del citoesqueleto (Chian *et al*, 2014).

Estudios realizados en el grupo de trabajo indican una optimización de la vitrificación empleando una mezcla de EG y DMSO a diferentes concentraciones como CPAs permeables y a la sacarosa como CPA impermeable (Casillas *et al*, 2014; 2015).

Sistemas de almacenamiento

Otro requerimiento importante para la vitrificación es el uso de sistemas de almacenamiento que permitan altas tasas de enfriamiento y calentamiento para reducir la formacion de cristales de hielo. La elección del dispositivo adecuado de acuerdo al tipo de muestra a vitrificar permitirá

garantizar la viabilidad e integridad durante el almacenamiento en el N_2L (Fahy y Wowk, 2015; Son *et al*, 2019).

Si la muestra está en contacto directo con el N_2L, los sistemas de almacenamiento se clasifican en dos tipos: sistema 'abierto' y 'cerrado'. Los sistemas abiertos alcanzan tasas de enfriamiento extremadamente altas debido al contacto directo con el N_2L , sin embargo, los riesgos de posible contaminación y la transmisión de enfermedades a través del N_2L aumentan cuando el almacenamiento es a largo plazo. De manera contraria, los sistemas cerrados pueden evitar el contacto directo con el N_2L y con ello reducir la posibilidad de contaminación de la muestra (Cai *et al*, 2018). En la Tabla 2 se muestran algunos sistemas de almacenamiento utilizados para la vitritficación y sus características.

Tabla 2. Sistemas de almacenamiento

Sistema de almacenamiento	Tipo de sistema	Volúmen	Tasa de enfriamiento (ºC/min)	Tasa de calentamiento (ºC/min)
Cryolock	Cerrado	< 0.1 µL	3,324	29,718
Cryotop	Abierto	< 0.1 µL	23,000	42,000
Cryoloop	Abierto	~ 0.5 µL	>20,000	45,000
CryoTip	Cerrado	-1 µL	12,000	24,000
OPS (*open pulled straw*)	Abierto	0.5-1 µL	17,700	13,900
Criotubos	Cerrado	1–5 mL	180-250	113

(Fahy y Wowk, 2015; Youm *et al*, 2017; Son *et al*, 2019).

El uso del ‘Cryolock’ ha ido en ascenso en la reproducción asistida debido a que es posible manejar volúmenes mínimos (< 0.1 µL), permitiendo la exposición a altas concentraciones de CPAs a volúmenes bajos, que representa ventaja durante la vitrificación de ovocitos y embriones

al influir en la eficiencia del enfriamiento para reducir el riesgo de toxicidad y de formación de cristales de hielo (Casillas *et al*, 2014).

1.4 Eficiencia de la vitrificación

La eficiencia y éxito de la vitrificación depende de factores técnicos y de factores propios del ovocito. Los primeros se relacionan con los diferentes protocolos, la naturaleza de los CPAs y los sistemas de almacenamiento a utilizar; los segundos están relacionados con la especie animal, sobre la etapa de desarrollo del ovocito (VG o MII) y la presencia o ausencia de las CC durante la realización de la técnica (Fahy, 2016; Ortiz-Escribano *et al*, 2016). Respecto al último factor, se han reportado algunas diferencias entre la vitrificación de ovocitos en VG o en MII y estas se deben principalmente a las características estructurales de cada etapa de maduración. La criopreservación de ovocitos en VG puede tener ventajas sobre aquellos en MII debido a que al no presentar un huso meiótico sensible a la temperatura y al contar con una membrana nuclear se podría proteger a la CR (Son *et al*, 2019), además de obtener mayores beneficios en los centros de reproducción asistida humana y en la producción animal al poder incrementar la cantidad de ovocitos colectados en esta etapa. Sin embargo, al presentar alto contenido de lípidos en la membrana celular puede ocasionar baja permeabilidad de los CPAs ocasionado por la alta concentración de colesterol que provoca rigidez de la membrana celular interfiriendo en la rehidratación celular durante el calentamiento del ovocito (Somfai *et al*, 2020) (Tabla 3).

Se ha descrito que la criopreservación de ovocitos en MII presenta varias dificultades debido a su gran tamaño, su alta sensibilidad a bajas temperaturas, son extremadamente frágiles, presentan alto contenido de agua, una baja relación superficie/volumen, la presencia del huso y otros orgánulos celulares, y que la membrana plasmática no cuenta con una permeabilidad

óptima a los CPAs y al agua (Konc *et al*, 2014). Sin embargo, la ventaja de vitrificar ovocitos en MII se debe principalmente a que no requieren de la comunicación entre el ovocito y las CC, ya que estos ya han obtenido los nutrientes y moléculas reguladoras para su maduración (Conti y Franciosi, 2018) (Tabla 3).

Tabla 3. Ventajas y desventajas de la vitrtificación de ovocitos en VG y MII según sus características estructurales y prácticas

Características	Etapa de VG	Etapa de MII
Estructurales		
Huso meiótico	(+) No presenta uso meiótico.	(-) Cromosomas unidos al uso meiótico.
Membrana nuclear	(+) Presencia, protege la cromatina.	(-) Sin presencia. Cromatina expuesta.
Membrana celular (contenido de lípidos)	(-) Alto contenido. Baja permeabilidad de CPAs.	(+) Alta permeabilidad de CPAs.
Células del cúmulo (uniones comunicantes)	(-) Integridad indispensable.	(+) No obligatoria.
Prácticas		
Facilidad de obtención de la muestra	(+) Recuperación de ovocitos en cualquier momento del ciclo menstrual o estral.	(-) Requiere estimulación ovárica.
Número de muestra	(+) Mayor número de muestra por ciclo.	(-) Número de muestra limitada por ciclo.

VG: vesícula germinal; MII: metafase II; (+): ventaja; (-): desventaja; CPAs: agentes crioprotectores.

Se ha mencionado que remover las CC podría mejorar la permeabilidad de los CPAs hacia el citoplasma del ovocito (Conti y Franciosi, 2018) y facilitar la adecuada hidratación y deshidratación durante la vitrificación disminuyendo la posibilidad de formación de cristales de hielo (Ortiz-Escribano *et al*, 2016); no obstante, se ha reportado que la presencia de estas células

protege al ovocito contra cambios osmóticos bruscos durante la eliminación de los CPAs en el calentamiento (Brambillasca *et al*, 2013).

Se presentan algunos estudios en diferentes especies comparando parámetros de competencia celular como la viabilidad, la maduración, la fertilización y el DE en relación a la etapa de maduración al momento de la vitrificación, además, se considera la presencia o ausencia de las CC que rodean al ovocito al momento de la vitrificación para el mejoramiento de los resultados (Tabla 4).

Tabla 4. Parámetros relacionados con la competencia celular de ovocitos vitrificados en VG o MII y en presencia o ausencia de las células del cúmulo

Modelo animal	Estado nuclear	Células del cúmulo Presencia (+) Ausencia (-)	Tasa de viabilidad (%)	Tasa de maduración (%)	Tasa de fertilización (%)	Tasa de desarrollo embrionario (%)	Referencia
Humano	VG MII	- -	94 91	51 83	NE	NE	Kasapi *et al*, 2017
Porcino	VG	+	78	40	31	33	López *et al*, 2021 b
Ovino	VG MII	+ - + -	NE	NE	NE	16 8 22 15 De 2 células	Dos Santos-Neto *et al*, 2020
Equino	VG MII MII	+ + -	79 79 83	44 46 20	NE	MII+ (0.03%) único con formación de un blastocisto	Ángel *et al*, 2020
Bovino	MII MII	+ -	83 94	NE	53 24	5 3 Blastocistos	Ortiz-Escribano *et al*, 2016

VG: vesícula germinal; MII: metafase II; (+): presencia; (-): ausencia; NE: no evaluado.

La vitrificación de ovocitos en MII presenta mejores resultados en términos de MIV, fertilización y DE, además, los parámetros mejoran cuando los ovocitos se encuentran rodeados o en presencia de las CC debido a que proporcionan a los ovocitos de nutrientes esenciales (AMPc, iones, piruvato, etc.) y precursores metabólicos (aminoácidos y nucleótidos) para la maduración meiótica, la ovulación y la fertilización, induciendo la capacitación espermática, la reacción acrosomal y la penetración del espermatozoide al ovocito (Casillas *et al*, 2020; Gershon y Dekel, 2020; Dos Santos-Neto *et al*, 2020; Turathum *et al*, 2021). No obstante, la vitrificación de ovocitos inmaduros (en VG) sigue siendo reto debido a las dificultades propias de las características de esta etapa y sus ventajas prácticas.

1.5 Daños estructurales causados por la vitrificación

1.5.1 Efecto de la vitrificación en las mitocondrias

Las mitocondrias son el orgánulo más estudiado en relación con la competencia del desarrollo del ovocito. Sus funciones esenciales en esta célula incluyen la síntesis de trifosfato de adenosina (ATP), homeostasis de Ca^{2+} y generación de especies reactivas de oxígeno (ERO). Se ha relacionado la calidad de los ovocitos con el número de mitocondrias, la distribución mitocondrial citoplasmática uniforme y el potencial de membrana mitocondrial (Reader *et al*, 2017; Gualtieri *et al*, 2021). Dado que las mitocondrias se localizan en áreas donde se requiere de señalización de ATP o Ca^{2+}, su reubicación durante el proceso de maduración puede ser crucial para la función de los ovocitos. Además, la distribución uniforme es importante antes de la escisión al garantizar que cada blastómero reciba suficientes mitocondrias para sobrevivir a la embriogénesis temprana (Van Blerkom *et al*, 2000; Reader *et al*, 2017).

En la cerda prepúber el ovocito presenta mitocondrias compartimentadas y con gránulos, en el ovocito pospúber, más competente, se describen como en forma de concha sin gránulos

(Pedersen *et al*, 2016). Los ovocitos maduros de vacas y cerdos con mayor potencial de desarrollo tienen más mitocondrias distribuidas uniformemente por todo el citoplasma que sus cohortes de mala calidad (Brevini *et al*, 2005). Los ovocitos de mala calidad se caracterizan por tener matrices más densas, la presencia de gránulos o vacuolas y la formación de mitocondrias encapuchadas (Reader *et al*, 2017).

La vitrificación de ovocitos de ratón puede alterar la distribución mitocondrial y reducir el potencial de membrana mitocondrial (Lei *et al*, 2014), además, reduce el contenido de ATP en ovocitos bovinos, humanos, conejos, murinos y porcinos (Gualtieri *et al*, 2021). Por el contrario, no se ha observado alguna alteración importante en la distribución mitocondrial entre los ovocitos humanos criopreservados antes y después de la MIV (Liu *et al*, 2017).

En la congelación lenta de ovocitos de ratón, tanto el EG como el PROH transportan Ca^{2+} del medio externo, sin embargo, el PROH aumenta de manera prolongada el Ca^{2+} intracelular provocando el endurecimiento de la zona pelúcida, la activación inadecuada de proteasas y fosfolipasas, degradación y apoptosis, por lo que se recomienda utilizar medios libres de Ca^{2+} durante esta técnica (Larman *et al*, 2007). El DMSO libera Ca^{2+} de las reservas internas del retículo endoplásmico aumentando sus niveles por exocitosis parcial de los gránulos corticales ocasionando el endurecimiento prematuro de la zona pelúcida (Larman *et al*, 2007; Gualtieri *et al*, 2011; 2021).

Las aberturas prolongadas del poro de transición de la permeabilidad mitocondrial debido a la sobrecarga de Ca^{2+} desencadenan la producción de ERO, la liberación de Ca^{2+}, la pérdida del potencial de membrana mitocondrial, disminución del contenido de ATP y la liberación de citocromo C, lo que puede ocasionar daño al ADN y apoptosis (Gualtieri *et al*, 2021). Esto se ha asociado con desregulación de genes relacionados con la apoptosis en ovocitos porcinos que fueron vitrificados (Dai *et al*, 2015).

1.5.2 Efecto de la vitrificación en la membrana plasmática

La estructura y composición de la membrana plasmática determinan los principales eventos celulares que tienen lugar durante la vitrificación debido a la dinámica los CPAs sobre esta. Su actividad durante el enfriamiento y calentamiento definirá los índices de supervivencia de la célula (Ávila-Portillo *et al*, 2006). La membrana plasmática tiene proteínas que pueden unirse a otros componentes membranales y a proteínas del citoplasma, como el citoesqueleto que está constituido por microfilamentos (MF), microtúbulos y filamentos intermedios que participan en la transducción de señales (Cereijido *et al*, 2003; Reece *et al*, 2011).

La membrana plasmática es una bicapa fosfolipídica de estructura dinámica, que continuamente incorpora o retira componentes de su conformación (lípidos y proteínas); por ejemplo, durante la diferenciación y el ciclo celular. La estructura de las colas de ácidos grasos de los fosfolípidos es importante para determinar la fluidez de la membrana. En condiciones normales, mayor concentración de colesterol en la membrana ocasiona rigidez y reduce la permeabilidad. A temperaturas bajas, las colas rectas de los ácidos grasos saturados pueden unirse estrechamente produciendo una membrana densa y rígida, sin embargo, en estas condiciones el aumento de la concentración de colesterol favorece la fluidez de la membrana al evitar el empaquetado hermético (Reece *et al*, 2011).

La membrana plasmática presenta canales iónicos que funcionan como poros acuosos para el intercambio de iones específicos, involucrados en la división celular, el reconocimiento celular y el equilibrio electroquímico (Cereijido *et al*, 2003). La membrana plasmática presenta, en menor proporción que los lípidos y las proteínas, carbohidratos que están presentes en la superficie externa, éstos están unidos a proteínas formando glicoproteínas o a lípidos formando glicolípidos (Reece *et al*, 2011). Se presenta cambio dinámico en la composición de los

triacilgliceroles, así como aumento en el metabolismo de los ácidos grasos en la membrana durante la MIV de ovocitos porcinos (Pirro *et al*, 2014).

La membrana plasmática es la estructura que sobrelleva el mayor daño durante la vitrificación debido a la pérdida de fluidez de sus componentes lipídicos, disminuyendo su capacidad de expansión durante la rehidratación, con ello, se considera que las etapas críticas para la supervivencia celular son la fase inicial del enfriamiento y el periodo de retorno a condiciones fisiológicas durante el calentamiento (Ávila-Portillo *et al*, 2006). Los ovocitos inmaduros porcinos presentan mayor contenido lipídico en comparación con los ovocitos maduros y embriones, así como los de otras especies (McEvoy *et al*, 2000). Lo anterior ocasiona que la permeabilidad de la membrana plasmática del ovocito se encuentre disminuida y sea difícil su vitrificación ante los CPAs, donde probablemente la alta concentración de colesterol esté jugando un papel importante (McEvoy *et al*, 2000; Somfai *et al*, 2012).

1.5.3 Efecto de la vitrificación en el citoesqueleto

La reorganización del citoesqueleto participa en la comunicación entre el ovocito y las CC durante la maduración del ovocito, en la fertilización en mamíferos (Bogliolo *et al*, 2015), así como en procesos como la citocinesis y la cariocinesis durante la división celular. Además, el citoesqueleto participa en el transporte intracelular y constituye al huso mitótico para la segregación de cromosomas y el desplazamiento de centriolos durante la mitosis (Benítez *et al*, 2003).

El citoesqueleto está formado por un complejo de estructuras constituidas por proteínas filamentosas presentes en la matriz citoplásmica de las células eucariótas, MF, microtúbulos, filamentos intermedios y una red microtrabecular (Benítez *et al*, 2003). Los MF son

componentes constituidos por actina, la proteína esquelética más importante en la mayoría de las células. La actina filamentosa es importante en la distribución de algunos organelos como el complejo de Golgi, el retículo endoplásmico liso y las mitocondrias, así como del posicionamiento espacial de RNAm (Bogliolo *et al*, 2015). El citoesqueleto de actina en los ovocitos de mamíferos es el sistema dinámico que dirige eventos durante la maduración y la fertilización, como el establecimiento de la polaridad de los ovocitos, la orientación, la migración y el desarrollo pronuclear del embrión (Cooper y Hausman, 2007).

Los microtúbulos son estructuras tubulares constituidas por repeticiones de dímeros de α- y β-tubulina. Participan en el transporte intracelular, movimiento de cilios y flagelos, constituyen el huso para la segregación de cromosomas y el desplazamiento de centriolos durante la mitosis (Benítez *et al*, 2003). Los microtúbulos y MF del citoesqueleto modulan el reordenamiento del retículo endoplásmico, regulando los canales iónicos, lo que ocasiona una mayor competencia en la generación de oscilaciones de Ca^{2+} intracelular, fundamentales para la activación del ovocito (Coticchio *et al*, 2015 a; Amdani *et al*, 2015).

Los filamentos intermedios son requeridos en diferentes etapas de la embriogénesis, en la organización nuclear, en la replicación del ADN, en el ensamble de la envoltura nuclear, para el transporte de colesterol, en la proliferación y migración celular (Benítez *et al*, 2003; Cooper y Hausman, 2007).

Durante la vitrificación de ovocitos porcinos, se ha observado mayor tasa de sobrevivencia de ovocitos vitrificados en MII que los vitrificados en VG. No obstante, los ovocitos vitrificados en VG presentan mayor integridad del huso y de la actina filamentosa cortical que los vitrificados en MII (Egerszegi *et al*, 2013). La vitrificación de ovocitos de ratas en VG provoca la pérdida del anillo perinuclear, así como aumento de actina globular en el citoplasma y una reducción de actina filamentosa en la zona cortical de los ovocitos (Kim *et al*, 2014). La

vitrificación de ovocitos de ovinos en MII altera la actina filamentosa cortical en los ovocitos expuestos únicamente a concentraciones de CPAs y vitrificados, sin diferencia entre ambos grupos. Con esto, se ha atribuido que el daño causado al citoesqueleto por la vitrificación se debe a la exposición a los CPAs más que al descenso de temperatura (Bogliolo *et al*, 2015). En ovocitos de cerdos en MII expuestos únicamente a 3 % de DMSO, puede alterar los husos y llevar a la desalineación cromosómica (Li *et al*, 2016). Sin embargo, en ovocitos de humanos vitrificados en MII, no hay diferencia en relación con el porcentaje de husos defectuosos y cromosomas entre la MIV de células frescas contra las vitrificadas (Lei *et al*, 2014). Se propone que 10 min de equilibrio post-vitrificación de ovocitos de ratón puede mejorar la configuración normal del huso meiótico y de los cromosomas (Jung *et al*, 2014).

1.5.4 Efecto de la vitrificación en la cromatina

La CR está formada por ADN eucariótico y proteínas, principalmente histonas (proteínas básicas ricas en arginina y lisina), además de una variedad de proteínas cromosómicas diferentes a las histonas. Existen 5 tipos importantes de histonas: la H1, H2A, H2B, H3 y H4 (Benítez *et al*, 2003; Cooper y Hausman, 2007). Morfológica y fisiológicamente se distinguen dos tipos de CR, la eucromatina y la heterocromatina. La primera tiene aspecto claro y está poco condensada, suele corresponder a regiones del ADN transcripcionalmente activas por lo que realiza una mayor expresión génica, se presenta mayormente en células en interfase. La heterocromatina, tiene aspecto oscuro y se encuentra muy condensada, en su mayoría es transcripcionalmente inactiva, sólo alrededor del 10 % de la CR en interfase es de este tipo, estando relacionada mayormente con células en mitosis. La heterocromatina a su vez se divide en facultativa (que en algún momento del ciclo celular también puede ser eucromatina), y en constitutiva, a la que se atribuye una función mayormente estructural (Cooper y Hausman, 2007).

Las configuraciones específicas de la CR son importantes para la reanudación meiótica y la competencia del desarrollo del ovocito. La integridad genética y los cambios que ocurren en el compartimento citoplásmico tienen implicaciones importantes para la maduración y la calidad de los ovocitos. Los MF, microtúbulos y la CR interactúan en la segregación de cromosomas y en el establecimiento de la asimetría celular que permite la extrusión del cuerpo polar con mínima pérdida de citoplasma (Coticchio *et al*, 2015a).

1.6 Estrategias para reducir los daños causados durante la criopreservación

La implementación de estrategias para mejorar los parámetros de competencia de ovocitos y embriones vitrificados, la mayoría se ha enfocado en la suplementación de medios de vitrificación, de calentamiento, de equilibrio pre/post-vitrificación, de MIV y de DE (Tabla 5).

Tabla 5. Suplementación de medios como estrategia para incrementar la eficiencia de la vitrificación en ovocitos y embriones

Suplementación de medios de cultivo	Modelo animal	Estado de desarrollo	Resultados	Referencias
Resveratrol (2 µM) en medio de pre-incubación, vitrificación, calentamiento y pos-calentamiento	Ratón	Ovocitos MII	Mejoró la viabilidad (94 vs 92%), fertilización (64 vs 43 %) y formación de blastocistos (68 vs 50 %).	Chen *et al*, 2019
Melatonina (10^{-7} mol/L) en medio de MIV	Ratón	Ovocitos VG	Aumentó la MIV, el potencial de membrana mitocondrial, distribución del huso meiótico y contenido de ATP y GSH. Disminuyó el porcentaje de ERO.	Wu *et al*, 2019
N-acetilcisteína (pre y post vitrificación)	Ratón	Ovocitos MII	Suplementación post-vitrificación, tasa de blastocistos similar al grupo control.	Matilla *et al*, 2019

IGF-I (100 ng.mL) en medio de MIV, Glutation (2 mM) en medio de calentamiento	Porcino	Ovocitos MII	IGF-I y GSH aumentaron las tasas de viabilidad.	Pereira *et al*, 2019 a
Resveratrol (0.5 µM) en medio de desarrollo embrionario	Bovino	Embriones tempranos (2 células)	Mejoró el desarrollo embrionario. Redujo número de mitocondrias dañadas y los niveles de ERO.	Hara *et al*, 2018

VG: vesícula germinal; MII: metafase II; MIV: maduración *in vitro*; ATP: adenosín trifosfato; GSH: glutatión; ERO: especie reactiva de oxígeno; IGF-I: factor de crecimiento parecido a la insulina I.

Entre los compuestos más utilizados para la suplementación se encuentran antioxidantes como el resveratrol (Hara *et al*, 2018; Chen *et al*, 2019) y factores de crecimiento (Pereira *et al*, 2019a), estos compuestos han mejorado parámetros de competencia celular como la viabilidad, la MIV, la fertilización y el DE. La mayoría de los estudios evalúan la influencia de la suplementación en la disminución de la producción de ERO (Wu *et al*, 2019; Hara *et al*, 2018), ya que puede ser una fuente importante de daño, en particular del ADN. La generación de ERO, es un problema inherente del cultivo celular y por la exposición a los CPAs durante la vitrificación.

Otras estrategias están enfocadas en modificaciones de los protocolos de vitrificación y de cultivo de células de la granulosa. Como el empleo de un co-cultivo de CC frescas para la MIV de ovocitos porcinos y ovinos después de la vitrificación de COCs, el cual mejora la tasa de MIV y DE en comparación con la ausencia de dicho co-cultivo (Casillas *et al*, 2014; Casillas *et al*, 2020; Dos Santos-Neto *et al*, 2020). Aún se requieren estudios para determinar el mecanismo de la participación de las CC durante el co-cultivo. Se han propuesto protocolos de vitrificación específicos para las células de la granulosa y CC (Pereira *et al*, 2019b) similares a la criopreservación de células somáticas, con concentraciones más bajas de CPAs y temperaturas más altas que las utilizadas para ovocitos y embriones. Así mismo, protocolos de

criopreservación para cultivos primarios de células de la granulosa, como la nucleación controlada del hielo, aunque los resultados solo han sido probados en la viabilidad celular (Daily *et al*, 2020). Otros más plantean el uso de compuestos naturales para la sustitución de CPAs (Alfoteisy *et al*, 2020) y la eliminación de CPAs permeables (Pereira *et al*, 2019b) que aunque se produce fragmentación severa del ADN de las CC periféricas, estas protegen a los ovocitos contra la fragmentación del ADN, lo que destaca la necesidad del uso de CPAs (Tabla 6).

Tabla 6. Estrategias para mejorar la eficiencia de la vitrificación

Estrategia	Modelo animal	Tipo celular	Resultados	Referencias
Co-cultivo de CC frescas para la MIV y FIV	Ovino	MI y MII	Mayor tasa de desarrollo embrionario de 2 células en co-cultivos (MI 14%, MII 28%).	Dos Santos-Neto *et al*, 2020
Nucleación controlada del hielo como técnica de criopreservación	Bovino	Cultivos primarios de CG	Nucleación controlada presentó alta tasa de viabilidad post-descongelación (58%)	Daily *et al*, 2020
Compuesto natural (miel) como CPA no permeable	Bovino	COCs VG	Porcentaje de blastocistos mayor (13%) que vitrificación con sacarosa, 0.5 M (3%).	Alfoteisy *et al*, 2020
Vitrificación libre de CPAs permeables (con sacarosa, 1 M)	Equino	COCs VG	Aumento de la tasa de fragmentación total del ADN de las CC (21%).	Pereira *et al*, 2019b
Vitrificación de CG	Equino	CG	Sin diferencia significativa en la fragmentación del ADN entre protocolos. P1= - 196 ºC / -80 °C (7%) P2= - 196 ºC/ -80 °C + CPAs (7.5% de EG + DMSO) (8%) P3= - 80 °C (7%) P4 = - 80 °C + CPAs (8%) Control (7%)	Pereira *et al*, 2019c

VG: vesícula germinal; MI: metafase I; MII: metafase II; CC: células del cúmulo; CG: células de la granulosa; COCs: complejos ovocitos-células del cúmulo; MIV: maduración *in vitro*; FIV: fertilización *in vitro*; CPA: agente crioprotector; EG: etilenglicol; DMSO: dimetilsulfóxido.

1.7 Fertilización *in vitro*

Algunas de las TRA importantes y más utilizadas, tanto para la reproducción asistida humana como para la producción animal, son la FIV convencional, la inyección intracitoplasmática de espermatozoide (ICSI; por sus siglas en inglés) y la inyección fisiológica intracitoplasmática de espermatozoide (PICSI; por sus siglas en inglés). Aunque estas dos últimas han ido en aumento, la FIV convencional sigue siendo indispensable en laboratorios de reproducción asistida, ya que no requiere de equipo costoso ni sofisticado, sino de la obtención de una muestra buena de ovocitos y espermatozoides para interactuar en condiciones de cultivo (Tanihara *et al*, 2013; Kitaji *et al*, 2015).

En la especie porcina, se ha presentado más eficiencia en la FIV (81%), sin embargo, gran parte es polispérmica (42%) (Abeydeera *et al*, 2000), siendo de los principales obstáculos durante la FIV en porcinos. Esto se ha atribuído al empleo del número excesivo de espermatozoides (100 000 espermatozoides/mm^3, por 300-400 mL de eyaculado; dilución final de la muestra para inseminar de $10x10^5$ espermatozoides/mL), condiciones subóptimas de la MIV y FIV, así como la penetración simultánea de los espermatozoides y el retraso de la reacción cortical de la zona pelúcida (Sano *et al*, 2010; Kitaji *et al*, 2015), por lo que se ha reducido la concentración de espermatozoides para la FIV para disminuir la polispermia, pero a su vez, se disminuye la tasa de FIV, por lo que se puede considerar que la cantidad de espermatozoides capacitados en el sitio de la fertilización es importante para reducir la incidencia de polispermia en el porcino (Kitaji *et al*, 2015). La alternativa a este problema ha sido la implementación de agitación constante de la dilución final de espermatozoides para la inseminación (simulación de las condiciones oviductales *in vivo*), así como del uso de clasificador espermático microfluídico

para aislar espermatozoides móviles mediante flujo laminar (sin centrifugación) (Sano *et al*, 2010; Tanihara *et al*, 2013; Kitaji *et al*, 2015).

1.8 Las células del cúmulo

Las CC son un grupo de células de la granulosa que se encuentran rodeando al ovocito y aquellas en contacto directo se denominan como corona radiada. Estas son las encargadas de proporcionar los nutrientes esenciales (monofosfato de adenosina cíclico, AMPc; iones, piruvato, etc.) a los ovocitos para concluir la primera división meiótica y para las etapas de desarrollo posteriores como la fertilización y el DE, además, regulan el mecanismo de detención y reanudación meiótica de los ovocitos. Por lo que, garantizar y mantener la viabilidad de estas células es fundamental para la maduración de los ovocitos después de la vitrificación (Betancourt *et al*, 2003; Casillas *et al*, 2020). La comunicación entre las CC y el ovocito está establecida por la comunicación intercelular por uniónes gap o también llamadas uniones comunicantes, estas uniones están directamente involucradas en la detención y reanudación meiótica de los ovocitos (Casillas *et al*, 2020).

Los folículos primordiales en los ovarios de mamíferos están formados por un ovocito detenido en la profase de la primera división meiótica, rodeado por una sola capa de células pregranulosas. Las células foliculares emiten proyecciones citoplásmicas hacia el ovocito estableciendo uniones comunicantes. Al final del período de crecimiento del folículo terciario, el ovocito deja de crecer y permanece rodeado por varias capas de células foliculares conocidas a partir de ese momento como CC, formando el complejo ovocito-células del cúmulo o también llamado COC (Casillas *et al*, 2020).

El ácido hialurónico de manera natural se encuentra en el moco cervical del tracto reproductor femenino durante la ovulación y también es sintetizado por las CC, es indispensable para la maduración del ovocito y el DE (Parmegiani *et al*, 2010; Appeltant *et al*, 2015, Casillas *et al*, 2015).

1.8.1 Importancia de las células del cúmulo en la maduración *in vitro*

Durante la ovogénesis, los ovocitos adquieren gradualmente la maduración nuclear y citoplasmática durante su crecimiento. La competencia meiótica, que es la capacidad de los ovocitos para reanudar la meiosis y alcanzar la madurez nuclear, se adquiere durante la foliculogénesis y está relacionada con la formación del antro, donde los ovocitos alcanzan aproximadamente el 80 % de su tamaño final. El desarrollo de la competencia del ovocito está relacionado con la maduración citoplasmática y se refiere a su capacidad para ser fecundado y desarrollar un embrión capaz de continuar su desarrollo a término y producir un individuo vivo. La maduración citoplasmática se adquiere después de que el ovocito es meióticamente competente (Casillas *et al*, 2020).

El ovocito que ha adquirido competencia meiótica no necesariamente adquiere maduración citoplásmica. La maduración meiótica implica varios procesos en cascada, iniciada por el aumento de LH (hormona luteinizante; por sus siglas en inglés), promoviendo el progreso del ovocito a MII y a la extrusión del primer cuerpo polar. Después del pico de LH, las CC producen ácido hialurónico en respuesta a las gonadotropinas y ocurre la expansión de estas células foliculares para la reanudación de la meiosis (Casillas *et al*, 2020).

Las CC que rodean al ovocito son las responsables de la detención meiótica mediante la producción y liberación de moléculas como AMPc o purinas como adenina e hipoxantina en el

citoplasma del ovocito. La inhibición de estas moléculas causado por la separación de las CC puede transmitirse al ovocito a través del líquido folicular a través de las uniones comunicantes. Los altos niveles de AMPc, suministrados principalmente por las CC, permiten que el ovocito se mantenga en la profase I y solo se libere del efecto inhibidor cuando se interrumpe la comunicación celular y los niveles de AMPc disminuyen. Esto inactiva a la proteína cinasa dependiente de AMPc que llevará a la activación de una fosfatasa que desfosforila al factor promotor de la maduración. Posteriormente, las gonadotropinas se unen a sus receptores en las CC produciendo una disminución en la concentración de AMPc en el ovocito resultando en la reanudación meiótica (Casillas *et al*, 2020).

1.8.2 Las células del cúmulo y el daño en el ADN causado por la vitrificación

Diversos estudios han reportado daños causados por la vitrificación en ovocitos y embriones, la mayoría de ellos a nivel estructural en organelos celulares y en años más recientes se ha realizado el análisis de la expresión génica (Tabla 7).

Tabla 7. Estudios de evaluación de daño en ovocitos y embriones vitrificados

Enfoque del estudio	Modelo animal	Estado de desarrollo	Resultados	Referencia
Análisis de la expresión génica	Porcino	Mórulas	Genes expresados diferencialmente (DEG) regulados a la alza: involucrados en la degradación de glicosaminoglicanos, las vías metabólicas y las vías KEGG del metabolismo del triptófano. DEG regulados a la baja: involucrados en la glucólisis/gluconeogénesis, la exportación de proteínas y la elongación de ácidos grasos.	Cuello *et al*, 2021

Análisis del citoesqueleto	Porcino	COCs VG	Disminuyó la viabilidad celular, MIV, desarrollo embrionario temprano y número de embriones con actina cortical.	López *et al*, 2021 b
Análisis de la cromatina	Porcino	COCs VG	Mayor producción de embriones con cromatina dispersa o sin desarrollo embrionario.	López *et al*, 2021 b
Análisis mitocondrial	Ratón	Ovocitos MII	Baja polarización mitocondrial y disminución de la tasa de blastocistos y blastómeros. No afectó los niveles de ERO.	Matilla *et al*, 2019
Análisis del huso meiótico	Porcino	Ovocitos MII	Exposición única al DMSO (3%) alteró los husos y la alineación cromosómica.	Li *et al*, 2016

DMSO: dimetilsulfóxido; COCs: complejos ovocitos-células del cúmulo; VG: vesícula germinal; MII: metafase II; MIV: maduración *in vitro*; ERO: especie reactiva de oxígeno.

Sin embargo, pocos estudios que investigan algún tipo de daño causado por la criopreservación en las células de la granulosa o en las CC causados por la vitrificación de los COCs (Tabla 8), debido a que las células de interés son principalmente el ovocito y el embrión, no obstante, es importante considerar a las CC debido a la participación que tienen durante el desarrollo celular.

Tabla 8. Tipos de daños en las células del cúmulo causados por la vitrificación

Enfoque del estudio	Modelo animal	Estado nuclear COCs	Resultados	Referencias
Daño al ADN	Porcino	MII	Viabilidad de CC menor que de ovocitos. Disminuyó las tasas de fertilización, desarrollo embrionario y blastocistos.	López *et al*, 2021 c
Daño en uniones comunicantes	Porcino	VG	Alteración de las uniones comunicantes entre los ovocitos-CC, redujo las tasas de MIV.	Casillas *et al*, 2020
Apoptosis	Porcino	VG	En las CC aumentó cascada apoptótica (por la vía de CASP3) por exposición a CPAs. Regulación positiva del gen antiapoptótico Bcl-XL en embriones.	Somfai *et al*, 2020
Expansión de las CC	Camello	VG	Menor expansión en las CC de COCs vitrificados vs grupo control.	Fathi *et al*, 2018

Maduración nuclear y citoplásmica	Porcino	VG	Progresión meiótica y maduración nuclear prematuras. Maduración citoplásmica procedió normalmente.	Appeltant *et al*, 2017

VG: vesícula germinal; MII: metafase II; CC:células del cúmulo; COCs: complejos ovocitos-células del cúmulo.

Para la evaluación del daño en el ADN se ha utilizado la electroforesis unicelular o también nombrado ensayo cometa de dos dimensiones. En éste se realizan dos tipos de electroforesis, la primera es una electroforesis neutra, con ella se producen desplazamientos a la derecha para la detección de rompimientos en el ADN de doble hebra (ds-ADN) y la segunda electroforesis es de tipo alcalina, con ella se realiza el desplazamiento de manera perpendicular a la primera electroforesis para la detección de rompimientos en el ADN de una sola hebra (ss-ADN) (Barcena *et al*, 2015). Con esta técnica se contemplan dos tipos de rdaños sobre el ADN pudiendo diferenciar la presencia de uno sobre el otro. Es importante mencionar que también se puede realiza la electroforesis unicelular alcalina y neutra de manera separada.

Otros estudios, incluyen la evaluación del daño del ADN por medio de la prueba de dispersión de la CR o también llamado 3D-Max en la que se puede medir el grado de daño celular. Así mismo, la prueba “In Situ Nick Translation” detecta la fragmentación del ADN por medio de la incorporación de nucleótidos marcados con fluoróforo, que mediante fluorescencia es posible detectar los dos tipos de rompimientos en el ADN. Ambos métodos, 3D-Max y “In Situ Nick Translation” utilizan el principio de la electroforesis (Barcena *et al*, 2015).

La vitrificación de COCs en VG de porcinos, no desencadena la fragmentación del ADN en los ovocitos en VG y en los posteriores embriones, sin embargo, en las CC aumenta la cascada apoptótica en aquellas que fueron expuestas a CPAs (Somfai *et al*, 2020) ocasionando muerte

celular, sin embargo, podemos inferir que las CC son mayormente afectadas durante la vitrificación de COCs pero que podrían ayudar a proteger al ovocito durante este proceso.

Se han presentado algunas alternativas para la reducción del daño de las células de la granulosa causados por la vitrificación, como el empleo de co-cultivo de células de la granulosa para la MIV y protocolos de vitrificación específicos para estas células foliculares (Tabla 6).

II. ANTECEDENTES

En los últimos años se han desarrollado diversos métodos de criopreservación a través de la vitrificación de ovocitos inmaduros (Casillas *et al*, 2018) y maduros (López *et al*, 2021c) así como para embriones en diferentes etapas de desarrollo. En general, los estudios han demostrado que los embriones tienen mayores probabilidades de supervivencia después de la vitrificación que los ovocitos inmaduros y maduros.

La vitrificación de ovocitos inmaduros en mujeres es conveniente debido a que se pueden obtener en cualquier fase del ciclo menstrual para posteriormente realizar la maduración *in vitro* y así evitar la hiperestimulación ovárica. Además, en otras especies de mamíferos, es posible recuperar un mayor número de ovocitos en VG que en MII. Para evaluar la calidad de los embriones producidos *in vitro* a partir de ovocitos inmaduros vitrificados, deben considerarse varios aspectos durante la maduración de los ovocitos, la fertilización y el DE, el uso de CPAs, sistemas de almacenamiento, procedimientos para el calentamiento y el empleo de co-cultivos de ovocitos con CC frescas (Casillas *et al*, 2015; Kopeika *et al*, 2015; de Munk y Vajta, 2017). Se han reportado algunos nacimientos vivos derivados de ovocitos inmaduros vitrificados y aunque hay avance en el conocimiento de la vitrificación de los ovocitos, la tasa de embriones

que alcanzan a desarrollarse a las etapas de mórula y de blastocisto permanece baja (Somfai *et al*, 2010).

Es importante resaltar que se ha evaluado el efecto de la vitrificación de ovocitos en VG y MII, cigotos y blastocistos mostrando resultados en la misma etapa de desarrollo en el que fueron vitrificados, sin embargo, aún es poco claro si los efectos producidos por la vitrificación de ovocitos en VG o en MII seguirán presentes durante las siguientes etapas de desarrollo y si repercutirán en la formación de alteraciones estructurales irreversibles que puedan comprometer la culminación del DE pre-implantacional. Por lo que en este estudio, el efecto de la vitrificación de ovocitos se evaluó en el posterior DE temprano. Estudios en cigotos y blastocistos analizan el papel de los MF de actina y la CR con base en el éxito de la fertilización y la producción de embriones (Wu *et al*, 2006; Somfai *et al*, 2010; Egerszegi *et al*, 2013), sin embargo, la distribución y las características morfológicas de estas estructuras en los blastómeros durante el DE temprano no ha sido evaluada. Por tanto, el presente estudio proporciona información importante que revela el daño causado por la vitrificación en ovocitos inmaduros y su posterior DE temprano.

Se ha realizado la evaluación del huso meiótico en ovocitos y mostrando alineamiento anormal de los cromosomas en la placa ecuatorial después de ser vitrificados (79.5 % frescos vs. 10.1 % VG vitrificados) (Wu *et al*, 2006). El porcentaje de ovocitos en VG y MII con distribución normal de actina filamentosa se reduce después de la vitrificación (72.3 % frescos vs. 16.9 y 37.2 % vitrificados en VG y MII, respectivamente) lo que sugiere que el daño irreversible al citoesqueleto de ovocitos porcinos en VG y MII después de la vitrificación podría ser un factor importante que afecte la competencia durante el desarrollo (Wu *et al*, 2006).

Se ha reportado que la exposición de los blastocistos porcinos a los CPAs sin enfriamiento aumenta la fragmentación del ADN de los núcleos y disminuye la tasa de supervivencia (Rajaei

et al, 2005), con lo que el daño citotóxico se puede adjudicar a los CPAs. De manera contraria, se ha indicado que la vitrificación de blastocistos humanos de quinto día de desarrollo no afecta la tasa de supervivencia y solo 4 % de los blastocistos frescos presentan algún tipo de conformación aberrante (anormal, multipolar y monopolar) en el huso mitótico, en comparación con 22 % de blastocistos vitrificados. Esto está asociado con la formación de cromosomas rezagados o falla en la unión a los cinetocoros de los cromosomas que puede llevar a la generación de aneuploidias (Chatzimeletiou *et al*, 2012).

Las CC desempeñan varias funciones importantes en la maduración y fertilización de los ovocitos (Van Soom *et al*, 2002), así mismo, se han propuesto como biomarcadores de la calidad de los ovocitos (Ekart *et al*, 2013). Se ha informado que los ovocitos vitrificados en MII rodeados con las CC dieron lugar a tasas de FIV más altas en comparación con los ovocitos desnudos (Kohaya *et al*, 2011), así como a mayores tasas de escisión, formación de blastocistos y eclosión que aquellos que se vitrificaron desnudos, además, se observó que un co-cultivo de CC frescas para la MIV mejora estos parámetros, incluso comparados a los que se vitrificaron en MII rodeados de las CC, esto en un modelo ovino (Dos Santos-Neto *et al*, 2020).

Las CC protegen a los ovocitos en MII contra el endurecimiento de la zona pelúcida y el daño citoplasmático durante el calentamiento por vitrificación (Tharasanit *et al*, 2009). Además, las CC previenen el daño criogénico de los ovocitos al preservar la estructura de los principales orgánulos después de la vitrificación (Taghizabet *et al*, 2018). Estas células se exponen en primer lugar a los CPAs, lo que previene el choque osmótico, facilita la deshidratación de los ovocitos y reduce el daño en ellos (Tharasanit *et al*, 2009). Debido a esto, la viabilidad de las CC disminuye considerablemente después de la vitrificación de los COCs (Tharasanit *et al*, 2009; Jin *et al*, 2012; Casillas *et al*, 2014). Por el contrario, otros estudios apoyan que la vitrificación de los COCs reduce la penetración de los CPAs conduciendo a la inadecuada

deshidratación, con lo que aumenta la formación de cristales de hielo en los ovocitos y disminuye su viabilidad (Ortiz-Escribano *et al*, 2016). Sin embargo, la información sobre los daños, en particular del ADN, que produce la vitrificación en las CC porcinas usados como modelo de investigación, es limitada.

Dado que la evaluación directa de los ovocitos mediante métodos invasivos puede perjudicar su desarrollo, el análisis de las CC puede reflejar la competencia del desarrollo de los ovocitos. La comunicación bidireccional entre las CC y el ovocito es necesario para el desarrollo y las funciones de ambos tipos celulares (Su *et al*, 2009). Los ovocitos influyen en el desarrollo y proliferación de las células foliculares mediante factores paracrinos y modulan las actividades metabólicas por promoción de la expresión génica (Su *et al*, 2009). Por lo tanto, evaluar si la vitrificación es capaz de generar daño al ADN en las CC es de gran importancia para la fertilización del ovocito y el DE. Se ha reportado que la vitrificación de COCs de equino genera mayor daño al ADN en las CC periféricas que en las internas, debido a que son las que se encuentran en primer contacto con los CPAs (Pereira *et al*, 2019b). Además, se ha informado de daños en el ADN de las CC generados después de la criopreservación en humanos (Lindley *et al*, 2001; Raman *et al*, 2001), bovinos (Urrego *et al*, 2005) y equinos (Pereira *et al*, 2019b; Tharasanit *et al*, 2009), sin embargo, es necesario indargar en el análisis en porcinos.

III. PREGUNTAS DE INVESTIGACIÓN

- ¿La vitrificación de ovocitos porcinos en VG repercutirá en el DE al producirse embriones con alteraciones a nivel estructural en los MF y la CR?
- ¿El daño al ADN de las CC causado por la vitrificación de COCs maduros es causado por los CPAs o es derivado por el descenso de la temperatura durante la vitrificación?

IV. HIPÓTESIS

La desorganizacion de la distribución cortical de los MF y de la distribución anormal de la CR de embriones derivados de ovocitos vitrificados en VG afectará el potencial del desarrollo y el DE temprano.

Dado que la vitrificación de ovocitos puede alterar la organización de estructuras como los MF y la CR, la vitrificación de COCs maduros dañará el ADN de las CC debido a la exposición directa de estas células a las concentraciones de CPAs.

V. OBJETIVOS GENERALES

Determinar las alteraciones en la estructura de los MF y la CR inducidas por la vitrificación de ovocitos porcinos en VG y su repercusión en el DE temprano.

Determinar el grado del daño en el ADN que la exposición a los CPAs puede causar en las CC de COCs maduros vitrificados.

VI. OBJETIVOS PARTICULARES

- Evaluar el efecto de la vitrificación en las tasas de viabilidad, MIV, FIV y DE.
- Evaluar el efecto de la vitrificación a nivel estructural en MF y CR en embriones tempranos (2-8 células).
- Evaluar el daño del ADN de las CC de COCs maduros expuestos únicamente a concentraciones de CPAs y vitrificados.

VII. METODOLOGÍA

Al menos que se indique lo contrario, los reactivos utilizados corresponden a la marca Sigma Aldrich Chemical Co. (EUA).

7.1 Diseño experimental

Las muestras de ovarios se obtuvieron de cerdas prepúberes sacrificadas en el rastro, fueron transportadas al laboratorio en solución salina de NaCl 0.15 M a 25 °C. Después de la selección de los COCs (descrito en la sección 7.2) se dividieron en: 1) grupo control: ovocitos frescos en VG para MIV, fertilizados mediante FIV y llevados hasta DE temprano y 2) grupo experimental, vitrificación: ovocitos vitrificados en VG que después del calentamiento se sometieron a MIV, FIV y posteriormente llevados hasta DE temprano.

Las evaluaciones primarias se realizaron en ambos grupos. Se realizó la evaluación morfológica de ovocitos y embriones mediante microscopio estereoscópico, la viabilidad por tinción con MTT y el análisis de desarrollo celular como la MIV, FIV y el DE temprano por tinción con Hoechst. Posteriormente, se realizaron las evaluaciones secundarias en el DE temprano de ambos grupos, que consistieron en el análisis de la distribución de MF por ensayo de inmunofluorescencia con isotiocianato de fluoresceína (FITC) conjugada con faloidina y de CR por tinción con Hoechst. En la evaluación de MF el número total de embriones evaluados en los grupos control y vitrificación fue 61 y 35, para la evaluación de CR de 64 y 33, respectivamente. El análisis estadístico se realizó en GraphPad Prism 8.2.1 (Graphpad Software Inc.) mediante la prueba no paramétrica de U de Mann-Whitney, con nivel de confianza cuando $p < 0.05$ para la diferencia de los datos de los grupos control y vitrificación. Los valores porcentuales fueron presentados como la media ± desviación estándar (DS) El resumen del diseño experimental se puede apreciar en la Figura 2.

Para el análisis del daño al ADN de las CC de los COCs que fueron vitrificados en una etapa madura (MII), tanto en el grupo control como de vitrificación se consideraron cuatro variables de evaluación:

a) Control (negativo al daño)

b) H_2O_2 (positivo al daño)

c) Toxicidad (expuestos únicamente a concentraciones de CPAs)

d) Vitrificación (vitrificados)

Se analizó el total de 928 células entre los cuatro grupos evaluados. El análisis estadístico de los grupos control y vitrificados, tanto de la viabilidad como del ensayo cometa se trataron como datos no paramétricos, posteriormente se analizaron mediante ANOVA seguido de un análisis de Duncan con una $p < 0.05$ para todas las comparaciones (Figura 2).

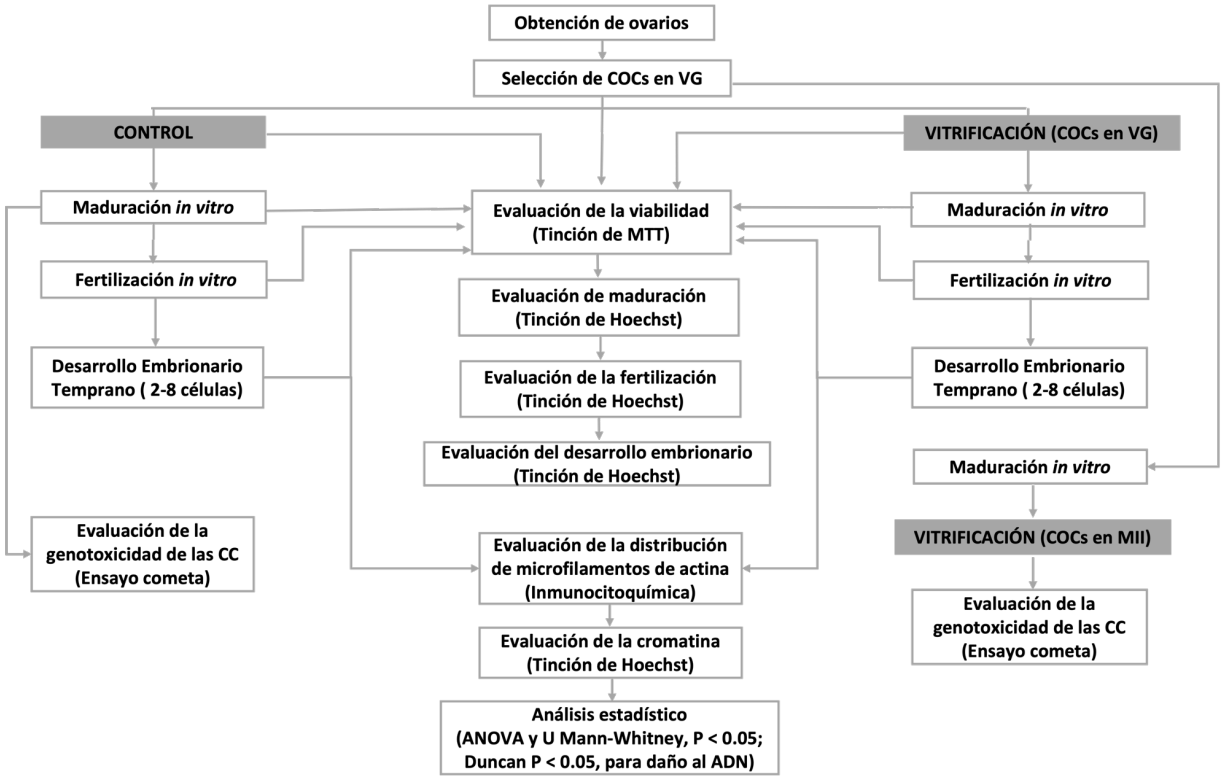

Figura 2. Diseño experimental de la vitrificación de ovocitos porcinos y su efecto a nivel estructural en embriones y en el ADN de las CC.

7.2 Selección de complejos ovocito-células del cúmulo

Se seleccionaron y puncionaron los folículos ováricos con diámetro de 3 a 6 mm para recuperar el líquido folicular, se sedimentó durante 20 min para obtener el paquete celular donde están presentes los COCs y se realizaron dos lavados de 15 min cada uno con medio TL HEPES-PVA 10 mM, suplementado con lactato de sodio 10 mM y 0.1% de alcohol polivinílico a pH de 7.3-7.4. Posteriormente, el paquete celular se colocó en cajas Petri para la colecta de los COCs bajo microscopio estereoscópico. Se seleccionaron los que presentaban ovocitos en VG con citoplasma uniforme y rodeados por al menos tres capas de CC (Ducolomb *et al*, 2005).

7.3 Vitrificación

En una caja Petri de cuatro pocillos (NUNC, EUA), en los pocillos 1 y 2 se agregaron 850 µL de medio base "Holding Medium" (HM, por sus siglas en inglés; HEPES al 0.59%, PVA al 0.1% y glutamina al 0.2 M en medio TCM-199) suplementado con sacarosa al 28.5 %. En el pocillo 3, para el primer medio de vitrificación, se agregaron 850 µL de HM, 75 µL de DMSO al 99.5 % y 75 µL de EG al 99 %. Finalmente, en el pocillo 4, para el segundo medio de vitrificación se añadieron 660 µL de medio sacarosa 0.4 M, 20 µL de HM, 160 µL de DMSO y 160 µL de EG. La caja se incubó al menos por 2 h para equilibrar el pH (Casillas *et al*, 2014).
Se transfirieron de 6 a 8 COCs a los pocillos 1 y 2, la exposición al pocillo 3 fue de 3 min y la exposición al pocillo 4 no fue mayor a 1 min. Finalmente fueron colocados en el Cryolock que fue sumergido de horizontalmente en el N_2L para el almacenamiento durante 30 min (Sánchez-Osorio *et al*, 2010; Casillas *et al*, 2014).

7.4 Calentamiento

Se retiraron los Cryolock del N_2L y se sumergieron verticalmente en una caja de cuatro pocillos que contenía una única concentración de sacarosa al 0.4 M en medio TCM-199, los COCs se expusieron a esta concentración durante 5 min (Sánchez Osorio *et al*, 2010; Casillas *et al*, 2014).

7.5 Maduración *in vitro*

Después de la colecta en el grupo control y después del calentamiento en el grupo vitrificación, los COCs se transfirieron a una caja Petri de cuatro pocillos con medio de maduración (TCM-199 con sales de Earle, suplementado con bicarbonato de sodio 26.2 mM, PVA al 0.1%, D-glucosa 3.05 mM, piruvato de sodio 0.91 mM, cisteína 0.57 mM y 10 ng/ml de factor de crecimiento epidérmico; In Vitro, México), suplementado con 5 µL (0.5 µg/mL) de hormona luteinizante (LH, por sus siglas en inglés; núm. Catálogo L5269) y 5 µL (0.5 µg/mL) de hormona folículo estimulante (FSH, por sus siglas en inglés; núm. Catálogo F2293). La incubación se realizó a 38.5 °C con 5 % de CO_2 y humedad a saturación durante 44 h (Ducolomb *et al*, 2005).

7.6 Fertilización *in vitro*

Posterior a la MIV, los ovocitos fueron denudados por acción mecánica con ayuda de una micropipeta de 100 µL. Para la FIV, se colocaron de 40 a 50 ovocitos en gotas de 50 µL de medio TBMm (medio de FIV) cubiertas con aceite mineral e incubados a 38.5 °C con 5 % de CO_2 y humedad a saturación por 1 h hasta la inseminación (Ducolomb *et al*, 2005).

La muestra de semen se obtuvo de verraco mediante el método de la mano enguantada, en una granja comercial, la muestra se diluyó 1:10 con diluyente de semen (MR-A, Kubus, S.A., UE). Se transportó al laboratorio a una temperatura de 16 °C. Cinco mL de la muestra de semen se agregaron a 5 mL de solución salina de fosfatos (PBS-Dulbecco. Gibco; dilución 1:1)

suplementada con 0.1 % de albúmina sérica bovina fracción V, 0.1 µg/mL (0.01 %) de penicilina potásica G y 0.08 µg/mL (0.008 %) de sulfato de estreptomicina. Se centrifugó a 61 xg durante 5 min. El sobrenadante se diluyó 1:1 con PBS-Dulbecco y se centrifugó a 1900 xg durante 5 min. Se retiró el sobrenadante hasta obtener únicamente el paquete celular el cual fue resuspendido en 10 mL de PBS-Dulbecco y se centrifugó nuevamente a 1900 xg durante 5 min. Finalmente se obtuvo el paquete celular y se resuspendió con 100 µL de TBMm. Se realizó una dilución 1:1000 para el conteo de espermatozoides en cámara de Neubauer.

Para llevar a cabo la solución final de espermatozoides, se realizaron las diluciones pertinentes con medio TBMm para obtener una concentración final de $5x10^5$ espermatozoides/mL. Finalmente se agregaron 50 µL de la solución final de espermatozoides a las gotas del medio de FIV con los ovocitos. La co-incubación se mantuvo durante 6 h para la fertilización (Ducolomb *et al*, 2005).

7.7 Desarrollo embrionario

Después del periodo de co-incubación para la FIV, los ovocitos se lavaron en medio North Carolina State University-23 (NCSU-23) (Petters y Wells, 1993) suplementado con BSA al 0.4 %, estos se colocaron en gotas de 500 µL del mismo medio y fueron cubiertas con aceite mineral para su incubación a 38.5 °C, 5 % de CO_2 y humedad a saturación durante 40 h para la evaluación del DE temprano (Ducolomb *et al*, 2005).

7.8 Evaluación de la distribución de la cromatina y los microfilamentos (tinción de Hoechst e inmunofluorescencia)

Para la evalución de la CR se realizó la tinción de Hoechst, la evalución de los MF se realizó por ensayo de inmunofluorescencia con FITC conjugada con faloidina (P5282; 1:350).

Después del periodo de incubación para el DE temprano, los embriones fueron lavados. En una caja de cuatro pozos se colocaron los embriones con la mínima cantidad de medio, se agregaron 300 µL de Hoechst y se mantuvieron a 4 °C por 45 min, posteriormente fueron lavados. Se les agregaron 200 µL de solución fijadora de paraformaldehído al 4 %, se conservaron a 4 °C toda la noche para entonces ser lavados. Posteriormente se expusieron a 200 µL de solución permeabilizante de PBS-Tritón X-100 al 1 % a 4 °C por 2 h y se lavaron. Se añadieron 200 µL de solución bloqueadora que contenía 20 mg/mL de PBS-BSA, 20 mg/mL de leche descremada y 11 mg/mL de glicina diluidos en PBS), se mantuvieron por 1 h a temperatura ambiente para después ser lavados.

Para el marcado de los MF, se agregaron 200 µL del anticuerpo conjugado con FITC-faloidina y se mantuvieron a 4 °C por 2 h, posteriormente fueron lavados. Para la obtención de las imágenes, se realizó el montaje de los embriones en portaobjetos con solución PBS/glicerol 9:1 y fueron cubiertos con cubreobjeto sellado con barniz transparente.

En cada tiempo de exposición con los anticuerpos respectivos, la caja que contenía los embriones fue cubierta con papel aluminio. Los embriones se lavaron en tres ocasiones con 500 µL de PBS-BSA. Bajo estas condiciones se realizaron todos los lavados.

Las imágenes fueron obtenidas por microscopia confocal (Carl Zeiss, modelo LSM780). El análisis de la imagen confocal se realizó por captura en pila de serie Z, considerando cuatro secciones que abarcaban a todo el embrión. Las imágenes se adquirieron secuencialmente mediante la línea de 488 nm de láser de argón para FITC utilizado para la visualización de MF (en coloración verde) y láser de argón-UV para DAPI para la visualización de la CR (en coloración azul). Se observaron a aumento de 200x. La evaluación de las imágenes se realizó mediante el procesador de imágenes, Image J (Abràmoff, 2004).

En la evaluación de la distribución de los MF, se clasificaron como embriones con actina cortical (AC) a los que presentaron un patrón uniforme de la actina en su periferia, estos embriones se consideraron de buena calidad y con alto potencial de DE. Se evaluaron como embriones con actina dispersa (AD) a los que presentaron actina globular en el citoplasma, estos fueron considerados como embriones de baja calidad, con menor potencial de desarrollo. Finalmente se evaluaron como embriones con actina cortical dispersa (ACD) a los que presentaron patrones actina interrumpida en la periferia con disperción de actina en el citoplasma, estos se consideraron como indicador de embriones de calidad media.

Para la evaluación de la CR, se consideraron dos clasificaciones: embriones sin daño (SD) y embriones con daño (D). Los primeros fueron aquellos que presentaron núcleos bien definidos, estos se relacionaron con buena calidad y con una alta probabilidad de éxito en el DE; mientras que los embriones con daño (D), a los que se identificaron con una o más estructuras de CR anormal (CRA), estos se relacionaron con un menor potencial de desarrollo.

7.9 Evaluación del daño en el ADN (electroforesis unicelular alcalina)

Después de la MIV, grupos de 8 a 10 COCs fueron tratados de la misma forma y concentraciones de CPAs que las descritas en el apartado de vitrificación, a excepción del almacenaje en N_2L. De igual forma, se expusieron a las concentraciones de CPAs descritas en el apartado de calentamiento (Casillas *et al*, 2018).

El daño al ADN generado por la exposición a CPAs y a la vitrificación se evaluó únicamente en las CC mediante electroforesis unicelular alcalino (ensayo cometa) siguiendo el protocolo de Einaudi y colaboradores (2013). Se prepararon agarosa de bajo punto de fusión (0.5 %) y de punto de fusión normal (0.1 %) en PBS (NaCl al 0.8 %, KCl al 0.02 %, KH_2PO_4 al 0.02 % y NaH_2PO_4 al 0.15 % en agua destilada). Los portaobjetos esmerilados se cubrieron con agarosa

de punto de fusión normal a temperatura ambiente durante al menos 24 h hasta su solidificación. Las CC se resuspendieron en la agarosa de bajo punto de fusión y se extendieron sobre un portaobjetos previamente tratado con agarosa de punto de fusión normal, se mantuvieron a 4 ºC en en oscuridad durante 10 min e inmediatamente después se añadió otra capa de agarosa de bajo punto de fusión. Los portaobjetos se sumergieron en solución de lisis durante 24 h a 4 ºC. Luego se colocaron en electroforesis horizontal y se equilibraron en solución amortiguadora durante 15 min; posteriormente, se realizó la electroforesis a 25 V y 300 mA durante 15 min. Al término de la electroforesis, las preparaciones se neutralizaron en solución de Tris (0.4 M) durante 10 min. Después se sumergieron en etanol al 70 % durante 5 min y se dejaron secar a temperatura ambiente durante aproximadamente 3 h.

Para evaluar el daño del ADN, las preparaciones se colocaron sobre papel absorbente húmedo y se tiñeron con 75 µL de bromuro de etidio (20 µL/mL) durante 10 min (Berthelot-Ricou *et al*, 2011), luego se analizaron al microscopio de epifluorescencia (Zeiss Axiostar) con el filtro Tex Red (561 o 594 nm) observando y capturando imágenes de los cometas a 400x. Las imágenes de los cometas se analizaron con el programa CromaGen (ODP, México).

Se obtuvieron las evaluaciones de la genotoxicidad celular mediante tres parámetros:

a) Longitud de la cola del cometa (LCC), la cual se refiere a la extensión del daño en el ADN y está dado en micrómetros.
b) Integridad del ADN (% ADN), que indica el porcentaje de fragmentación del ADN.
c) El "tiempo momento" de la cola (OTM, por sus siglas en inglés), el cual representa el producto de la LCC y el porcentaje de fragmentación del ADN.

El OTM es proporcional al daño genotóxico (a mayor OTM el daño genotóxico es superior) y es de las medidas más confiables para evaluar el daño al ADN celular.

7.10 Evaluación de la viabilidad en ovocitos, embriones y células del cúmulo

Los COCs se denudaron mecánicamente para separar los ovocitos de las CC. La viabilidad de los ovocitos y embriones se analizó en todos los casos por medio de la tinción con MTT, añadiendo 100 µL de 0.5 mg/mL de MTT diluido en PBS. Después de 30 min de incubación se analizaron con un microscopio óptico (Zeiss Axiostar), las células en coloración púrpura fueron considerados como vivas y aquellas sin coloración fueron consideradas como muertas.

En la evaluación de las CC, a 10 µL de azul de metileno se transfirieron 10 µL del medio de maduración con las CC de la denudación. Esta gota de 20 µL fue colocada en una cámara de Neubauer para el conteo en microscopio óptico. Las células incoloras se consideraron viables y las que presentaron una coloración azul como no viables.

7.11 Evaluación de la maduración *in vitro*, la fertilización *in vitro* y el desarrollo embrionario

Para determinar la influencia de cada grupo de estudio sobre los parámetros de maduración, fertilización y DE, las células se tiñeron con 500 µL de 10 µg/mL de bisbenzimida (Hoechst 33342) diluida en PBS durante 40 min. Las células se fijaron con glutaraldehído al 2 % en PBS y se montaron en portaobjetos con medio de montaje cubiertos con cubreobjetos y sellado con barniz transparente. Las células se analizaron en un microscopio de epifluorescencia (Zeiss Axiostar) a 400x (Ducolomb *et al*, 2005).

Para la evaluación de la maduración, se clasificaron como inmaduros a los ovocitos en GV, en vias de maduración a ovocitos en MI y como madurados aquellos en MII con su primer cuerpo polar.

La evaluación de los parámetros de fertilización se evaluaron 16 h después de la FIV, considerando como: ovocito activado, a los que tenían un pronúcleo (PN); cigoto

monospérmico, a los que tenían dos PN; ovocito polispérmico, a los que presentaron más de dos PN o 2 cabezas de espermatozoides descondensados; finalmente fueron considerados como ovocitos no fertilizados a los que estaban en alguna fase de la meiosis. El DE temprano se evaluó después de las 40 h de cultivo posterior a la fertilización, se consideraron como embriones tempranos a los que presentaban de 2-4 y de 6-8 células con núcleos bien definidos (Casillas *et al*, 2018).

VIII. RESULTADOS

8.1 Viabilidad de ovocitos y embriones tempranos

Se realizaron 5 experimentos para el grupo control y 3 para el grupo vitrificación. El 97 % de los ovocitos control se encontraron vivos inmediatamente después de su colecta (T 0), este porcentaje se redujo significativamente al 78 % en el grupo vitrificado (después de la vitrificación). Los ovocitos del grupo control después de las 44 h del cultivo para la MIV (T 44 h-MIV) presentaron 89 % de viabilidad, este porcentaje se redujo significativamente en el grupo de vitrificados a 63 % de viabilidad. Posteriormente, el 94 % de los embriones tempranos del grupo control se encontraron vivos después de las 40 h de cultivo contrario al 54 % del grupo vitrificación, siendo estadísticamente significativa esta diferencia (Figura 3 B).

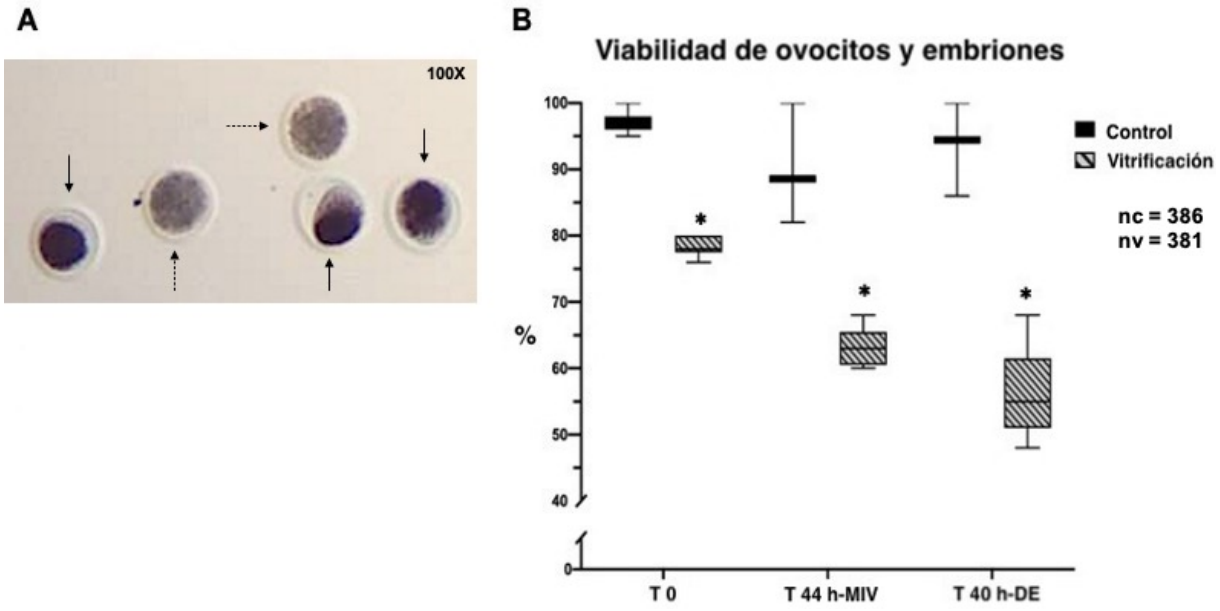

Figura 3. A) Criterios de evaluación de la viabilidad en ovocitos y embriones. Tinción con MTT. Microscopía de campo claro a 100x. B) Porcentaje de la viabilidad de ovocitos y embriones. T 0, tiempo 0 h de cultivo (células en VG); T 44 h-MIV, tiempo 44 h de cultivo para la maduración (células en MII); T 40 h-DE, tiempo 40 h de cultivo para el DE temprano; nc, número de células evaluadas del control; nv, número de células evaluadas de vitrificación. * p< 0.05, comparado con su control.

8.2 Maduración *in vitro* de ovocitos

Se realizaron 6 experimentos para el grupo control y 3 para el grupo vitrificación. La maduración del grupo vitrificación (40 %) se vio reducida significativamente en comparación con el grupo control (79 %), por lo que, el número de VG se presentó en mayor porcentaje en el grupo vitrificación (58 %) en comparación al grupo control (12 %). Los ovocitos en MI, aunque fueron los que se presentaron en menor cantidad en ambos grupos, fueron significativamente diferentes entre el grupo control (12 %) y el grupo vitrificación (4 %) (Figura 4 B).

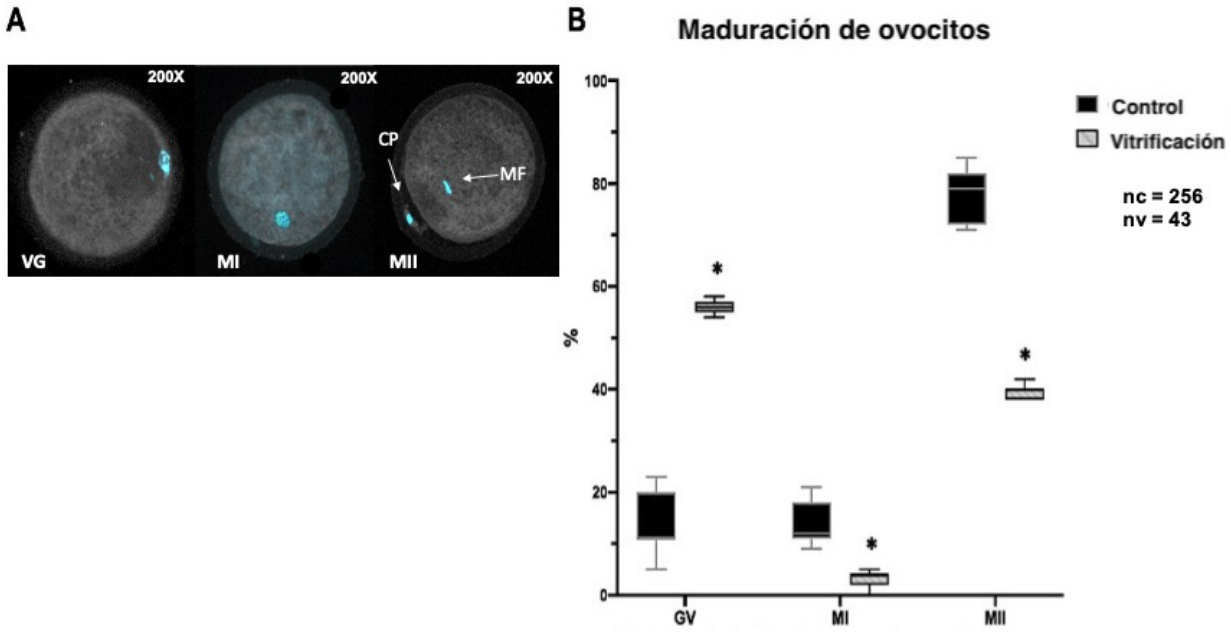

Figura 4. A) Criterios de evaluación de la MIV de ovocitos. Tinción con Hoechst. Microscopía de fluorescencia a 400x. B) Porcentaje de la MIV de ovocitos. VG, vesícula germinal; MI, metafase I; MII, metafase II; CP, cuerpo polar; MF, metafase; nc, número de células evaluadas del control; nv, número de células evaluadas de vitrificación. * p< 0.05, comparado con su control.

8.3 Fertilización *in vitro*

Se realizaron 4 experimentos para el grupo control y 3 para el grupo vitrificación. En la FIV monospérmica (con dos pronúcleos), considerado el mejor parámetro para la evaluación de la calidad de la fertilización, no hubo una diferencia significativa entre el grupo control (36 %) y el grupo vitrificación (31 %) siendo el único parámetro que no se vio afectado. En el caso del grupo vitrificación, se presentó en mayor porcentaje en los parámetros de activación (con un pronúcleo) y no fertilizados (sin formación de pronúcleo) en comparación con el grupo control (42 vs 27 % y 20 vs 13 %, respectivamente). Finalmente, en la fertilización polispérmica (más de dos pronúcleos o cabezas de espermatozoides descondensadas) derivada

de ovocitos vitrificados fue significativamente menor en comparación con el obtenido en el grupo control (8 vs 36 %, respectivamente) (Figura 5 B).

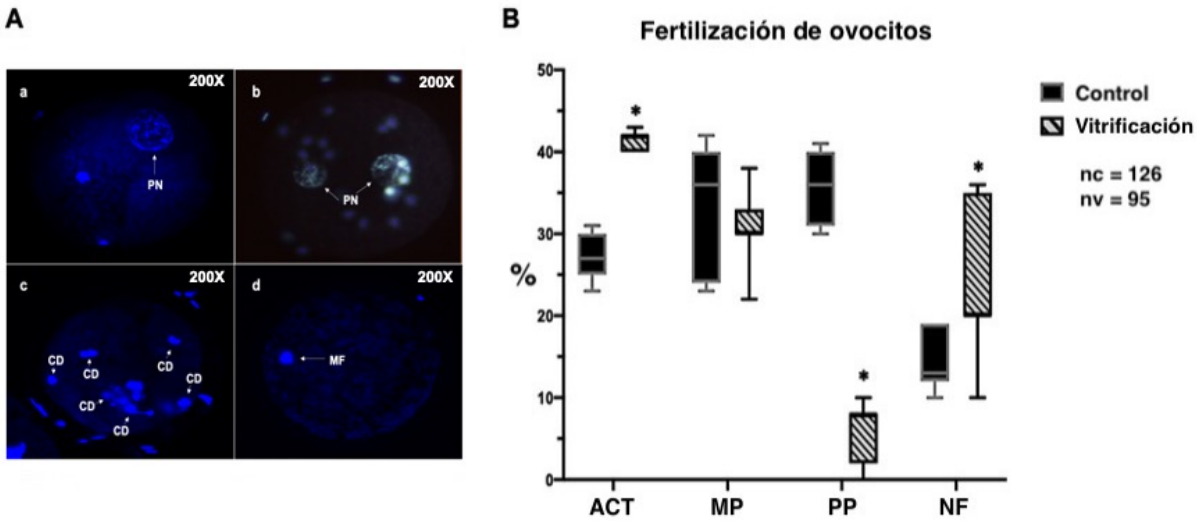

Figura 5. A) Criterios de evaluación de la FIV. Tinción con Hoechst. Microscopía de fluorescencia a 400x. B) Porcentaje de la FIV de ovocitos. ACT, activado; MP, monospérmico; PP, polispérmico; NF, no fertilizado; PN, pronúcleo; CD, cabeza descondensada; nc, número de células evaluadas del control; nv, número de células evaluadas de vitrificación. * p< 0.05, comparado con su control.

8.4 Desarrollo embrionario

Se realizaron 7 experimentos para el grupo control y 4 para el grupo vitrificación. El DE evaluado a las 40 h de incubación presentó disminución significativa del porcentaje de embriones de 2-4 células derivados del grupo vitrificación en comparación con los obtenidos en el grupo control (33 % vs 58 %, respectivamente). Así mismo, en el grupo vitrificación se presentó incremento del porcentaje de células no divididas en comparación con las obtenidas en el control (63 % vs 33 %, respectivamente). Hubo una tendencia en ambos grupos de menor

número de embriones desarrollados en ambos grupos fue de 5-8 células (9 % control vs 4 % grupo vitrificación) (Figura 6 B).

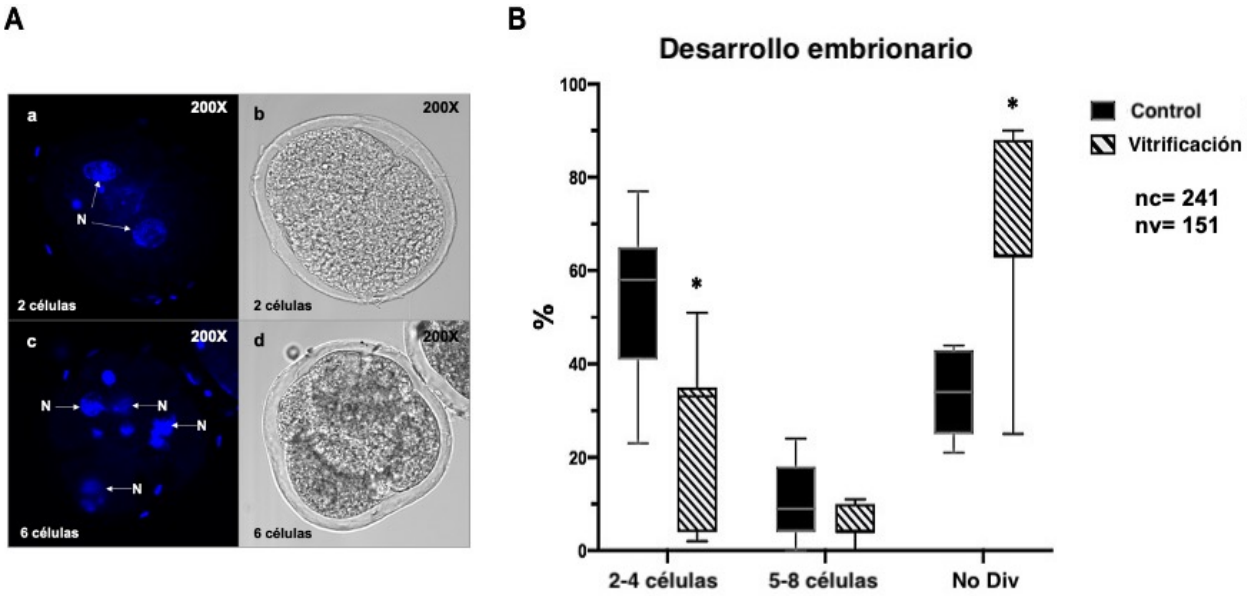

Figura 6. A) Criterios de evaluación del DE. Tinción con Hoechst. Microscopía de fluorescencia a 400x. B) Porcentaje de DE. N, núcleo; nc, número de células evaluadas del control; nv, número de células evaluadas de vitrificación. * p< 0.05, comparado con su control.

8.5 Análisis del citoesqueleto en embriones tempranos

Se realizaron 6 experimentos para el grupo control y 4 para el grupo vitrificación. El criterio de los diferentes patrones de distribución de los MF de actina encontrados en los embriones tempranos de ambos grupos se presentan en la Figura 7 A. El grupo de vitrificación disminuyó el porcentaje de embriones tempranos con actina cortical en comparación al grupo control (29 vs 67 %, respectivamente), en tanto que en el grupo de vitrificación aumento el porcentaje de embriones tempranos con actina cortical dispersa seguido de actina disgregada en comparación

con el control (40 y 32 % vs 23 y 11 %, respectivamente), las diferencias de los tres patrones evaluados fueron significativas respecto al grupo control (Figura 7 B).

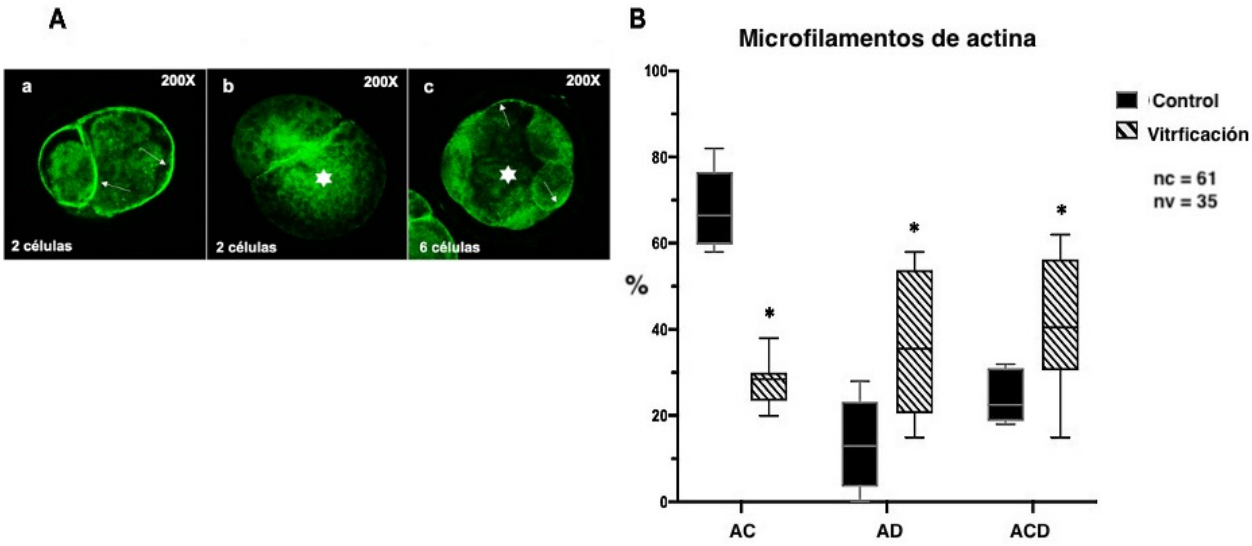

Figura 7. A) Criterios de evaluación de la distribución de MF de actina en embriones tempranos. Tinción con FITC-faloidina. Microscopía confocal láser de barrido a 200x. B) Porcentaje de la distribución de MF de actina en embriones tempranos. AC, actina cortical; AD, actina dispersa; ACD, actina cortical dispersa; nc, número de células evaluadas del control; nv, número de células evaluadas de vitrificación. * p< 0.05, comparado con su control.

8.6 Análisis de la integridad de la cromatina en embriones tempranos

Se realizaron 6 experimentos para el grupo control y 4 para el grupo vitrificación. El porcentaje de embriones tempranos derivados del grupo vitrificación presentó un aumento significativo de embriones dañados en la CR con una o más estructuras anormales, como se muestra en la imagen *b* de la Figura 8 A, e incluso, algunos de ellos no presentaron DE, esto en comparación con el grupo control (61 vs 23 %, respectivamente). En el caso contrario, el grupo control presentó mayor porcentaje de embriones con la CR sin daño (imagen *a* de la Figura 8 A), con

diferencia significativa respecto al grupo vitrificación (77 vs 41 %, respectivamente) (Figura 8 B).

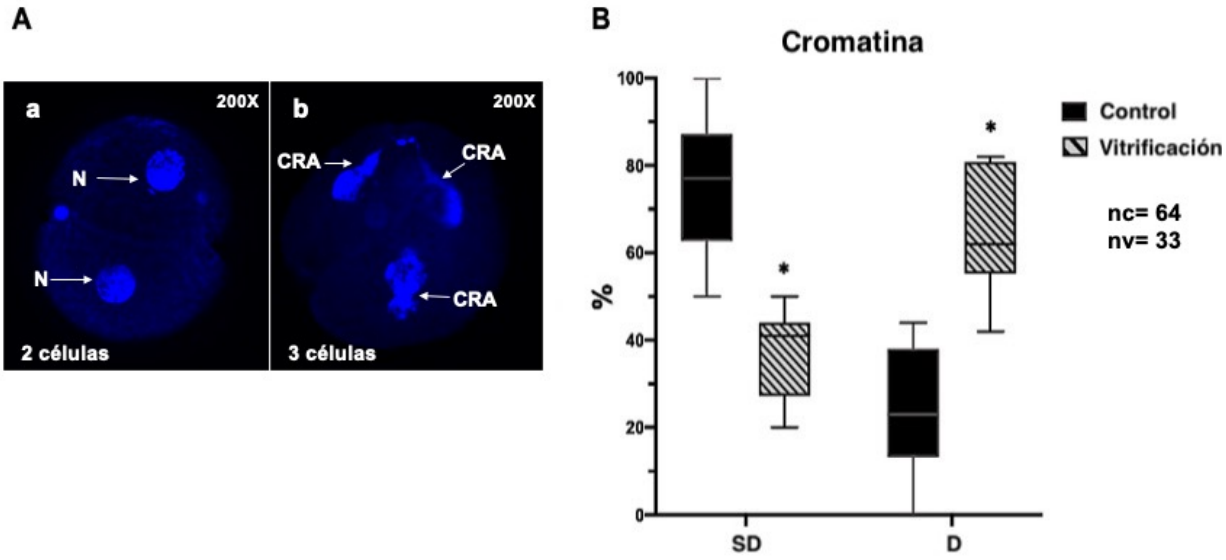

Figura 8. A) Criterios de evaluación de la integridad de la CR en embriones tempranos. Tinción con Hoechst. Microscopía confocal láser de barrido a 200x. B) Porcentaje de embriones tempranos con daño o sin daño en la CR. P< 0.05. SD, sin daño; D, dañado; N, núcleo; CRA, cromatina anormal; nc, número de células evaluadas del control; nv, número de células evaluadas de vitrificación. * p< 0.05, comparado con su control.

La Figura 9 muestra las imágenes en superposición de MF y CR en embriones tempranos. Las imágenes a, b y c corresponden al grupo control y las imágenes d, e y f al grupo de vitrificación. Algunos embriones muestraron blastómeros dañados y otros no se dividieron. Parece que la distribución cortical de los MF de actina y la integridad de la CR están relacionadas con la calidad del embrión. Los ovocitos con actina dispersa también mostraron una distribución anormal de la CR e incluso ausencia de división celular. Por el contrario, los embriones de control mostraron una conformación de CR sin daño, con actina cortical o con algún grado de dispersión de actina.

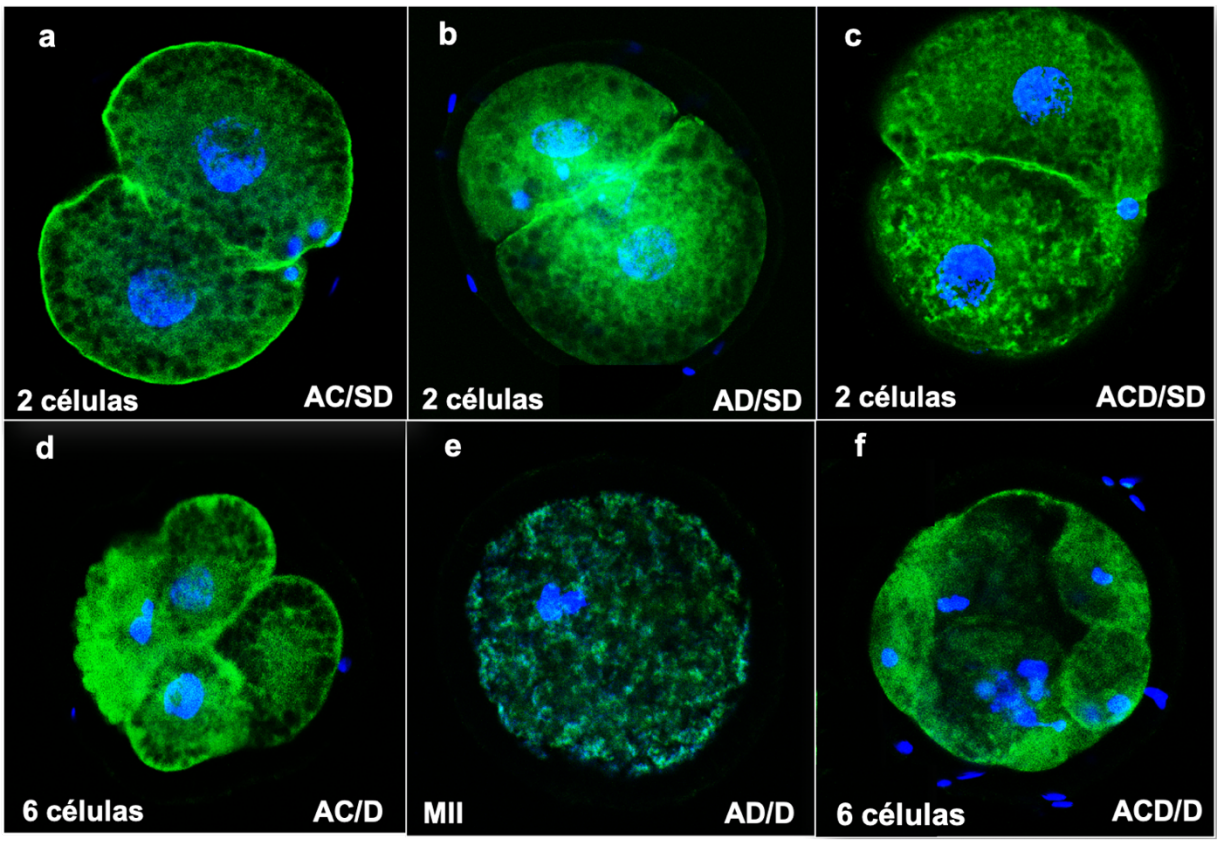

Figura 9. Superposición de las imágenes de los MF y la CR en embriones tempranos. (a, b, c) grupo control; (d, e, f) grupo vitrificación. (AC/SD) actina cortical sin daño en la CR, (AD/SD) actina disgregada sin daño en la CR, (ACD/SD) actina cortical disgregada sin daño en la CR, (AC/D) actina cortical con daño en la CR, (AD/D) actina disgregada con daño en la CR, (ACD/D) actina cortical disgregada con daño en la CR. Microscopía confocal láser de barrido. 200x.

8.7 Daño en el ADN de las células del cúmulo de complejos ovocitos-células del cúmulo vitrificados en metafase II

El daño en el ADN de las CC se evaluó apartir de COCs que fueron vitrificados en etapa madura (MII). Las condiciones de las variables a evaluar fueron descritas en el apartado del diseño experimental.

8.7.1 Viabilidad celular de ovocitos y de células del cúmulo

En la Figura 10, la viabilidad de los ovocitos y las CC fue evaluada después de la MIV (controles, negativo y positivo) y después de la vitrificación. En el caso de los ovocitos (Figura 10 A *a* y *b*), los criterios fueron descritos previamente en el apartado 7.10, para la evaluación de las CC se consideraron como muertas a las que presentaron una coloración azul y como vivas a las que no estaban teñidas (Figura 10 A *c* y *d*).

En la Figura 10 B se puede observar que la viabilidad disminuyó tanto en ovocitos como en las CC de todos los grupos en comparación con el control negativo ($P < 0.05$). Además, se puede observar que la viabilidad de las CC se redujo significativamente en comparación con los ovocitos en todos los grupos. Con lo que se puede afirmar que la vitrificación afectó principalmente la supervivencia de las CC en comparación con el control negativo, además de ser las principales células en ser afectadas durante el proceso de la vitrificación ($p < 0.05$).

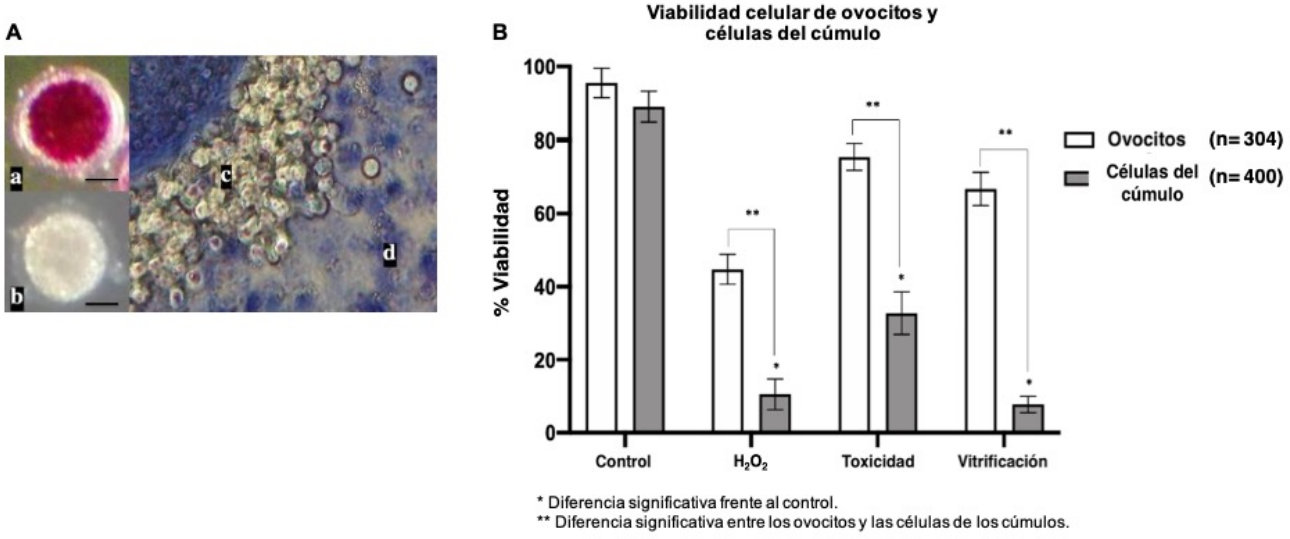

Figura 10. A) Criterios de evaluación de la viabilidad en ovocitos y CC. Tinción con MTT (a y b), tinción con azul de metilo (c y d). Ovocito viable, coloración púrpura (a); ovocito no teñido, no viable (b); CC viables, no teñidas (c); CC teñidas, no viables (d). Microscopía

estereoscópica a 400x. B) Porcentaje de la viabilidad celular de ovocitos y las CC. H_2O_2, control positivo a daño; toxicidad, exposición a concentraciones de CPAs; vitrificación, COCs vitrificados; n, número de células evaluadas. * p< 0.05, comparado con su control; ** p< 0.05, comparación entre ovocitos y las CC.

8.7.2 Genotoxicidad de las células del cúmulo expresado por la longitud de la cola del cometa, la integridad del ADN y el "Olive Tail Moment".

En la Figura 11 A se presentan los criterios de clasificación de la genotoxicidad por el ensayo cometa de las CC evaluadas de todos los grupos. En la Figura 11 B se presenta la evaluación de la genotoxicidad mediante la LCC y el % ADN y en la Figura 11 C mediante el OTM.

En cuanto a la LCC, se observa incremento de la LCC en los grupos tratados. El grupo H_2O_2 (control positivo) presenta los valores más altos LCC, seguido por el grupo Toxicidad y, posteriormente, el grupo vitrificación; sin embargo, es importante mencionar que no presentaron diferencia significativa entre ellos (Figura 11 B).

La integridad del ADN, representado por el % ADN (porcentaje de ADN), fue mayor en el control negativo en comparación con todos los grupos. Así mismo, los grupos de H_2O_2, toxicidad y vitrificación fueron estadisticamente diferentes en comparación con el control negativo. Además, el grupo toxicidad presentó mayor integridad del ADN, seguido por el de vitrificación y H_2O_2; sin diferencia entre ellos ($p < 0.05$) (Figura 11 B).

Los resultados del OTM indican que los grupos con tratamiento son significativamente diferentes en comparación con el control negativo ($P< 0.05$). El grupo H_2O_2 no fue diferente en comparación con los grupos toxicidad y vitrificación, lo que sugiere que la producción del daño en el ADN se debe a la exposición a los CPAs y la vitrificación. Aunque no hubo una diferencia entre los grupos de toxicidad y vitrificación, sí se presentó una tendencia de mayor daño en el

grupo de toxicidad en comparación del grupo de vitrificación, con lo que se puede suponer que el daño ocasionado al ADN celular se debe principalmente a la exposición a los CPAs más que al descenso de temperatura, propia del proceso de vitrificación ($p < 0.05$) (Figura 11 C).

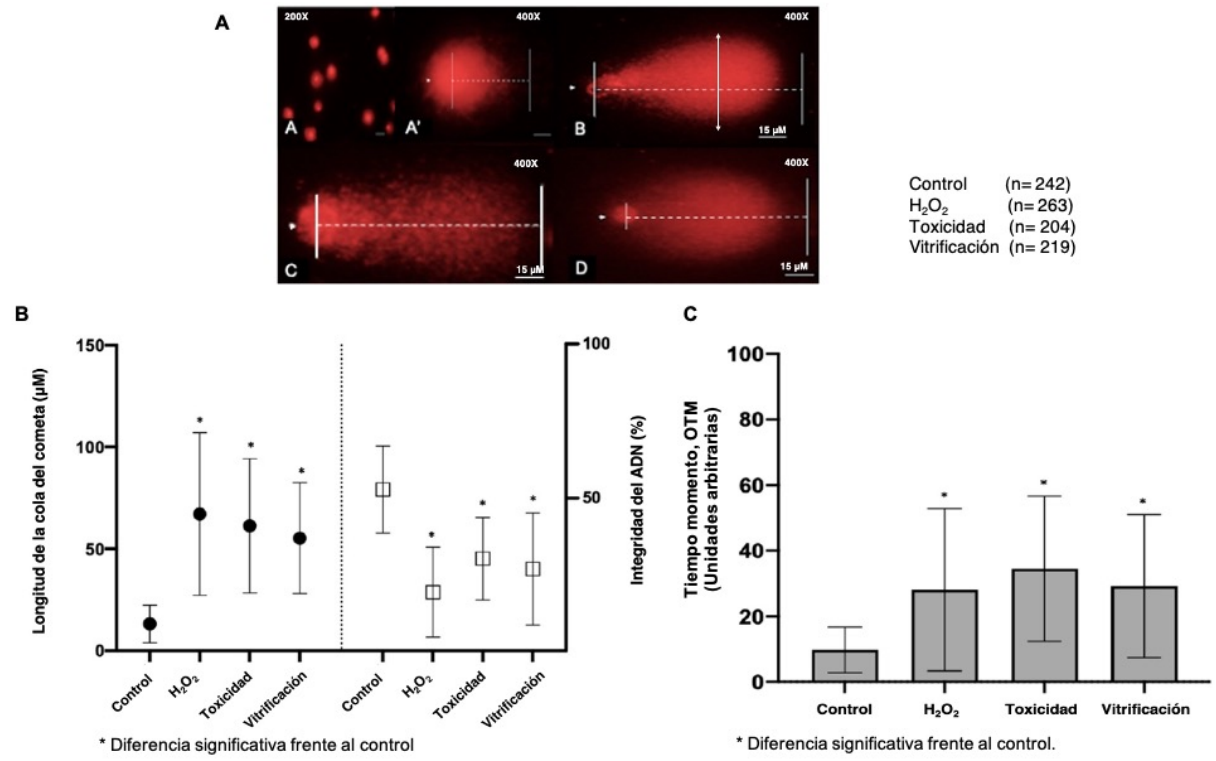

Figura 11. A) Clasificación de la genotoxicidad de las CC mediante el ensayo cometa. Tinción con bromuro de etidio. Microscopía de fluorescencia A 400x. La línea punteada indica la LCC, la doble flecha indica la extensión de la integridad del ADN (% ADN) y la cabeza de la flecha el nucleoide de la CC. B) Evaluación de la genotoxicidad de las CC expresada por la LCC (izquierda) y por % ADN (derecha). C) Evaluación de la genotoxicidad de las CC expresado por el OTM. H_2O_2, control positivo a daño; toxicidad, exposición a concentraciones de CPAs; vitrificación, COCs vitrificados; n, número de células evaluadas. * p< 0.05, comparado con su control.

IX. DISCUSIÓN

9.1 Viabilidad de ovocitos y embriones

Uno de los principales parámetros afectados por la vitrificación fue la viabilidad de ovocitos y embriones. Después de 44 h de MIV en el grupo de vitrificación, este parámetro descendió (63 %) en comparación con el grupo control (89 %). Además, los resultados indican que la viabilidad disminuyó significativamente en embriones derivados de ovocitos vitrificados en comparación con la del grupo de control (54 vs 94 %, respectivamente). Esto indica que la vitrificación afecta la diferenciación de los ovocitos para alcanzar el DE, debido a sus características celulares, el ovocito es más sensible a los efectos ocasionados por la vitrificación, por lo que se ha atribuido que la formación de cristales de hielo intracelular, el volumen celular mínimo y la transición de fase de las membranas de los lípidos, que está dado por la temperatura a la que está expuesta la membrana celular, pueden ser los principales elementos responsables de la lesión criogénica causado por rompimiento de estructuras celulares, mediante el aumento del grado de deshidratación celular y el cambio de la velocidad de enfriamiento y/o calentamiento que repercuten en la viabilidad celular (Kopeika *et al*, 2015).

El contenido lipídico de la célula varía entre especies, la especie porcina presenta alta cantidad de triglicéridos en comparación con otras, esto reduce la permeabilidad celular a los CPAs. La VG es muy sensible a la vitrificación debido a la alta cantidad de lípidos como fosfolípidos en la membrana plasmática y triglicéridos en el citoplasma, así como el recubrimiento por las CC causan que los ovocitos en esta etapa tengan menor permeabilidad a los CPAs lo que puede ocasionar procesos de nucleación del hielo durante el calentamiento que aumentan la probabilidad de rompimiento de estructuras celulares internas y la muerte celular (Jin *et al*, 2012; Casillas *et al,* 2014; 2015 a). También se ha atrubuido el origen de peroxidación lipídica

en la membrana celular de ovocitos causado por la generación de ERO ocasionado por los CPAs que puede resultar en muerte celular (Somfai *et al*, 2012).

Además, se ha sugerido que los CPAs puede causar daños irreversibles en organelos como las mitocondrias de los ovocitos y la consecuente generación de estrés oxidante. Tanto el EG como el PROH transportan Ca^{2+} del medio externo aunque el PROH de forma más potente y permanente, mientras que el DMSO libera Ca^{2+} de las reservas internas del retículo endoplásmico, lo que conduce a un aumento anormal de los niveles de Ca^{2+} citosólico y mitocondrial. Esta sobrecarga mitocondrial de Ca^{2+} y el estrés oxidante ocasionan la apertura del poro de transición de permeabilidad mitocondrial, cuya apertura aumenta la permeabilidad de la membrana mitocondrial interna a los solutos de hasta 1500 Da, afectando la homeostasis del potencial de membrana mitocondrial, Ca^{2+}, ATP y la producción de ERO, generando daño y muerte celular, comprometiendo de esta manera la viabilidad de los ovocitos y embriones vitrificados (Gualtieri *et al*, 2021).

9.2 Maduración *in vitro*, fertilización *in vitro* y desarrollo embrionario

Maduración *in vitro*

En este estudio se presentó una disminución en la tasa de MIV de ovocitos vitrificados en comparación con los del grupo control (40% vs 79%, respectivamente), estos resultados fueron similares a los reportados previamente en el grupo de trabajo (Casillas *et al*, 2020). Acorde a la literatura, algunos estudios que evaluaron la MIV en ovocitos vitrificados han informado resultados diferentes, esto puede deberse a la etapa de maduración del ovocito antes de la vitrificación (como puede ser la VG), sistemas de almacenamiento, tipos de CPAs o temperatura del enfriamiento y calentamiento (Fernández-Reyes *et al*, 2012; Casillas *et al*, 2014).

Las mitocondrias de los ovocitos se encuentran activas y su producción de ATP es necesaria para eventos esenciales como la maduración del ovocito, el ensamblaje del huso, la extrusión del cuerpo polar, la segregación cromosómica, la fertilización y el DE. Sin embargo, se ha encontrado que la vitrificación reduce significativamente el contenido de ATP en ovocitos bovinos, humanos, de conejo, murinos y porcinos probablemente debido al daño en las mitocondrias por el uso de altas concentraciones de CPAs (Gualtieri *et al*, 2021).

Los ovocitos maduros (MII) tienen mayor capacidad de supervivencia durante la criopreservación que los ovocitos inmaduros (VG) (Somfai *et al*, 2012), sin embargo, se ha observado que los ovocitos en VG que sobreviven a la vitrificación recuperan su capacidad de desarrollo durante la MIV, posiblemente debido a la restauración de la competencia meiótica y a la recuperación de la habilidad para acumular GSH; esto sugiere la existencia de un mecanismo de recuperación durante la MIV, el cual, aún necesita ser esclarecido (Somfai *et al*, 2012). Se ha señalado la relación de los niveles de GSH citoplásmico sintetizado por las CC intactas con la eficiencia de la maduración de los ovocitos, la fertilización y el DE, mediante la transferencia de este compuesto al citoplasma del ovocito (Maedomari *et al*, 2007).

Otro punto que se ha señalado son las altas concentraciones de CPAs utilizados durante la vitrificación de COCs que producen daño en las CC, estas células son indispensables para proporcionar los nutrientes esenciales a los ovocitos para su desarrollo, además, regulan los mecanismos de detención y reanudación meiótica y participan en la fecundación a través de la activación enzimática por parte del espermatozoide teniendo un impacto positivo en el DE (Ortiz-Escribano *et al*, 2016; Casillas *et al*, 2020; Sadat *et al*, 2020). Por lo que, mantener la viabilidad de estas células después de la vitrificación es fundamental para la maduración de los ovocitos y su posterior potencial de desarrollo.

Fertilización *in vitro*

El porcentaje de ovocitos fertilizados monospérmicos fue similar en ambos grupos, siendo el único parámetro que no se vio afectado por la vitrificación. Como se ha mencionado anteriormente, las mitocondrias de los ovocitos están implicadas en la homeostasis del Ca^{2+} y en las oscilaciones del Ca^{2+} en la fecundación y éstas, a su vez, regulan el correcto DE. Además, si consideramos que para la fertilización participa un nuevo factor como lo es el gameto masculino, los transitorios de Ca^{2+} citosólico desencadenados por el espermatozoide estimulan directamente la actividad mitocondrial, necesaria para mantener los niveles basales de Ca^{2+} citosólico en el ovocito no fertilizado y para recuperar el nivel basal después de cada transitorio de Ca^{2+} en la fertilización. Apoyando este punto, se ha encontrado que en los ovocitos de ratón, los CPAs permeables utilizados en la congelación lenta y la vitrificación provocan aumentos de Ca^{2+} intracelular comparables al aumento inicial desencadenado de manera natural durante la fertilización (Gualtieri *et al*, 2021), lo que puede explicar que la FIV no sea afectado por la vitrificación.

El aumento significativo en la tasa de activación en el grupo vitrificación comparado al del grupo control (42% vs 27%) puede estar causada por la alteración o daño de los organelos que regulan el Ca^{2+}, las mitocondrias y el retículo endoplasmático. Puede deberse a dos procesos, por el choque osmótico causado por el cambio de temperatura o por la degeneración de los MF de actina cortical, ya que estas estructuras reorganizan los orgánulos involucrados en la fertilización, como la extrusión del segundo cuerpo polar y la reorganización del retículo endoplásmico liso para la generación de Ca^{2+} intracelular durante la activación del ovocito (López *et al*, 2021b).

Otro parámetro a resaltar en el estudio es el porcentaje de polispermia, menor en el grupo de ovocitos vitrificados en comparación con el grupo control. Esto se puede atribuir a que la

vitrificación desplaza las mitocondrias y gránulos corticales del área pericortical al citoplasma más profundo, comprometiendo la respuesta fisiológica de Ca^{2+} promoviendo la reacción cortical por exocitocis de los gránulos corticales y con ello el endurecimiento de la zona pelúcida, bloqueando el acceso al ovocito de varios espermatozoides al mismo tiempo (Gualtieri *et al*, 2011; Egerszegi *et al*, 2013). En ovocitos de ratón, el PROH, DMSO y EG causan elevaciones de la concentración de Ca^{2+} intracelular, así como endurecimiento de la zona pelúcida y, en ovocitos de humanos, el PROH y DMSO induce exocitosis por GC (Gualtieri *et al*, 2021).

Desarrollo embrionario

La etapa temprana del DE (desde 2 hasta 8 blastómeros) es considerada la etapa más crítica, ya que son células totipotenciales que cuentan con el potencial para diferenciarse en células embrionarias o extraembrionarias, con capacidad para formar un nuevo organismo. Por lo que, de presentarse algún tipo de daño durante el desarrollo del ovocito, éste debe ser reparado para evitar la replicación y transmisión del defecto al nuevo organismo, o de lo contrario llevar a la muerte celular. La transmisión de defectos en el embrión tiende a replicarse, ya que el ciclo celular de embriones tempranos consiste en la alternancia de las fases S (síntesis) y M (mitosis) con puntos de restricción, por lo que el daño al ADN se replica rápidamente. Se menciona que si el embrión temprano logra superar esta etapa, tiene más posibilidad de culminar su desarrollo hasta blastocisto (Martin *et al*, 2017).

A las 40 h posteriores a la fertilización se considera como DE temprano, en este tiempo se espera un mayor número de embriones con 2-4 blastómeros, sin embargo, un pequeño número de ellos puede presentar de 4-8 blastómeros. En los resultados obtenidos se puede observar que el grupo

control presentó mayor tasa de embriones con divisiones de 2-4 células, comparado con el grupo vitrificación en el cual se presentó una disminución significativa de embriones en esta etapa (58% vs 33%, respectivamente). Algunos embriones mostraron de 6-8 blastómeros en ambos grupos, sin diferencia significativa entre ellos (9% y 4%), además, alto porcentaje de ovocitos vitrificados no presentaron división celular, lo que indica que la vitrificación de ovocitos en VG afectó el DE temprano.

La señalización de Ca^{2+} en la activación de los ovocitos controla eventos tanto tempranos como tardíos del DE como la exocitosis de los gránulos corticales, la reanudación del ciclo celular y la entrada a la primera división mitótica, además, el reclutamiento de ARNm materno, así como, la capacidad de los blastocistos para implantarse o desarrollarse después de la implantación (Gualtieri *et al*, 2021). La pausa del flujo de Ca^{2+} intracelular coincide con la activación del ciclo de reparación del ADN y con la regulación de la maquinaria de protección, asegurando las condiciones óptimas del cigoto para la embriogénesis (Martin *et al*, 2017). Como se mencionó, algunos CPAs (PROH, DMSO y EG) utilizados durante la vitrificación causan elevación de la concentración de Ca^{2+} intracelular, con lo que este ciclo de reparación de daño no podría llevarse acabo, proporcionando una posible explicación del porqué el DE temprano de ovocitos vitrificados se ve comprometido. Aunado a esto, la disposición de la actina filamentosa cortical determina la distribución de organelos implicados en las ocilasiones de Ca^{2+} intracelular (como mitocondrias, retículo endoplásmico y GC), por lo que su disgregación, podría causar alteraciones en las concentraciones de Ca^{2+} intracelular.

Además, los niveles de ATP en los ovocitos se han correlacionado positivamente con las tasas de fertilización y de desarrollo del embrión, se necesita un contenido de ATP $\geq$ 2 pmol/ovocito para apoyar el desarrollo normal del embrión (Gualtieri *et al*, 2021). Si consideramos, como se mencionó anteriormente, que la vitrificación reduce significativamente el contenido de ATP en

ovocitos debido al daño en mitocondrias por el uso de altas concentraciones de CPAs, entonces la capacidad para culminar eficazmente el DE se verá comprometida.

9.3 Análisis del citoesqueleto en embriones tempranos

La actina es un componente esencial del citoesqueleto que realiza funciones como la migración, división celular, y la regulación de la expresión génica, que son procesos básicos para el DE. En los ovocitos de mamíferos, en la VG la distribución de los MF de actina no se encuentra polarizada, de manera contraria, en MII la distribución cortical de los MF de actina se encuentra polarizada, lo que se evidencia por una protuberancia cerca el eje de la metafase y de menor manera en el dominio cortical opuesto. Esto indica que la actina filamentosa tiene una reestructuración para la polimerización durante la maduración de los ovocitos (Coticchio *et al*, 2014, 2015a). Los MF están implicados en la inmovilización de las mitocondrias en la corteza celular o en sitios de alta utilización de ATP (Gualtieri *et al*, 2011). Algunos estudios han evaluado el efecto de la vitrificación de ovocitos sobre el citoesqueleto, mostrando una interrupción en el red de MF corticales, así como la desorganización del huso de los microtúbulos que conduce a la dispersión cromosómica; además, no se presentó diferencia entre los ovocitos que fueron expuestos únicamente a CPAs y aquellos que pasaron por todo el proceso de vitrificación, por lo que el daño se adjudicó principalmente a los CPAs (Rojas *et al*, 2004; Chatzimeletiou *et al*, 2012).

Se ha propuesto que el efecto tóxico ocasionado por los CPAs permeables (particularmente el DMSO) ocasiona la desnaturalización de proteínas debido a su interacción con el agua, alterando la capa de solvatación (capa de moléculas de agua) que rodea a la superficie proteica impidiendo su hidratación (Fahy, 2016). Esta capa es crítica para la estructura y la función de

las proteínas, debido a que participa en su plegamiento, en la actividad enzimática y en el reconocimiento molecular (Fogarty y Laage, 2014; Laage *et al*, 2017). Además, hace posible la hidratación de las células y tejidos en los seres vivos, por lo que cualquier factor que modifique la interacción de la proteína con el agua, la pérdida total o parcial de esta envoltura acuosa o la ruptura de puentes de hidrógeno, disminuirá su estabilidad ocasionando la desnaturalización de la proteína (Laage *et al*, 2017). La mayoría de las proteínas pueden desnaturalizarse cuando son expuestas a temperaturas menores de 10-15 °C, esto podría explicar los cambios en la polimerización y agregación de actina dependiente de la temperatura y de los microtúbulos que puede desestabilizar el huso meiótico y disgregar la CR (Fahy y Wowk, 2015; Fahy, 2016). La despolimerización de los microtúbulos o la ausencia del huso pueden comprometer la capacidad del ovocito de ser fertilizado, de formar el segundo cuerpo polar, la migración de los pronúcleos y de llevar a cabo el DE normal previo a la implantación del blastocisto (Konc *et al*, 2014).

Estos datos podrían explicar el posible mecanismo de daño derivado de la vitrificación de ovocitos inmaduros y la producción de embriones derivado de estos, los cuales presentaron diferencias significativamente mayores en el número de embriones tempranos con algún tipo de disgregación en la actina (actina dispersa o actina cortical dispersa) comparado con el grupo control (31% vs 8%, 41% vs 23%, respectivamente. Esto puede deberse a que la vitrificación inhibe la correcta polimerización de la actina globular, que se ve reflejada en una ruptura de MF en el citoplasma y su disrupción en el área cortical (Baarlink *et al*, 2017; Misu *et al*, 2017).

Los blastómeros que lograron alcanzar el DE temprano, un bajo porcentaje de ellos presentó actina cortical (distribución considerada en embriones de buena calidad) comparada con el grupo control (28% vs 69%, respectivamente).

9.4 Análisis de la integridad de la cromatina en embriones tempranos

En el grupo de vitrificación, hubo una alta tasa de embriones no divididos. El porcentaje de los que alcanzaron a dividirse de 5 a 8 células, también fue más baja después de la vitrificación que los de 2 a 4 células. Esto podría estar relacionado con la falla de mecanismos de reparación de daño en los ovocitos. El aumento en la fragmentación del ADN de los blastómeros en blastocistos se ha atribuido al uso de los CPAs (Rajaei *et al*, 2005), además, la citotoxicidad de estos agentes es mayor en células con alta actividad metabólica, como los ovocitos inmaduros y embriones (Lawson *et al*, 2011). Si consideramos, como se mencionó anteriormente, que los CPAs pueden ocasionar la despolimerización y desorganización del huso acromático conduciendo a la dispersión cromosómica, esto puede resultar en daño en la CR y el desencadenamiento de aneuploidías durante el DE (Rojas *et al*, 2004; Chatzimeletiou *et al*, 2012). La despolarización y la desorganización del citoesqueleto evitará la externalización de la organización meiótica hacia la zona cortical y la alineación de los cromosomas en el eje ecuatorial de los ovocitos (Gualtieri *et al*, 2011).

La fertilización y la capacidad de división celular después de la vitrificación pueden verse modificadas negativamente cuando el ADN, las nucleoproteínas (principalmente histonas) y el ADNm fueron afectados por este proceso (Kopeika *et al*, 2015). El ADN es susceptible a una variedad de factores físicos y químicos que provocan alteraciones en su conformación como resultado de errores producidos durante la replicación, la recombinación y la reparación de la molécula (Coticchio *et al*, 2015b). Por ejemplo, el estrés hiperosmótico producido durante los ciclos de deshidratación-rehidratación de ovocitos por los CPAs, puede afectar la arquitectura molecular del huso meiótico y de la CR dañándolo o inhibiendo su reparación, ocasionando la formación de aberraciones cromosómicas. Esto puede deberse a la perturbación de la

distribución y la funcionalidad de componentes citoplásmicos como los microtúbulos y los microfilamentos que interactúan con las mitocondrias (Gualtieri *et al*, 2011).

Si consideramos que los cambios en la CR son el resultado de enzimas modificadoras de histonas, que alteran su actividad postranscripcional y los complejos remodeladores de la CR dependientes de ATP que utilizan la energía para cambiar los contactos ADN-histona e inducir deslizamiento o acumulación de ADN en los nucleosomas (Farrants *et al*, 2008), la reducción de la producción de ATP en ovocitos humanos vitrificados puede causar fallas en el sistema de reparación del ADN, lo que lleva a desordenes en la CR (Manipalviratn *et al*, 2011).

En el presente estudio, los mecanismos descritos pueden ayudar a entender el alto porcentaje de embriones derivados de ovocitos vitrificados que presentaron algún tipo de daño en la CR, el cual fue significativamente mayor a lo obtenido en el grupo control (59% vs 23%, respectivamente) que posiblemente presentaron un mecanismo íntegro en la maquinaria de reparación de daño al ADN, que se vio reflejado en la alta tasa de embriones sin daño en la CR comparado a los del grupo vitrificados (77% vs 41%, respectivamente). Esto puede estar ligado a la integridad de la actina cortical y a las altas tasas de DE temprano. Es importante señalar que, los mecanismos sugeridos son resultado de la participación coordinada de las estructuras evaluadas en este estudio, por lo que, el efecto de una estructura no puede ser desligada del funcionamiento de la otra ya que forman parte de la misma maquinaria celular.

9.5 Daño en el ADN de las células del cúmulo de complejos ovocitos-células del cúmulo vitrificados en metafase II

9.5.1 Viabilidad celular de ovocitos y de las células del cúmulo

Las CC son importantes en todos los procesos de desarrollo del ovocito, desde la maduración hasta el desarrollo del embrión. Nuestros resultados demuestran que la exposición a los CPAs y a la vitrificación disminuyeron la viabilidad de las CC y de los ovocitos. En comparación con los ovocitos, las CC son de menor tamaño y son las primeras en contacto con los CPAs, lo que implica que estas células reciben inicialmente las altas concentraciones haciendolas menos criotolerantes. Se informó que el DMSO inhibe la expansión de las CC de forma dependiente de la concentración, resultando en la muerte celular por apoptosis (Li *et al*, 2016), comprometiendo la viabilidad de las CC disminuyendo considerablemente después de la vitrificación de los COCs (Tharasanit *et al*, 2009; Jin *et al*, 2012; Casillas *et al*, 2014). Sin embargo, pueden ayudar a prevenir el daño criogénico de los ovocitos después de la vitrificación al preservar la estructura de los principales orgánulos después de la vitrificación (Taghizabet *et al*, 2018) al prevenir el choque osmótico, facilitando la deshidratación de los ovocitos (Tharasanit *et al*, 2009).

Aunque la viabilidad disminuyó en las CC (7.7 ± 2.21 %), también se redujo hasta 66.7 ± 4.57 % en ovocitos en MII vitrificados. En este sentido, la etapa nuclear de la célula antes de la vitrificación es un factor clave a considerar. Los ovocitos en VG (Kharche *et al*, 2005) o MII tienen menos permeabilidad a los CPAs y al agua que los cigotos y los embriones en etapa tardía.

La vitrificación de ovocitos desnudos en MII podrían generar alteraciones en la membrana plasmática, distribución mitocondrial, huso meiótico y cromosomas. Cuando los ovocitos se vitrifican con las CC, estas células pueden prevenir la criolesión de los ovocitos al preservar la estructura de sus principales orgánulos después de la vitrificación (Taghizabet *et al*, 2018). En el presente estudio, la reducción de viabilidad de los ovocitos hasta 75.5 ± 3.69 % después de la

exposición a CPAs y 66.7 ± 4.57 % después de la vitrificación podría deberse a las posibles lesiones de los ovocitos provocadas por los CPAs.

Una relación importante entre la permeabilidad de las CC, los ovocitos y los CPAs podría tener un impacto en las tasas de supervivencia de los COCs después de la vitrificación. Se ha señalado que la presencia de las CC disminuye la entrada de los CPAs al ovocito, haciendo ineficiente el proceso de re-hidratación durante el calentamiento dañando principalmente a la membrana celular del ovocito por estrés mecánico y el efecto tóxico en el citoesqueleto, esto tendría como consecuencia la interrupción de las uniones comunicantes entre ovocito-CC resultando en pérdida de la viabilidad de las CC (Brambillasca *et al*, 2013; Casillas *et al*, 2020).

9.5.2 Genotoxicidad de las células del cúmulo expresado por la longitud de la cola del cometa, la integridad del ADN y por el "Olive Tail Moment"

En la genotoxicidad celular causada por la exposición y vitrificación de CPAs, el ensayo cometa se ha utilizado generalmente como método de evaluación. Aunque otros criterios se han considerado para evaluar el daño del ADN mediante esta técnica, la mayoría de los estudios consideran la LCC como un indicador de la extensión del daño (Gunasekarana *et al*, 2015) y el % ADN como la fragmentación; sin embargo, el OTM se considera el valor más fiable (Greco *et al*, 2015). La LCC está relacionada con el porcentaje de integridad del ADN, valores altos indican una menor integridad del ADN. En el presente estudio, se realizó el ensayo cometa alcalino para detectar diferentes tipos de lesiones de ADN, incluidos los rompimientos de cadena sencilla (SSB, por sus siglas en inglés) y rompimientos de cadena doble (DSB, por sus siglas en inglés), sin embargo, la versión alcalina no permite la discriminación simultánea entre SSB y DSB. Los SSB representan el tipo más común de daño al ADN y no reparadas pueden alterar la replicación y transcripción del ADN que se han asociado a enfermedades. Por el

contrario, los DSB son una de las formas más graves de daño en el ADN y pueden causar muerte celular, aberraciones cromosómicas o pérdida de material genético (Hossain *et al*, 2018).

Altas concentraciones de CPAs se han utilizado en diversos estudios (16-50 %), produciendo efectos perjudiciales en las células, especialmente daño del ADN tanto en ovocitos como en las CC (Tharasanit *et al*, 2009; Rojas *et al*, 2004; Stachowiak *et al*, 2009; Lindley *et al*, 2001; Berthelot-Ricou *et al*, 2011; Berthelot-Ricou *et al*, 2013 (a); Berthelot-Ricou *et al*, 2013 (b); Kopeika *et al*, 2015). Los resultados demuestran que la exposición a los CPAs y la vitrificación generó daño al ADN en las CC. Este daño puede generarse porque las concentraciones de CPAs utilizadas durante la vitrificación son muy altas para este tipo de células. Generalmente, en la mayoría de los protocolos de vitrificación, estas concentraciones se calculan considerando las características de los ovocitos pero no las de las CC. Por lo tanto, esto puede hacer que las CC sufran más daño por la vitrificación que los ovocitos. Como ha sido informado por Taghizabet y colaboradores (2018), las CC crean un escudo protector natural alrededor del ovocito contra las agresiones físico-químicas debidas a la vitrificación. Además, el daño al ADN generado en las CC después de la vitrificación también podría deberse al aumento de la producción de ERO por los CPAs (Somfai *et al*, 2007; Sharma *et al*, 2016). Se ha reportado que las ERO pueden causar mutaciones genéticas y cromosómicas a través de las DSB del ADN (Sharma *et al*, 2016). Por ejemplo, el H_2O_2 causa roturas de la cadena de ADN después de la conversión al radical hidroxilo (Berthelot-Ricou *et al*, 2011), es por esto que se utilizó en el presente estudio como inductor de daño al ADN (control positivo). Los niveles de ERO pueden afectar la integridad, viabilidad y función, contribuyendo al estrés oxidativo generando mutaciones y daño al ADN (Goud *et al*, 2008; Kang *et al*, 2017). Las principales consecuencias de la reducción de la viabilidad de las CC y el aumento del daño al ADN son fallas en la maduración de los ovocitos, la fertilización y la producción de embriones.

En este estudio se presentó mayor tendencia de daño en el ADN de las CC causado por la exposición a los CPAs, más que al enfriamiento durante la vitrificación, esto se puede atribuir a que estas células reciben inicialmente las altas concentraciones de CPAs, produciendo mayor daño citotóxico en ellas (Taghizabet *et al*, 2018). Este tipo de daño puede repercutir en el aumento de la cascada apoptótica por efecto de los CPAs (Somfai *et al*, 2020) adjudicado a la producción de ERO (Shaeib *et al*, 2016), ya que en condiciones patológicas las CC y el ovocito intercambian antioxidantes enzimáticos (catalasa, GSH peroxidasa) y no enzimáticos (ác. ascórbico y GSH reducido) para protegerse frente a las ERO (Shaeib *et al*, 2016), por lo tanto, al verse afectada la comunicación bidireccional entre ambos tipos celulares el efecto antioxidante puede comprometerse. Sin embargo, aunque las CC presenten daño en el ADN, pueden proteger al ovocito contra las altas tasas de fragmentación del ADN (Pereira *et al*, 2019c). Es importante mencionar que en los protocolos de vitrificación, las concentraciones de CPAs están consideradas para las características de los ovocitos, pero no de las CC. Garantizar y mantener la viabilidad de estas células es fundamental para el desarrollo posterior de los ovocitos (Casillas *et al*, 2020). Por lo tanto, se sugiere que la vitrificación de ovocitos porcinos debería llevarse a cabo sin eliminar las CC ya que con su presencia se obtiene una mayor viabilidad de los ovocitos. Sin embargo, dado que la integridad en las CC se compromete, se recomienda el uso de un sistema de co-cultivo con CC frescas después de la vitrificación.

X. APLICACIONES PRÁCTICAS

Los resultados obtenidos en este proyecto ayudarán a ampliar la información sobre algunos efectos producidos por la vitrificación en una etapa temprana de desarrollo y la repercusión que puedan tener en el DE. El uso del porcino como modelo experimental, debido a sus similitudes,

permite que la información pueda ser aplicada en estudios en humanos, siendo un sector importante en el que la aplicación de la vitrificación de ovocitos se realiza de manera habitual en centros de reproducción asistida. Además, la evaluación del daño al ADN en las CC de COCs vitrificados puede ser implementado como un análisis predictivo no invasivo de la calidad de la producción de los embriones.

XI. PERSPECTIVAS

- Evaluar la viabilidad celular en la FIV (16 h) para analizar la correlación entre el descenso de la viabilidad con la etapa de desarrollo celular y el tiempo de incubación.
- Evaluar el efecto de la vitrificación de ovocitos en VG sobre los microtúbulos en embriones de desarrollo temprano, para determinar si existe una relación entre la distribución de los microtúbulos con la integridad de la CR y de los microfilamentos, sobre la calidad de la producción embrionaria.
- Implementar el uso de marcadores que revelen la presencia de roturas de ADN de doble cadena y de la activación de una respuesta de reparación del ADN (γH2AX y Rad51, respectivamente), para puntualizar sobre el tipo de daño ocurrido en el ADN.
- Evaluar mediante electroforesis unicelular la genotoxicidad de los ovocitos producida por la vitrificación de COCs, para esclarecer si existe una relación entre el daño al ADN de las CC con la del ovocito.

XII. CONCLUSIÓN

La vitrificación de ovocitos porcinos en etapa de VG alteró parámetros relacionados con la competencia celular, como la viabilidad de ovocitos, cigotos y embriones, además de la MIV y

el DE temprano. Contrariamente, el único parámetro que no se vió afectado por la vitrificación fue la tasa de la FIV monospérmica.

En el análisis del citoesqueleto y de la CR en embriones tempranos se observó que la distribución cortical de los MF de actina y la integridad de la CR pueden estar relacionadas con la calidad del embrión con base en la competencia del DE. Además, se obtuvo una correlación entre la distribución cortical de los microfilamentos de actina con la integridad de la CR (sin daño), ya que los ovocitos con actina dispersa mostraron distribución anormal de CR e incluso la ausencia de división celular. En el caso de los embriones del grupo control mostraron la presencia de CR sin daño, con distribución de actina cortical.

Respecto al daño en el ADN de las CC, se demuestra que la exposición de los COCs a los CPAs o a la vitrificación redujo la viabilidad de los ovocitos y de las CC pero de mayor manera en estas últimas células, además de generar daño al ADN en las CC.

La mayoría de los estudios han evaluado los efectos que produce la vitrificación en los ovocitos dejando de lado la importancia de las CC (Brambillasca *et al*, 2013; Lei *et al*, 2014). Estas células son de gran importancia debido a que juegan papeles importantes en los procesos de maduración y fertilización (Racowsky *et al*, 2018; Casillas *et al*, 2020), estableciendo comunicación bidireccional con los ovocitos para la adquisición de la competencia del desarrollo de los ovocitos y la finalización de la maduración nuclear y citoplasmática (Racowsky *et al*, 2018). Esta comunicación se realiza principalmente a través de las uniones comunicantes en las que las CC proporcionan a los ovocitos nutrientes y moléculas esenciales como metabolitos, iones, azúcares, segundos mensajeros y agua, regulando la detención y reanudación meiótica (Casillas *et al*, 2020).

Es importante señalar que los estudios reportados en la literatura han realizado la evaluación del efecto de la vitrificación en ovocitos en VG y MII, cigotos y blastocistos en la misma etapa de desarrollo en la que se han vitrificado, además, los estudios de vitrificación en cigotos o blastocistos miden el papel de los MF, microtúbulos y CR en cuanto al éxito que se tenga en la fertilización y en la producción embrionaria, y no en la distribución de estas estructuras en el DE temprano (Wu *et al*, 2006; Somfai *et al*, 2010; Egerszegi *et al*, 2013; Wan-Hafizah *et al*, 2015). Por lo que, aún es necesario ampliar los conocimientos en cuanto a los posibles efectos de la vitrificación en etapas tempranas y la progresión que estos tendrán a largo plazo. Ya que es posible que los parámetros morfométricos del citoesqueleto y la CR, así como de la evaluación del daño al ADN en las CC, puedan ser utilizados como marcadores predictivos de la competencia celular y de la calidad de los embriones tempranos.

XIII. REFERENCIAS BIBLIOGRÁFICAS

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Review Article

Vitrification and its impact on oocyte structures. A review

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Abstract

The vitrification of oocytes and embryos produces structural damage caused by the cytotoxicity of the cryoprotective agents (CPAs). Some of these damages occur in the cell plasma membrane, cytoskeleton and genetic material, which might result in the formation of aneuploidies. The toxicity produced by the use of CPAs during vitrification can cause alterations in microfilaments (MF), microtubules (MT) and chromatin (CR), affecting not only cell viability, maturation, fertilization and embryonic development (ED), but also the genetic integrity that will result in the formation of aneuploidies. The latter can result in spontaneous abortions and/or the absence of a term pregnancy, and if an opposite case arises, the birth of offspring with chromosomal aberrations will affect the organism development.

Introduction

Vitrification, as a method of cell cryopreservation, has been considered a very important tool for Assisted Reproduction Technologies (ART), improving the reproductive quality of economic important species for animal production and, in humans for subfertility treatments [1,2]. Vitrification is used in oocytes and embryos of different species such as pigs [3], sheep [4], cattle [5], humans [6,7], among others, since it has been superior to slow freezing [8,9].

Since the first vitrification success reported in 1985 [10], its effects on different domestic species and cell structures have been studied [11-15]. Although vitrification is a technique widely used for gametes preservation, multiple cell structures have been reported that, to a greater or lesser extent, suffer damage derived from the cryoprotective agents (CPAs). Therefore, the success of this method will be largely reflected by the reduction of the damage caused by the intracellular formation of ice crystals and the use of appropriate concentrations of CPAs.

Cell Structure Damage

Plasma membrane

It is important to highlight that the structure and composition of plasma membranes determine the main cellular events that take place during cryopreservation processes. The plasma membrane of the eukaryotic cell is mainly composed of amphipathic lipids, proteins and carbohydrates, with varying proportions according to cell type and animal species [16]. The CPAs used for the maintenance of cells at low temperatures must have the ability to penetrate the cell membranes in order to diffuse and take place inside the cell, remaining in a solid vitreous state. It is important to consider that the determination of the concentration of the CPAs is critical, since it will depend on that the role of protective agent, and thus, reducing its toxicity.

CPAs toxicity is mainly due to two causes. First is the chemical reaction performed in the cells before cryopreservation, and second is the chemical effect caused by the change of osmosis in the freezing solutions. In addition to this, the decrease in the freezing point (derived from the concentration of the CPAs mixture) is related to their permeability, which can cause osmotic stress before vitrification [17]. Therefore, when seeking to reduce the risk of toxicity damage, it is important to consider implementing the optimal CPAs, appropriate exposure time and temperature [18].

CPAs do not have the same membrane diffusion capacity in all cell types, which is why cell membranes are one of the structures that suffer the most damage during cryopreservation due to the

loss of fluidity of their lipid components, reducing their ability to expansion during rehydration [19]. It has been proposed that an important damage factor caused by CPAs in oocytes plasma membranes and embryos is derived from protein denaturation. It has been attributed that the toxic effects of vitrification solutions are due to the strong interaction between permeable CPAs, particularly dimethyl sulfoxide (DMSO) and water, which can cause damage by affecting the hydration of proteins [20]. Due to the interaction of these permeable agents with the hydration layer of water molecules (or solvation layer) that surrounds protein surfaces, is what makes possible the hydration of cells and tissues in living beings. Any factor that modifies the interaction of the proteins with water, the total or partial disappearance of this aqueous envelope or the breakdown of hydrogen bonds, will decrease its stability causing denaturation. Most proteins can be denatured when exposed to temperatures below 10°C and 15°C [21,22].

It has been reported that the vitrification of mature buffalo oocytes with 40% ethylene glycol (EG) or 40% DMSO, decreases morulae development rates up to 7.2% and 8%, compared to those vitrified with a mixture of 20% EG+DMSO up to 11.5% [23], suggesting that the use of CPAs mixtures reduces the specific toxicity of each CPA and replaces it with denaturation associated with high concentrations of aggregates [22].

Studies in humans have shown that vitrification decreases the potential of internal membrane in oocytes in metaphase II (MII) [6,24]. In addition, by means of transmission electron microscopy, the fracture of the plasma membrane [25] and the zona pellucida has been observed, as well as the separation of cumulus-oocyte complexes (COCs) [26], the rupture of the communicating junctions between cells and the interruption or disappearance of microvilli, as well as drops of non-homogeneous lipids surrounding large vacuoles [26].

In oocytes and embryos, the fracture of the cell membranes is caused by the mechanical effect of the solutions solidification, and the critical temperature at which this damage occurs is between -50 and -150°C [9]. It is important to highlight the importance of the structure and composition of the plasma membrane during the cryopreservation processes. Plasma membranes have proteins that can bind to other membrane components and cytoplasm proteins, in addition to other cellular structures such as the cytoskeleton, which is made up of microfilaments (MF), microtubules (MT), and intermediate filaments (IF) [27,28].

Interactions between proteins associated with the plasma membrane could explain the changes in the polymerization and aggregation of temperature-dependent actin and MT, which is an important factor in the destabilization of the meiotic spindle and, therefore, of the disintegration of chromatin (CR) [21,22].

Cytoskeleton

Another important structure responsible for maintaining the structural and functional integrity of the oocyte is the cytoskeleton. The reorganization of this structure is involved in the communication between the oocyte and the cumulus cells during the maturation process, the fertilization in mammals [24,29,30,31,32], as well as in processes related to division and cell cycle, such as cytokinesis and karyokinesis. In addition, they participate in intracellular transport and constitute the spindle for chromosome segregation and centriole displacement during mitosis [33].

Studies in MII human oocytes report the reorganization of the cytoskeleton, in addition to the alteration in mitochondrial function without presenting any significant change in the configuration of meiotic spindle [6, 24], as well as depolymerization of tubulin [7]. Therefore, the association between chromosomes and the organization of MT during mitosis [34] is essential for oocyte maturation. In ovine, abnormal patterns were presented in the organization of the F-actin of the MII oocyte cytoskeleton exposed to CPAs and vitrified [35].

Laser scanning confocal microscopy (LSCM) examined the cytoskeleton and observed a considerable reduction of oocytes with spindles with normal chromosomes alignment in the equatorial plate after vitrification (79.5% fresh vs. 10.1% GV vitrified) [26]. Another study indicated that the meiosis stages in oocytes are related to temperature differences and equilibrium time of depolymerization and repolymerization of spindles, for example, telophase I (TI) has less depolymerization of spindles at room temperature, 4°C and after vitrification than those found in MI or MII [36]. In addition, the percentage of oocytes in GV and MII with normal distribution of F-actin is reduced after vitrification (72.3% fresh vs. 16.9 and 37.2%, respectively) [26].

The importance of the plasma membrane and the cytoskeleton of the oocyte during fertilization has been demonstrated, since it contains coupling receptors for sperm plasma membrane proteins, without which the recognition of gametes would not be possible [37].

Genetic material damage

CR configurations are important for meiotic resumption and competence in oocyte development. The genetic integrity and changes that occur in the cytoplasmic compartment have important implications for oocyte maturation and quality. MF, MT and CR interact in the segregation of chromosomes and in the establishment of the cellular asymmetry that allows the extrusion of the polar body with a minimal loss of cytoplasm [38].

Exogenous and endogenous factors may be responsible for causing damage to the oocytes genetic material, thereby compromising the success of ART. Several studies have reported the impact that DNA damage would cause throughout cell development [39-41], however, most of these studies have caused damage by exposing oocytes to physical or chemical stimuli, such as microhaz dissection laser (LMD) used in assisted reproduction and bleomycin (BLM) [42]. These studies have focused on analyzing the damage of double-strand break (DSB) of DNA, and although they did not implement vitrification, they did demonstrate the importance of the genetic integrity of the oocyte in cell development.

One study performed in mice evaluated the effect that vitrification has on oocytes in MII and the resulting preimplantation embryos, it was measured the levels of reactive oxygen species (ROS), the accumulation of γ-H2AX (phosphorylation of histone variant H2AX in serine 139) sensitive marker for DSB, apoptosis, percentage of early embryonic development and gene expression related to DNA damage (Brca1 and 53bp1) in early embryos derived from in vitro fertilization. It was found that vitrification increased the expression of γ-H2AX in zygotes (in both pronuclei) and in embryos of two cells, but embryos of four and eight cells, as well as blastocysts were not affected. This was confirmed by Western blot analysis [43]. Because γ-H2AX is not a sensitive marker for the analysis of DSB in MII oocytes, it was opted for the implementation of the Comet Assay as a method of damage analysis, in which they found no results that involved vitrification as a producer of DSB [43]. This may be due to the fact that the MII oocyte being transcriptionally inactive, requires the reserves of endogenous proteins and/or transcripts of mRNA accumulated during oogenesis for DNA repair and the preservation of the integrity of the maternal genome such as homologous recombination (HR) and the final non-homologous union (NHEJ) [44].

The accumulation of γ-H2AX was attributed to the overproduction of ROS, because DNA damage can be induced by endogenous metabolites that may react with macromolecules such as lipids, proteins or nucleic acids, as well as exogenous stimuli such as ionizing and ultraviolet radiation, as is the production of ROS [43,45,46,47]. However, several studies report various functions related to the presence of γ-H2AX. Recent studies have shown that histone-H2AX phosphorylation (γ-H2AX) accumulates independently of the presence of DSB and functions as a regulator of cell cycle progression by inhibiting DNA replication, as well as to maintain the self-renewal of mouse embryonic stem cells [48-50].

It has been reported that exposure of porcine blastocysts to CPAs decreases survival rates, even without being vitrified, an increase in DNA fragmentation in the nuclei of these embryos was also found [51], thereby causing cytotoxic damage can be awarded to the exposure of the CPAs. This may be due to the fact that the cytotoxicity of these agents occurs in greater proportion in cells that perform a greater metabolic activity [52].

Through the analysis of DNA fingerprints based on microarrays, no evidence was found that vitrification of human MII oocytes increased the risk of aneuploidy production in arrested embryos than those properly obtained during an in vitro production system. Likewise, neither did the implantation potential decrease, however, IVF and viability were some of the factors that were affected [53]. Otherwise, in 2016, another study in humans found out that IVF rates were not affected by the vitrification of MII oocytes, however, embryonic development and viability were diminished due to this procedure. In the study by Forman et al. 2012, it was shown that there is no significant risk in the formation of aneuploidies caused by vitrification [54].

In both cases it can be explained that the percentage of IVF has not been affected because they used ICSI as a fertilization technique, which compensates for the disadvantage caused by vitrification with the hardening of the zona pellucida, which with a conventional fertilization would not be favored [55]. In addition, in both studies, oocytes were vitrified in the MII stage, in which the maternal genes are expressed and the embryos depend on the reserves of mRNA and the proteins stored in the cytoplasm of the oocyte, so, if they occur, some repair mechanism has already been activated when vitrification was performed [56]. However, it is not difficult to consider that the damage to the genetic material can cause genomic instability, leading to chromosomal aberrations such as aneuploidies.

Aneuploidy formation

Chromosomal abnormalities that occur in embryos are an important cause of pregnancy loss and greatly impair the normal ED and the fetus or lead to the birth of individuals suffering from various congenital abnormalities [57,58]. Structural and numerical chromosomal aberrations are important biological points in genotoxic studies. With the implementation of the fluorescent in situ hybridization (FISH) technique with chromosome-specific DNA probes, the sensitivity of detection of chromosomal aberrations can be increased.

Studies in farm animals are minimal compared to those in humans, however, numerical errors such as trisomy of particular chromosomes, monosomy of the X chromosome, polyploidy, as well as structural chromosomal abnormalities including Robertsonian translocations have been found in the number of chromosomes that arise from the union of two acrocentric chromosomes on a single metacentric (chromosome decreasing the haploid number) and reciprocals (segment exchange between two non-homologous chromosomes), or insertions similar to abnormalities present in humans [57-59].

The incidence and type of chromosomal abnormalities differ between gametes and embryos and even between species. In the pig there is a relatively high incidence of reciprocal translocations, in addition, the frequency of aneuploidy in oocytes or embryos varies due to different circumstances such as the different age of the animals used for experiments, methods used or by in vitro culture processes [57,59]. It was observed that, in early pig embryos obtained in vivo, 11 (14.3%) of the 77 embryos examined had aneuploidies, in chromosomes 8, 11, 12, 13, 17 and X being the most frequent, contrary to chromosomes 2, 9 and 18 that presented a lower frequency of chromosomal errors [57].

This is important in experimental research, since most of the studies that evaluate aneuploidies as a result of the production of genetic damage derived from vitrification have been performed in humans [60-63], due to the importance it has in clinical application. This is possible thanks to the scientific progress that has achieved the characterization of the predominant diseases derived from aneuploidies and polyploidies. However, it is necessary to extend

this knowledge in non-human species, since this can broaden the possibilities of the emergence of reproduction technologies in domestic species, which could also be applied in humans and that would have an advantage over the legal regulations involved in handling and experimentation with human cells.

Discussion

While it is true that the damage of oocytes and embryos caused by vitrification is produced, it is important to emphasize the lower cellular damage obtained by this technique compared to conventional slow freezing [9], so its use in ART remains promising. The vitrification of oocytes and embryos produces structural damage, which are inherent in the cytotoxicity of the CPA's used during the procedure. Several animal species and cell types have been studied (Table 1), in addition, the progress in microscopy has allowed the analysis and thus the implementation of various methodologies for its study. Some of these damages occur in the plasma membrane, the cytoskeleton and the genetic material, which result in the formation of aneuploidies in the resulting embryos (Table 1).

Table 1: Structural damage caused by vitrification in different species and meiotic stages.

Specie	Meiotic Stage	Vitrification Device	Cellular Injury	Injury Analysis Method	Reference
Human	MII	McGill Cryoleaf	Decreased inner membrane potential. Cytoskeletal reorganization. Alteration of mitochondrial function.	Fluorescence microscopy	[6]
	MII	Cryotop	Tubulin depolymerization.	Confocal microscopy	[7]
Porcine	GV, MII	Open pulled straw (OPS)	Spindles with misalignment of chromosomes. Abnormal distribution of F-actin.	LSCM	[26]
	Blastocyst	None	Fragmented-DNA	TUNEL	[51]
Ovine	MII	Cryotop	Abnormal organization of F-actin cytoskeleton.	Raman microspectroscopy	[35]
Mice	MI, MII	Cryotop	Spindles depolymerization.	Fluorescence microscopy	[36]

The increasing use of ART has increased the need to improve vitrification protocols, which although it is indisputable that they produce damage that naturally would not occur frequently, are necessary for the preservation of gametes, in particular the gamete female.

Derived from the need to continue implementing vitrification as one of the most used ART in clinical and experimental research, some alternatives have been considered to counteract the damage caused by this technique. As the use of glutathione (GSH) for the supplementation of the vitrification medium, which it has been observed that it promotes the development of mouse blastocysts derived from vitrified oocytes [64,65]. Resveratrol has been assigned the ability to decrease ROS levels, γ-H2AX accumulation and apoptotic production, in addition to improving embryonic development rates [43].

The importance of the structural integrity of the oocyte for the correct progressive development has been shown. Interactions between proteins associated with the plasma membrane play a crucial role in the polymerization and aggregation of the cytoskeleton protein structures. The polymerization of actin and microtubules will establish their distribution and rearrangement within the cell at different stages of development, participating in the stabilization of the meiotic spindle, therefore, the appropriate aggregation of chromatin. In addition to the presence of endogenous proteins and/or mRNA transcripts, such as HR and NHEJ, which participate in DNA repair and the preservation of the integrity of the maternal genome that can prevent the formation of chromosomal aberrations such as aneuploidies.

Conclusion

Oocytes and embryos manipulation, typical of the implementation of ART, results in structural and genetic changes have an impact on the normal cellular development. However, the need for the implementation of these technologies in society is increasing, not only for the application in human reproduction, but also for species of animals that coexist with it and that their manipulation represents an advantage for the human, either economically, production, research or preservation.

In order to guarantee the genetic maintenance of the individuals, the need to preserve the integrity, as much as possible, of the reserves of endogenous proteins and/or transcripts of mRNA of the oocytes for the repair of DNA is unquestionable, which is expected to result consequently in the integrity, proper functioning and correct distribution of other cellular structures such as cytoskeleton and plasma membrane. What will guarantee the progression of the cellular development of the oocytes produced in vitro until the ED, which not only must maintain the standards of development quality such as viability, maturation, IVF and ED, but guarantee the production of embryos that maintain their genetic integrity which will result in the implantation of the embryo, term pregnancy and the birth of offspring not only viable but with the absence of chromosomal aberrations that ensure an assertive and healthy development for the individual.

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Conflicts of Interest

No conflicts of interest.

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Effects of Porcine Immature Oocyte Vitrification on Actin Microfilament Distribution and Chromatin Integrity During Early Embryo Development *in vitro*

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Vitrification is mainly used to cryopreserve female gametes. This technique allows maintaining cell viability, functionality, and developmental potential at low temperatures into liquid nitrogen at −196°C. For this, the addition of cryoprotectant agents, which are substances that provide cell protection during cooling and warming, is required. However, they have been reported to be toxic, reducing oocyte viability, maturation, fertilization, and embryo development, possibly by altering cell cytoskeleton structure and chromatin. Previous studies have evaluated the effects of vitrification in the germinal vesicle, metaphase II oocytes, zygotes, and blastocysts, but the knowledge of its impact on their further embryo development is limited. Other studies have evaluated the role of actin microfilaments and chromatin, based on the fertilization and embryo development rates obtained, but not the direct evaluation of these structures in embryos produced from vitrified immature oocytes. Therefore, this study was designed to evaluate how the vitrification of porcine immature oocytes affects early embryo development by the evaluation of actin microfilament distribution and chromatin integrity. Results demonstrate that the damage generated by the vitrification of immature oocytes affects viability, maturation, and the distribution of actin microfilaments and chromatin integrity, observed in early embryos. Therefore, it is suggested that vitrification could affect oocyte repair mechanisms in those structures, being one of the mechanisms that explain the low embryo development rates after vitrification.

Keywords: vitrification, embryo development, immature oocytes, porcine (pig) model, actin microfilaments, chromatin, *in vitro* fertilization-embryos

Abbreviations: ACHR, abnormal chromatin; ACT, activated; ART, assisted reproduction techniques; CHR, chromatin; CA, cortical actin; CA/D, cortical actin with damage; CA/ND, cortical actin without damage; CPAs, cryoprotectant agents; COCs, cumulus-oocyte complexes; DH, decondensed sperm heads; DA, dispersed actin; DA/D, dispersed actin with damage; DA/ND, dispersed actin without damage; DCA, dispersed cortical actin; DCA/D, dispersed cortical actin with damage; DCA/ND, dispersed cortical actin without damage; ED, embryo development; FITC, fluorescein isothiocyanate; GV, germinal vesicle; IVF, *in vitro* fertilization; IVM, *in vitro* maturation; MM, maturation medium; MI, metaphase I; MII, metaphase II; MTT, methyl tetrazolium; MF, microfilaments; MSP, monospermic; N, nucleus; ND, nucleus without damage; PP, polyspermic; PN, pronuclei; mTBM, tris-buffer medium; UF, unfertilized; VW, vitrification warming.

INTRODUCTION

Currently, vitrification is mostly used to cryopreserve gametes and embryos. It is intended to maintain cell viability, functionality, and developmental potential when they are stored at low temperatures (Chian et al., 2004; Casillas et al., 2018). In recent years, vitrification has been a useful tool for assisted reproduction techniques (ART), so that the scientific and technical progress in this field has been developed for female gametes. In humans, it is considered an important resource in the treatment of reproductive conditions and infertility (Khalili et al., 2017), as well as to improve the reproductive capacity and gamete quality in economically important and endangered species (Mullen and Fahy, 2012). The cryoprotectant agents (CPAs) are substances that protect cells during cooling and warming. However, their use in high concentrations increases the risk of osmotic damage caused by their chemical components (Chian et al., 2004). Although substantial progress has been made to improve vitrification protocols by the use of co-culture systems (Jia et al., 2019), the Cryotech method (Angel et al., 2020; Nowak et al., 2020), the reduction of the volume of cryopreservation cell devices, and CPA selection (Sun et al., 2020), the recovery of intact morphophysiological gametes after vitrification are still low due to the damage generated in cell structures, mainly the plasma membrane, cytoplasm, nucleus, and DNA (Chang et al., 2019). In this regard, it was reported that the extent of the cell damage depends on the nuclear cell stage (Somfai et al., 2012; Egerszegi et al., 2013).

During vitrification, the addition of CPAs is required for cell protection, and it depends on the animal species, the cell type, and the chemical nature of the CPAs to select an appropriate vitrification procedure. In pigs, vitrification can cause alterations in actin microfilaments (MF) and chromatin (CHR), affecting oocyte viability, maturation, fertilization, and embryo development (ED). Previous studies have evaluated the effect of vitrification on germinal vesicle (GV), metaphase II (MII) oocytes, zygotes, and blastocysts in the same stage of development (Egerszegi et al., 2013). Other studies evaluated the role of MF and CHR based on the fertilization and ED rates, but they did not determine the alterations of these structures (Rajaei et al., 2005; Egerszegi et al., 2013). Therefore, this study was designed to evaluate how the vitrification of porcine immature oocytes affects early ED according to the distribution of actin MF and CHR integrity.

MATERIALS AND METHODS

Ethics Statement and Animal Care

This study was approved under the regulations of the Ethics Committee for care and use of animals, Metropolitan Autonomous University-Iztapalapa Campus.

Experimental Design

Five replicates were performed for all experiments. After selection, the cumulus–oocyte complexes (COCs) were divided into two groups: (a) control group, fresh GV oocytes underwent *in vitro* maturation (IVM), and subsequently fertilized *in vitro* (IVF) for early ED (two to four blastomeres) through 40 h and (five to eight blastomeres) through 68 h. (b) Experimental group, vitrified GV oocytes, then IVM in a co-culture with fresh granulosa cells, followed by IVF and early ED. In both groups, the viability in oocytes and embryos was evaluated by methyl tetrazolium (MTT) staining. IVM, IVF, and ED were evaluated by bisbenzimide (Hoechst 33342) staining. The analysis of actin MF distribution was carried out using phalloidin–fluorescein isothiocyanate conjugate (phalloidin–FITC), and CHR by Hoechst staining (Rojas et al., 2004).

Chemicals, Culture Media, and Culture Conditions

Unless otherwise stated, all reagents were purchased from Sigma Chemical Co. (St. Louis, MO, United States), and different culture media were prepared in the laboratory. For COCs collection and washing, Tyrode's medium containing 10 mM HEPES, 10 mM sodium lactate, and 1 mg/ml of polyvinyl alcohol (TL-HEPES-PVA) were used (Abeydeera et al., 1998).

For oocyte vitrification and warming, TCM-199-HEPES medium was supplemented with 0.5 mM L-glutamine and 0.1% 200 PVA (VW medium). To perform IVM, the maturation medium (MM) consisted of TCM 199 with Earle's salt medium, supplemented with 26.2 mM sodium bicarbonate, 0.1% PVA, 3.05 mM D-glucose, 0.91 mM sodium pyruvate, 0.57 mM cysteine, and 10 ng/ml of EGF (In Vitro, Mexico).

The medium for fertilization was Tris-buffered (mTBM) containing 3 mM KCl, 13.1 mM NaCl, 7.5 mM $CaCl_2$, 20 mM Tris, 11 mM glucose, and 5 mM sodium pyruvate, 0.4% fraction V bovine serum albumin (BSA), and 2.5 mM caffeine (Abeydeera and Day, 1997).

The medium for early embryo culture was North Carolina State University-23 (NCSU-23) medium supplemented with 0.4% BSA (Petters and Wells, 1993). All culture media and samples were incubated under mineral oil at 38.5°C with 5% CO_2 in air and humidity at saturation.

Oocyte Collection

Porcine ovaries were obtained from pre-pubertal Landrace gilts at "Los Arcos" slaughterhouse, Edo. de México and transported to the laboratory in 0.9% NaCl solution at 25°C. The slaughterhouse is registered in health federal law authorization under the number 6265375. For COCs collection, ovarian follicles between 3 and 6 mm in diameter were punctured using an 18-gauge needle set to a 10-ml syringe. Oocytes with intact cytoplasm and surrounded by cumulus cells were selected for the assays.

Vitrification and Warming

For vitrification, COCs were washed twice in VW medium and equilibrated in the first vitrification solution containing 7.5% dimethylsulfoxide (DMSO) and 7.5% ethylene glycol (EG) for 3 min. Then, COCs were exposed to the second vitrification solution containing 16% DMSO, 16% EG, and 0.4 M sucrose for 1 min, and at least nine oocytes were immersed in a 2-μl drop and loaded into the Cryolock (Importadora Mexicana de Materiales

para Reproducción Asistida S. A. de C.V. Mexico). Finally, in less than 1 min, the Cryolock was plunged horizontally into liquid nitrogen at −196°C, then COCs were vitrified for 30 min (Casillas et al., 2014). For warming, the one-step method was performed (Sánchez-Osorio et al., 2010). For COCs recovery, the Cryolock was immersed vertically in a four-well dish containing 800 μl of VW medium with 0.13 M sucrose. Immediately, oocytes were incubated in the same medium for 5 min, then recovered for IVM (Sánchez-Osorio et al., 2008).

In vitro Maturation

Control and vitrified-warmed COCs were washed in 500 μl of MM three times. Afterward, 30–40 oocytes were randomly distributed in a four-well dish (Thermo-Scientific Nunc, Rochester NY) containing 500 μl of MM with 0.5 μg/ml of LH and 0.5 μg/ml of FSH (Ducolomb et al., 2009) for 44 h (Casas et al., 1999). Vitrified oocytes were matured in MM with a co-culture with fresh granulosa cells for 44 h (Casillas et al., 2014). The total numbers of evaluated cells in the control and vitrification groups were 256 and 143, respectively.

In vitro Fertilization

After IVM, oocytes were denuded mechanically with a 100-μl micropipette. Then, the oocytes were washed three times in MM and three times in mTBM in 500-μl drops covered with mineral oil. For IVF, 40–60 oocytes were placed in 50-μl drops of mTBM covered with mineral oil and incubated at 38.5°C with 5% CO_2 and humidity at saturation for 1 h until insemination (Ducolomb et al., 2009).

Semen was obtained by the gloved hand method on a commercial farm; a 1:10 dilution was made with boar semen extender (MR-A, Kubus, S.A.) and transported to the laboratory at 16°C. Five microliters of semen was diluted in 5 ml of PBS-Dulbecco, Gibco, 1:1 dilution, supplemented with 0.1% BSA fraction V, 0.1 μg/ml of potassium penicillin G, and 0.08 μg/ml of streptomycin sulfate. It was centrifuged at 61 × *g* at 25°C for 5 min. The supernatant was diluted 1:1 with PBS-Dulbecco and centrifuged at 1,900 × *g* for 5 min. The supernatant was removed and suspended in 10 ml of PBS-Dulbecco and centrifuged at 1,900 × *g* for 5 min. The pellet was suspended with 100 μl of mTBM. From this solution, 10 μl was diluted 1:1,000 with mTBM to calculate a final concentration of 5×10^5 spermatozoa/ml. Finally, 50 μl of the sperm suspension was co-incubated for 6 h with the matured oocytes for fertilization at 38.5°C (Ducolomb et al., 2009). The fertilization rate was determined through pronuclei (PNs) formation, and the total numbers of evaluated cells in the control and vitrification groups were 126 and 95, respectively.

Embryo Development

After co-incubation, oocytes were washed three times in 50-μl drops of NCSU-23 medium (Petters and Wells, 1993) supplemented with 0.4% fatty acid–free BSA and placed in drops of 500 μl of the same medium covered with mineral oil in a four-well dish and incubated for 16 h. The evaluation of early ED was performed after 40 and 68 h of incubation (Ducolomb et al., 2009). The total numbers of evaluated cells in the control and vitrification groups were 241 and 151, respectively.

Evaluation of Oocytes and Embryo Viability

Viability was analyzed in oocytes and embryos with MTT staining at T 0 h after oocyte collection, and vitrification, after 44 h of IVM, and after 40 and 68 h of early ED (Mosmann, 1983). Oocytes and embryos were stained with 100-μl drops of 0.5 mg/ml of MTT diluted in mTBM. After 1.30 h, oocytes and embryos were observed under a light microscope (Zeiss Axiostar). Cells showing a purple stain were considered alive, and those colorless were considered dead (**Figure 1A**). The total numbers of evaluated cells in the control and vitrification groups were 386 and 381, respectively.

Evaluation of Oocyte Maturation

Maturation was evaluated by Hoechst stain. Oocytes were stained with 10 μg/ml of Hoechst for 40 min using a confocal scanning laser microscope (Zeiss, LSM T-PMT) for observation. Maturation was evaluated at 44 h of incubation; oocytes with a germinal vesicle (GV) or in metaphase I (MI) were considered as immature and those in metaphase II (MII) with the first polar body as mature (**Figure 2A**).

Evaluation of *in vitro* Fertilization

Zygotes and embryos were stained with 10 μg/ml of Hoechst for 40 min using Zeiss, LSM T-PMT for observation. To evaluate fertilization, oocytes with one pronucleus (PN) were considered activated (ACT) (**Figure 3Aa**) and those with two pronuclei as monospermic (MSP) (**Figure 3Ab**), and more than two decondensed sperm heads (DH) or more than two-pronuclei were considered polyspermic (PP) (**Figure 3Ac**). Oocytes in MII (first polar body) were considered as not fertilized (UF) (**Figure 3Ad**).

Evaluation of Embryo Development

Early ED was evaluated 40 h after IVF, two to four cell embryos (**Figure 4Aa**), and 68 h after IVF five to eight cell embryos (**Figure 4Ac**).

Hoechst Staining and Immunocytochemistry (Actin Microfilaments and Chromatin)

For CHR evaluation, embryos were stained with Hoechst and, for actin MF, by immunofluorescence with phalloidin-FITC, 1:350 in PBS. After 40 and 68 h of incubation, early embryos were washed three times to 500-μl drops of PBS-BSA; then, 300 μl of Hoechst was added and kept at 4°C for 45 min; afterward, 200 μl of 4% paraformaldehyde fixative solution was added and kept at 4°C overnight. After, 200 μl of PBS–Triton X-100 1% permeabilizing solution was added at 4°C for 2 h and washed. Next, 200 μl of blocking solution was added, with 0.02 g/ml of PBS–BSA, 0.02 g/ml of skimmed milk, and 0.011 g/ml of glycine diluted in PBS, for 1 h at room temperature. For MF labeling, 200 μl of the phalloidin–FITC was added and kept at 4°C for 2 h,

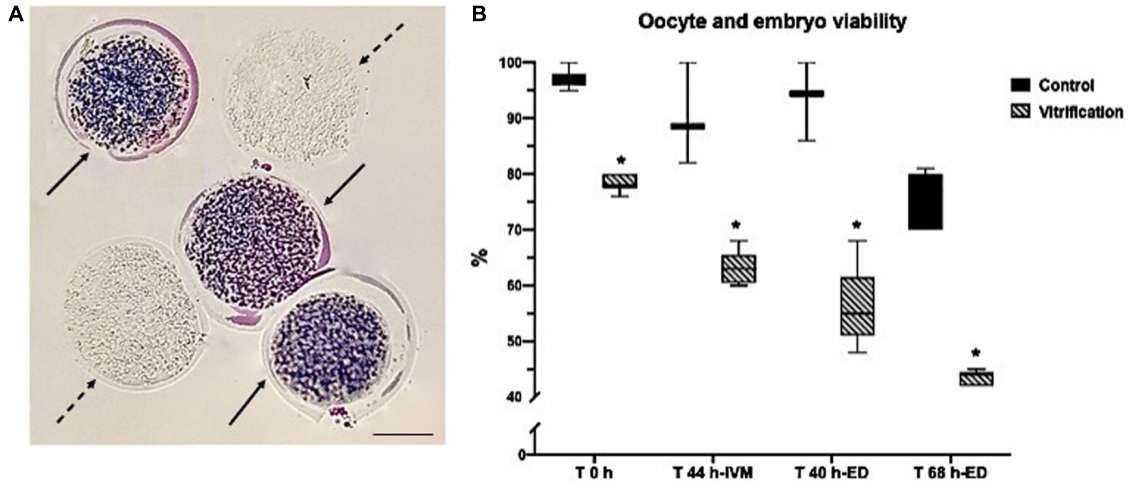

FIGURE 1 | Oocyte and embryo viability evaluation by methyl tetrazolium (MTT) staining in control and vitrified groups: **(A)** representative image for oocyte viability evaluation criteria (continuous arrows show purple living cells, and dotted arrows show colorless dead cells); 10×, scale bar = 50 μm; **(B)** percentage of oocyte viability at T 0 h, after T 44 h-IVM and early embryos after T 40 and 68 h-ED; the total numbers of evaluated cells in the control and vitrification groups were 386 and 381, respectively. *Significant difference vs. control. $P < 0.05$.

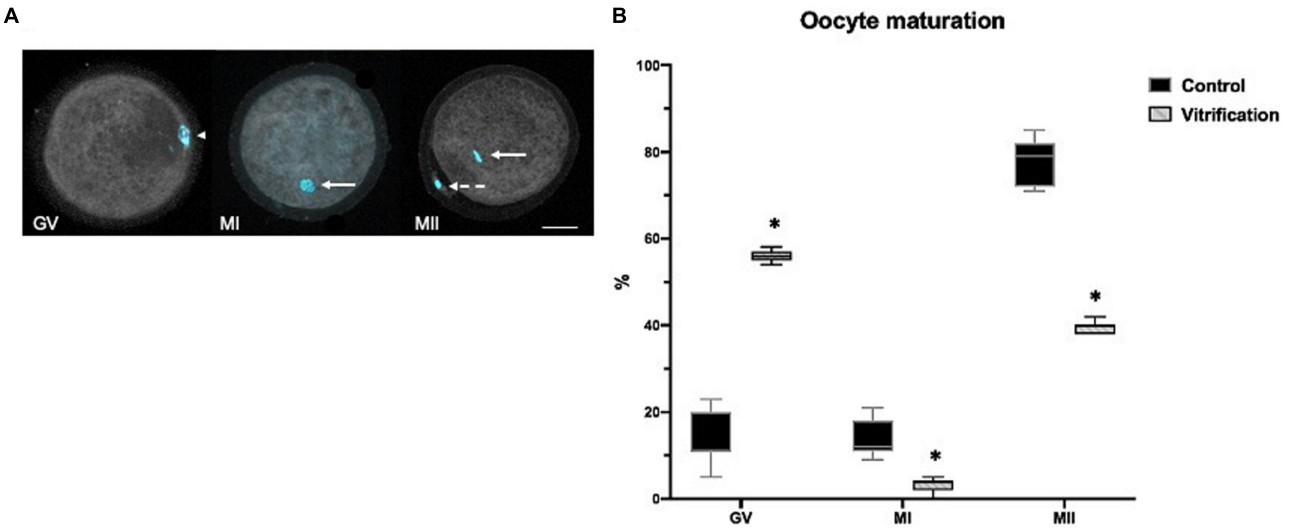

FIGURE 2 | Oocyte *in vitro* maturation (IVM) evaluation by Hoechst staining in control and vitrified groups: **(A)** representative image for oocyte IVM evaluation criteria (oocytes were classified as: arrowhead shows germinal vesicle (GV), continuous arrow shows metaphase I (MI), continuous arrow shows metaphase II (MII), and the dotted arrow show the polar body; 400×, scale bar = 30 μm; **(B)** percentage of oocyte IVM; the total numbers of evaluated cells in the control and vitrification groups were 256 and 143, respectively. *Significant difference vs. control. $P < 0.05$.

then, transferred three times to 500-μl drops. All the incubations were performed in the dark. The washing of the embryos was made with PBS–BSA. The slide mounting was performed with PBS/glycerol 1:9 on slides and covered with a coverslip and sealed with transparent nail polish (Rojas et al., 2004).

Images were obtained using Zeiss, LSM T-PMT. The analysis was carried out capturing Z stack series, through four sections covering the whole embryo. MF visualization (green) was by Phalloidin–FITC with an excitation wavelength of 490 nm and an emission of 525 nm. For CHR (blue), Hoechst had an excitation of 350 nm and an emission of 470 nm. The evaluation of images was performed using the Image J Processor.

For actin MF distribution evaluation, embryos were classified as embryos with cortical actin (CA) (**Figure 5Aa**), disperse actin (DA) (**Figure 5Ab**), and dispersed cortical actin (DCA) (**Figure 5Ac**). Embryos showing CA were considered with good quality and high developmental potential (**Figure 5Aa**); DCA was considered as a medium quality embryo indicator (**Figure 5Ac**) and DA as a low embryo quality, with less developmental potential (**Figure 5Ab**). For CHR evaluation, two classifications

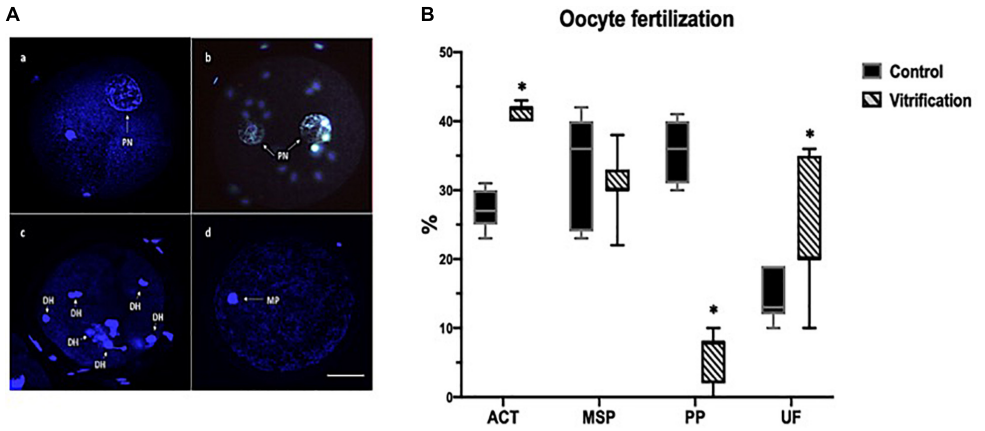

FIGURE 3 | Oocyte *in vitro* fertilization (IVF) evaluation by Hoechst staining in the control and vitrified groups. **(A)** Representative images for IVF evaluation criteria: (a) activated oocyte, arrow shows one PN; (b) monospermic zygotes, arrows show two PN; (c) polyspermic oocyte, arrows show decondensed sperm heads, and (d) unfertilized oocytes, arrow shows the metaphase I; pronuclei (PN), decondensed sperm heads (DH), metaphase (MP); **(B)** percentage of oocyte IVF; activated (ACT), monospermic (MSP), polyspermic (PP), and unfertilized oocytes (UF); the total numbers of evaluated cells in the control and vitrification groups were 126 and 95, respectively; 200×, scale bar = 30 μm. *Significant difference vs. control. $P < 0.05$.

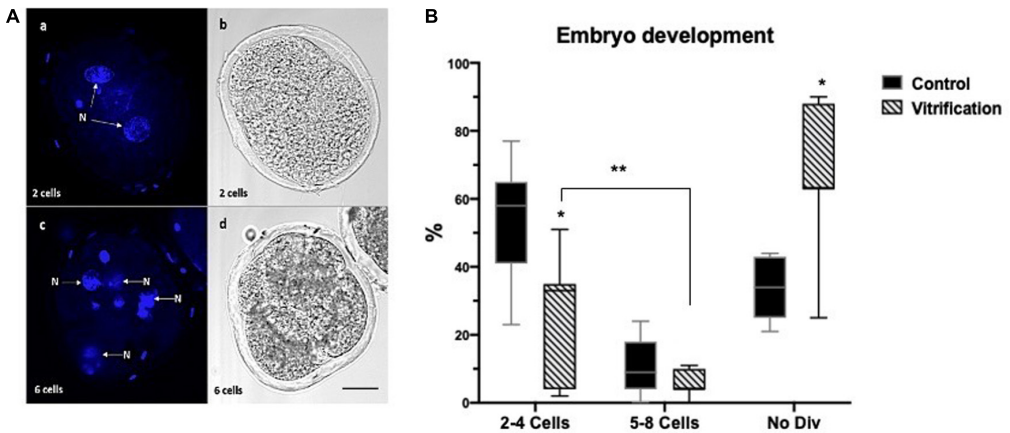

FIGURE 4 | Early embryo development (ED) evaluation by Hoechst staining in the control and vitrified groups. **(A)** Representative images for early ED evaluation criteria; (a,c) blastomeres stained with Hoechst; (b,d) clear field showing embryos blastomeres; (N) nucleus; the total numbers of evaluated cells in the control and vitrification groups were 241 and 151, respectively; 200×, scale bar = 30 μm; **(B)** percentage of early ED [two to four cells, five to eight cells, and non-divided (No Div)]; *Significant difference vs. control. $P < 0.05$. **Significant differences between groups. $P < 0.05$.

in both groups were considered: embryos without damage (ND) and with damage (D) (**Figure 6A**). The ND CHR embryos presented well-defined nuclei (**Figure 6Aa**). D CHR embryos were considered when one or more abnormal chromatin (ACHR) structures were identified (**Figure 6Ab**). ND CHR embryos are related to good quality with a high probability of successful ED. D CHR embryos have less embryo development potential. For MF evaluation, the total numbers of evaluated cells in the control and vitrification groups were 61 and 35, respectively. For CHR evaluation, the total numbers of evaluated cells in the control and vitrification groups were 64 and 33, respectively.

Statistical Analysis

Statistical analyses were carried out using GraphPad Prism 8.2.1 (Graphpad Software Inc.). Data from vitrified and control groups were compared with non-parametric Mann–Whitney *U* test with a confidence level of $P < 0.05$, and percentage data are presented as mean ± standard deviation (SD) values.

RESULTS

Evaluation of Oocytes, Embryo Viability, and Oocyte Maturation

After collection (T 0 h), 97% of the control oocytes were alive, but this percentage was reduced significantly (78%) after vitrification ($P < 0.05$). Oocyte viability after 44 h of *in vitro* maturation in the control was 89%, while in the vitrified group, it was 63% ($P < 0.05$). After 40 h of embryo development, 94% of embryos were alive in the control and decreased significantly in

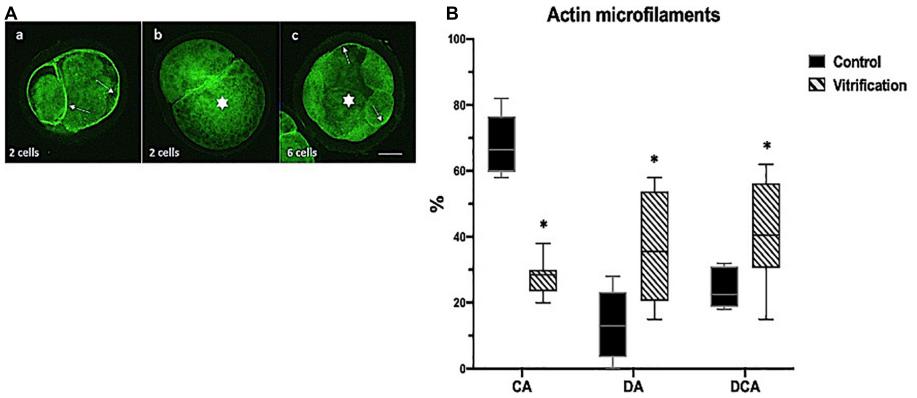

FIGURE 5 | Evaluation of actin microfilaments (MF) in early embryos by phalloidin-fluorescein isothiocyanate (FITC) staining in the control and vitrified groups; **(A)** representative images for actin microfilament evaluation criteria in early ED: (a) white arrows show cortical actin (CA); (b) white star shows dispersed actin (DA), and (c) white star shows dispersed cortical actin (DCA); the total numbers of evaluated cells in the control and vitrification groups were 61 and 35, respectively; 200×, scale bar = 30 μm; **(B)** percentage of actin microfilaments in early ED. *Indicate significant difference vs. control. $P < 0.05$.

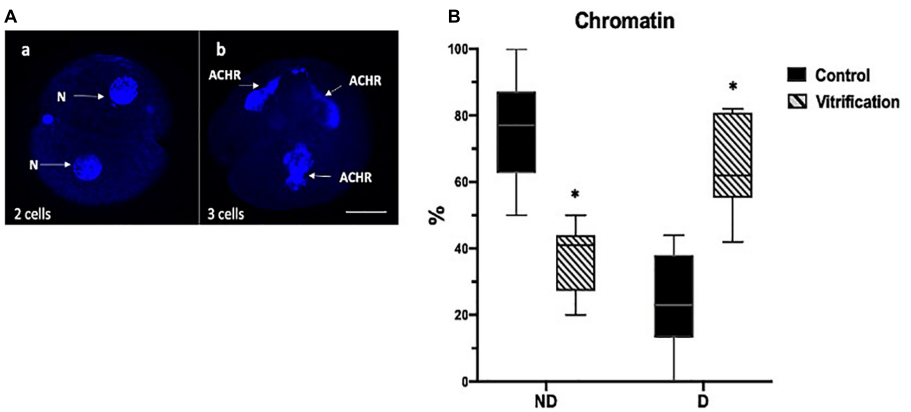

FIGURE 6 | Chromatin (CHR) evaluation in early ED by Hoechst staining in control and vitrified groups. **(A)** representative images for CHR evaluation criteria in early ED; (a) 2-Cell embryo, white arrows show nucleus without damage (ND), and (b) 3-Cell embryo, white arrows show abnormal chromatin (ACHR); the total numbers of evaluated cells in the control and vitrification groups were 64 and 33, respectively; 200×, scale bar = 30 μm; **(B)** percentage of CHR in early ED. *Significant difference vs. control. $P < 0.05$.

the vitrified group up to 54% ($P < 0.05$). After 68 h of embryo development, viability decreased up to 42% ($P < 0.05$) in the vitrification group compared with that of the control (**Figure 1B**).

Figure 2B shows the percentage of oocytes *in vitro* maturation in both groups. Maturation (metaphase II-first polar body) was significantly lower in the vitrification group 40% compared with the control 79% ($P < 0.05$). A higher germinal vesicle rate (58%) was obtained in the vitrification group ($P < 0.05$) compared with that of the control (12%). Also, the percentage of metaphase I oocytes was significantly lower in the vitrification group ($P < 0.05$) compared with that of the control (4 and 12%, respectively).

Evaluation of *in vitro* Fertilization and Embryo Development

In vitro fertilization results indicated that vitrified oocytes displayed a higher percentage ($P < 0.05$) of activated (one-pronucleus) and unfertilized (without pronuclei formation) rates compared with that of the control (42 vs. 27%, 20 vs. 13%, respectively); however, polyspermic fertilization (more than two-pronuclei) in vitrified oocytes was significantly lower ($P < 0.05$) than that of the control (8 vs. 36%, respectively). Meanwhile, monospermic fertilization (two-pronuclei) had no significant difference between both groups ($P > 0.05$) (36% control vs. 31% vitrified) (**Figure 3B**).

The percentage of early embryo development at 40 and 68 h of incubation in both groups are shown in **Figure 4B**. In the control group, a higher percentage of embryos with two to four cells (58%) was found compared with the vitrification group (33%) ($P < 0.05$). In vitrified oocytes, a higher percentage of undivided embryos (No Div) (63%) was obtained compared with that in the control (33%) ($P < 0.05$). However, both groups were not statistically different in the production of five to eight cell embryos ($P > 0.05$) (9% control vs. 4% vitrified). Also, the percentage of embryos that reached five to eight cells was

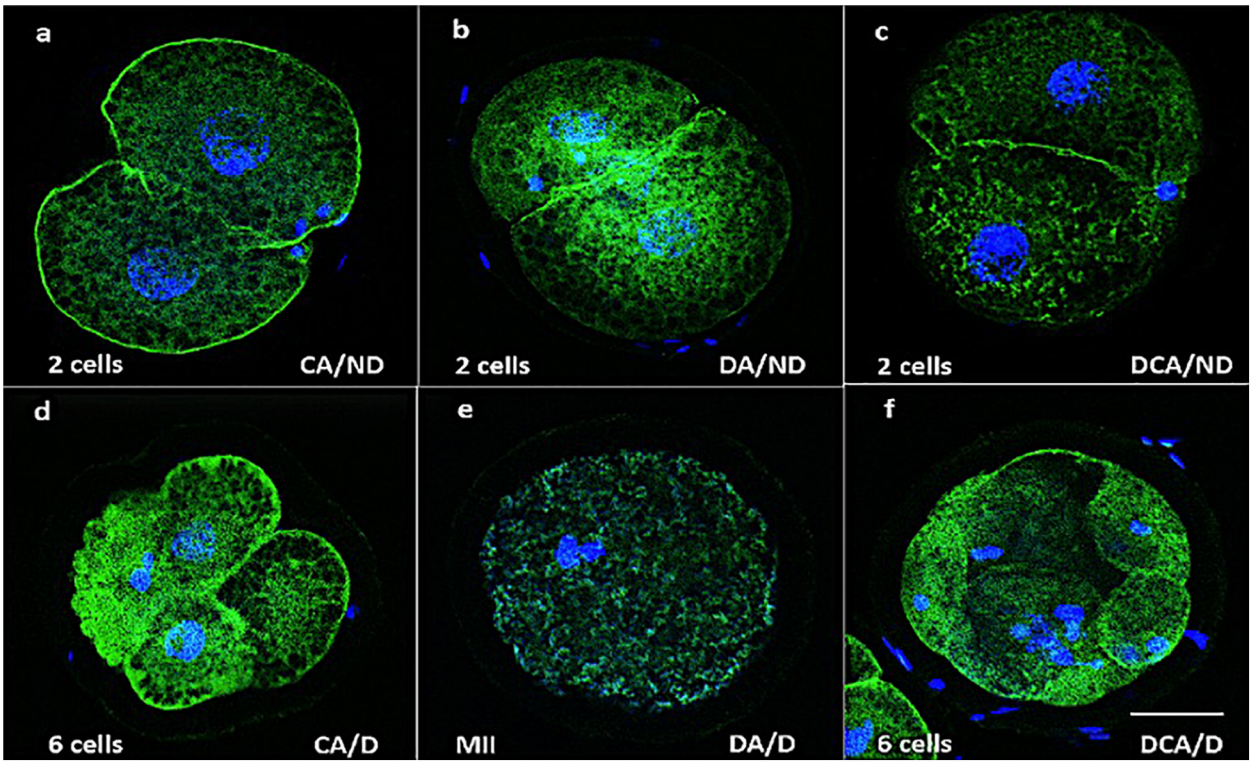

FIGURE 7 | Representative merged images of actin microfilaments (green) and chromatin (blue) in early ED. **(a–c)** Images correspond to embryos from fresh oocytes, and **(d–f)** images correspond to embryos from vitrified oocytes. **(a)** Cortical actin without damage (CA/ND); **(b)** dispersed actin without damage (DA/ND); **(c)** dispersed cortical actin without damage (DCA/ND); **(d)** cortical actin with damage (CA/D); **(e)** dispersed actin with damage (DA/D); **(f)** dispersed cortical actin with damage (DCA/D); 200×, scale bar = 30 μm.

significantly lower after vitrification than the two to four cells ($P > 0.05$).

Evaluation of Actin Microfilament Distribution and Chromatin Integrity in Early Embryos

Results in the control group showed a higher percentage of embryos with cortical actin compared with the vitrification group ($P < 0.05$) (67 vs. 29%). In the vitrified group, higher percentages of dispersed actin (32 vs. 11%) and dispersed cortical actin (40 vs. 23%) were obtained compared with the control ($P < 0.05$) (**Figure 5B**).

For chromatin evaluation, results indicate that in the control, a higher percentage of embryos with chromatin without damage, with well-defined nuclei was obtained compared with the vitrification group ($P < 0.05$) (77 and 41%, respectively). Also, there was a greater percentage of embryos in the vitrification group with chromatin damage, with one or more scattered chromatin structures and without embryo division (61 and 23%, respectively) (**Figure 6B**).

Figure 7 shows the merged images from microfilaments and chromatin evaluation in early embryos. Pictures a, b, and c correspond to the control group and images d, e, and f to the vitrification group. Some embryos show damaged blastomeres, and others did not divide. It seems that the cortical distribution of actin microfilaments and the integrity of the chromatin are related to embryo quality. Oocytes with dispersed actin also showed abnormal chromatin distribution and even the absence of cell division (**Figure 7a**). In contrast, control embryos showed normal chromatin conformation (ND), with cortical actin, or with some degree of actin dispersion (**Figures 7a,c**).

DISCUSSION

Over the past years, several methods for cryopreservation have been developed through the vitrification of immature (Casillas et al., 2018) and mature oocytes (Casillas et al., 2020) as well as for embryos in different developmental stages. In general, studies have shown that embryos have greater probabilities of survival after vitrification than immature and mature oocytes.

Immature oocyte vitrification in women is important because ovarian hyperstimulation can be avoided. Also, in other mammalian species, it is possible to recover a greater number of GV oocytes than MII. It has been reported that vitrification success in immature oocytes depends on the species and so different strategies are used. To evaluate the quality of embryos produced *in vitro* from vitrified immature oocytes, several aspects must be considered through the oocyte maturation, fertilization,

as well as ED, like CPAs, containers, warming procedures, and recently the use of cumulus cell–oocyte co-culture (Casillas et al., 2015; Kopeika et al., 2015; de Munck and Vajta, 2017).

Although there is great progress in the knowledge of oocyte vitrification, the rate of embryos reaching morulae and blastocyst stages remains low; therefore, a few births of live offspring from vitrified immature oocytes are reported (Somfai et al., 2010). Several studies have used different approaches to explain the possible causes for the decrease in viable embryos produced from vitrified oocytes. One of the main parameters affected by vitrification is oocyte viability. After 44 h of IVM, in the vitrification group, it was 63% lower than that of the control group, which was 89% (Casillas et al., 2014). According to the literature, this is the first study that directly evaluates the viability of embryos derived from vitrified immature oocytes. In several species, studies reported only the percentage of ED to consider this parameter (Rajaei et al., 2005; Somfai et al., 2010; Chatzimeletiou et al., 2012; Fernández-Reyes et al., 2012). In the present study, results indicate that viability decreased significantly in embryos derived from vitrified oocytes compared with that of the control group (54 vs. 94%, respectively). This indicates that vitrification affects oocyte differentiation and ED *in vitro*. Although oocytes survived during this process, they were affected to carry out an optimal ED. However, the percentage of fertilized oocytes was similar in both groups. The percentage of polyspermy was lower in vitrified oocytes compared to the control.

In this study, there was a decrease in the IVM rate in vitrified oocytes compared with the control group. Similar results were previously reported (Casillas et al., 2020). According to the literature, studies evaluating IVM in vitrified oocytes have reported different results. This may be due to the oocyte maturation stage before vitrification (GV), cell containers, types of cryoprotectants, temperatures, or different cooling and warming procedures (Fernández-Reyes et al., 2012; Casillas et al., 2014; Wu et al., 2017). In addition, Somfai et al. (2010) reported 77% of IVM in the control vs. 22% in the vitrified oocytes using the solid surface vitrification method. These results are similar to those obtained in the present study; however, they use maturation media supplemented with porcine follicular fluid.

Actin is an essential component of the cytoskeleton that achieves functions such as cell migration and division, and the regulation of gene expression, which are basic processes for ED. Besides, microfilaments rearrange the organelles involved in fertilization (Sun and Schatten, 2006), such as extrusion of the second polar body and reorganization of the smooth endoplasmic reticulum for the generation of intracellular Ca^{2+} during oocyte activation (Chankitisakul et al., 2010; Bunnell et al., 2011; Gualtieri et al., 2011; Egerszegi et al., 2013; Martin et al., 2017). In mammals, the cortical distribution of actin microfilaments in mature oocytes is polarized, which is evidenced by swelling near the metaphase axis and less in the opposite cortical domain. In immature oocytes, the actin microfilament distribution does not appear polarized. This indicates that F-actin has a restructuration for polymerization during oocyte maturation (Coticchio et al., 2014, 2015b). Some studies have evaluated the effect of oocyte vitrification in the cytoskeleton, showing an interruption in the cortical microfilaments network, as well as disorganization of the microtubule spindle, which led to chromosomal dispersion (Rojas et al., 2004; Chatzimeletiou et al., 2012).

These data could explain the possible mechanism of damage derived from immature oocyte vitrification. Our results showed significant differences in the percentage of early embryos with actin dispersion. Vitrification inhibits the correct polymerization of G-actin, reflected in an MF breakdown in the cytoplasm and its disruption in the cortical area (Baarlink et al., 2017; Misu et al., 2017).

In the vitrification group, there was a high rate of No Div embryos. The percentage of embryos that reached five to eight cells was also lower after vitrification than the two to four cells. This could be related to the lack of damage repair mechanisms in the oocytes. Some blastomeres in the early embryos showed lower cortical actin compared with that of the control group. Besides, some disrupted actin polymerization (dispersed actin and dispersed cortical actin). An increase in blastomere DNA fragmentation in blastocyst is attributed to the cryoprotectants (Rajaei et al., 2005). Also, it was reported that the cytotoxicity of these agents occurs in a greater proportion of cells with high metabolic activity as immature oocytes or embryos (Lawson et al., 2011). Cryoprotectants interrupt the cortical microfilament network, causing spindle depolymerization and disorganization, which leads to chromosomal dispersion that may trigger aneuploidies during the ED (Rojas et al., 2004; Chatzimeletiou et al., 2012).

Actin microfilaments also are involved in the immobilization of mitochondria to the cell cortex or sites with high ATP utilization (Boldogh and Pon, 2006). Depolarization and disorganization of the cytoskeleton will prevent the externalization of the meiotic organization toward the cortical zone and the alignment of the chromosomes on the equatorial axis in the oocytes (Gualtieri et al., 2011).

DNA is susceptible to a variety of chemical compounds and physical agents causing alterations in its conformation as a result of errors produced during replication, recombination, and repair (Coticchio et al., 2015a). Changes in chromatin are a result of histones modifying enzymes, which alter its post-transcription activities and ATP-dependent chromatin remodeling complexes (Farrants, 2008). Reduced production of ATP in human vitrified oocytes can be associated with depolymerization of actin microfilaments, causing failure in the DNA repair system, leading to chromatin disorders (Manipalviratn et al., 2011). In the present study, this mechanism may explain the high proportion of embryos derived from vitrified oocytes showing some type of damage in the chromatin affecting the DNA repair system.

It is important to highlight that several studies reported in the literature have evaluated the effect of vitrification in GV and MII oocytes, zygotes, and blastocysts at the same stages of development in which they were vitrified. In this study, the effect of vitrification was evaluated in the further early ED. Previous studies in zygotes or blastocysts analyzed only the role of actin microfilaments and chromatin in the success of the fertilization and embryo production (Wu et al., 2006; Somfai et al., 2010; Egerszegi et al., 2013); however, the distribution and the morphological characteristics of these structures in early ED

blastomeres has not been evaluated. Therefore, the present study provides important information that reveals the damage caused by vitrification in immature oocytes and their further early ED.

CONCLUSION

In conclusion, the results of this study indicate that the damage generated by the vitrification of immature oocytes affects viability, maturation, and the distribution of actin MF and CHR integrity, as observed in early embryos.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

This study was approved under the regulations of the Ethics Committee for care and use of animals; Metropolitan Autonomous University-Iztapalapa Campus.

AUTHOR CONTRIBUTIONS

AL developed the methodology, performed the experiments, analyzed the results, conducted the investigation, and prepared and wrote the original draft. YD also developed the methodology, performed the experiments, and analyzed the results. EC analyzed the results and reviewed and edited the manuscript. SR-M reviewed and edited the manuscript. MB and FC conceptualized the study and developed the methodology, software, data curation, prepared and wrote the original draft, conducted the visualization, investigation, supervision, validation of the study, reviewed, edited, and wrote the final manuscript. All authors contributed to the article and approved the submitted version.

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

Porcine Health Management

RESEARCH

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DNA damage in cumulus cells generated after the vitrification of in vitro matured porcine oocytes and its impact on fertilization and embryo development

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Abstract

Background: The evaluation of the DNA damage generated in cumulus cells after mature cumulus-oocyte complexes vitrification can be considered as an indicator of oocyte quality since these cells play important roles in oocyte developmental competence. Therefore, the aim of this study was to determine if matured cumulus-oocyte complexes exposure to cryoprotectants (CPAs) or vitrification affects oocytes and cumulus cells viability, but also if DNA damage is generated in cumulus cells, affecting fertilization and embryo development.

Results: The DNA damage in cumulus cells was measured using the alkaline comet assay and expressed as Comet Tail Length (CTL) and Olive Tail Moment (OTM). Results demonstrate that oocyte exposure to CPAs or vitrification reduced oocyte (75.5 ± 3.69%, Toxicity; 66.7 ± 4.57%, Vitrification) and cumulus cells viability (32.7 ± 5.85%, Toxicity; 7.7 ± 2.21%, Vitrification) compared to control (95.5 ± 4.04%, oocytes; 89 ± 4.24%, cumulus cells). Also, significantly higher DNA damage expressed as OTM was generated in the cumulus cells after exposure to CPAs and vitrification (39 ± 17.41, 33.6 ± 16.69, respectively) compared to control (7.4 ± 4.22). In addition, fertilization and embryo development rates also decreased after exposure to CPAs (35.3 ± 16.65%, 22.6 ± 3.05%, respectively) and vitrification (32.3 ± 9.29%, 20 ± 1%, respectively). It was also found that fertilization and embryo development rates in granulose-intact oocytes were significantly higher compared to denuded oocytes in the control groups. However, a decline in embryo development to the blastocyst stage was observed after CPAs exposure (1.66 ± 0.57%) or vitrification (2 ± 1%) compared to control (22.3 ± 2.51%). This could be attributed to the reduction in both cell types viability, and the generation of DNA damage in the cumulus cells.

Conclusion: This study demonstrates that oocyte exposure to CPAs or vitrification reduced viability in oocytes and cumulus cells, and generated DNA damage in the cumulus cells, affecting fertilization and embryo development rates. These findings will allow to understand some of the mechanisms of oocyte damage after vitrification that compromise their developmental capacity, as well as the search for new vitrification strategies to increase fertilization and embryo development rates by preserving the integrity of the cumulus cells.

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Keywords: Vitrification, Matured oocytes, Cumulus cells, DNA damage, Cryoprotectants, Porcine

Introduction

Oocyte vitrification has become an important tool for the improvement of assisted reproduction in humans and other mammalian species. The oocyte meiotic stage [1], the cryoprotectant agents (CPAs) selection, and the volume of the cell-storage device [2] are key factors associated with the success of vitrification. For vitrification, CPAs are used at high concentrations (16–50%), which causes detrimental effects in oocytes and compromises their further development. The toxicity and use of high CPAs concentrations have been a limiting factor for cryopreservation success. For this reason, cryoprotectant-free vitrification methods have been attempted in human spermatozoa [3] and equine oocytes [4] without success, and so far, it has never been performed in porcine. Moreover, results from previous studies demonstrated the need for CPAs [3–6], and recently new nontoxic CPAs has been proposed [7]. For oocyte cryopreservation, ethylene glycol (EG) and dimethylsulfoxide (DMSO) have been the most widely used permeable CPAs. It was reported that its use is safer than 1,2- propanediol (PROH) [8]. Somfai et al. [2] reported that the mixture of EG + propylene glycol (PG) is similar to EG + DMSO in blastocyst production after immature oocyte vitrification. Also, we reported that immature oocyte vitrification with EG + DMSO resulted in a 30% blastocyst formation [9]. Therefore, in the present study, EG + DMSO were used for metaphase II (MII) oocytes exposure or vitrification.

In humans and other mammalian species, oocytes are mostly recovered and vitrified at the MII stage [10–14] prior to in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI). At this stage, the cumulus cells (CCs) are only removed if ICSI is performed. However, studies have reported contradictory results, some of which highlight the beneficial effects of the CCs [15] surrounding the oocyte during vitrification [16], but another study reported that the removal of these cells before vitrification improves oocyte survival and maturation [17]. CCs play several major roles in oocyte maturation and fertilization [15] and have also been proposed as oocyte quality biomarkers [18]. In this regard, a study reported that vitrified MII oocytes with CCs resulted in higher IVF rates compared to denuded oocytes [19]. It was also reported that the CCs protect MII oocytes against zona pellucida hardening and cytoplasmic damage during vitrification-warming [20]. Additionally, the CCs prevent oocyte cryodamage by preserving the structure of major oocyte organelles after vitrification [21]. These cells are firstly exposed to the CPAs, preventing osmotic shock, facilitating oocyte dehydration, and reducing oocyte damage [20]. Because of this, it was reported that the CCs viability decreases considerably after cumulus-oocyte complexes (COCs) vitrification [20, 22, 23]. In contrast, other studies support that COCs vitrification reduces CPAs penetration and increases ice crystal formation in oocytes leading to inadequate dehydration, which affects oocyte survival [24, 25]. However, information about the alterations produced by vitrification in porcine CCs is limited.

The inefficiencies in determining the quality of the oocytes are a major issue that compromises successful fertilization rates. Since the direct evaluation of oocytes by invasive methods can impair their development, the study of CCs can reflect oocytes developmental competence. It has been reported that CCs and oocytes bidirectional communication is needed for the development and functions of both cell types [26]. Oocytes influence granulosa cells development by paracrine factors, and control metabolic activities by promoting gene expression in CCs [26]. Therefore, to evaluate if vitrification is capable of generating DNA damage in the CCs is of great importance for oocyte fertilization and embryo development (ED). For this purpose, DNA fragmentation can be measured by means of the comet assay [27]. Most studies have been carried out to evaluate the effects caused by vitrification on the oocytes leaving aside the importance of the CCs [28–30]. Stachowiak et al. [31] evaluated the DNA damage using the comet assay in bovine oocytes exposed to different vitrification methods. This study suggests that the vitrification of MII oocytes resulted in considerable DNA fragmentation. Also, DNA damage in CCs generated after cryopreservation has been reported in humans [32], bovine [33], and equine [20]. It was reported that after vitrification, greater DNA damage is generated in the peripheral CCs than in the inner CCs [4]. However, in pigs, this has not yet been evaluated. Pigs are an important experimental model since this species has anatomical, biochemical, and endocrine similarities with humans [34]. Therefore, in vitro studies may suggest some of the mechanisms of damage produced by vitrification and its possible application in humans. Thus, the evaluation of the DNA integrity after vitrification in CCs will be helpful in order to find new vitrification strategies that will increase IVF and ED rates. Therefore, the aim of this study was to determine if matured porcine COCs exposure to CPAs or vitrification affects oocyte and cumulus cells viability, and if DNA damage is generated in cumulus cells, affecting fertilization and ED.

Materials and methods

Experimental design

Seven replicates were performed. In each replicate all experiments were performed. In vitro matured COCs with a two-four-layer of CCs [23] were randomly distributed into four groups: (1) control (no treatment); (2) hydrogen peroxide (H_2O_2) was used as a DNA damage-inducer [35], positive control (COCs exposed to 2.2% of H_2O_2 for 5 min); (3) toxicity (COCs exposed to CPAs, EG + DMSO without vitrification); and (4) Vitrification (COCs exposed to CPAs, EG + DMSO and vitrified in Cryolock, Importadora Mexicana de Materiales para Reproducción Asistida S.A. de C.V., México). After treatments, viability was evaluated in oocytes and CCs. For this, CCs were separated from oocytes by COCs mechanical denudation. The DNA damage was evaluated only in the CCs. After treatments, to determine the importance of the CCs during IVF and ED, oocytes were fertilized in the absence (denuded oocytes, − CCs) or presence (intact COCs, + CCs) of the CCs. The number of evaluated oocytes and CCs for each experiment is shown in the description of the figure captions.

Ethics statement

This study was approved under the regulations of the Ethics Committee for the care and use of animals; Metropolitan Autonomous University-Iztapalapa Campus.

Oocyte collection and in vitro maturation

Ovaries were collected from F1 (Landrace X Yorkshire) pre-pubertal gilts at the "Los Arcos" slaughterhouse (State of Mexico) and transported to the laboratory in 0.9% NaCl solution at 25 °C in less than 2 h. The aforementioned facility has the animal health federal law authorization number 6265375. Ovarian follicles between 3 and 6 mm in diameter were punctured to obtain the follicular fluid. Follicular contents were left to sediment and washed twice with Tyrode modified medium supplemented with 10 mM sodium lactate, 10 mM HEPES and 1 mg/mL polyvinyl alcohol (PVA) (TL-HEPES-PVA) at pH 7.3–7.4 for COCs collection. Oocytes with uniform cytoplasm surrounded by a two-four-layer compact mass of CCs were selected. COCs were washed three times in 500 µL drops of maturation medium: TCM-199 with Earle's salts and 26.2 mM sodium bicarbonate (In Vitro, Mexico) supplemented with 0.1% PVA, 3.05 mM D-glucose, 0.91 mM sodium pyruvate, 0.57 mM cysteine and 10 ng/mL EGF, 0.5 µg/mL LH, and 0.5 µg/mL FSH. 30–40 COCs were placed in each well of a four-well dish (Thermo-Scientific Nunc, Rochester NY) containing 500 µL of maturation medium and incubated at 38.5 °C with 5% CO_2 in air and humidity at saturation for 44 h [9]. Maturation was evaluated by the Hoechst stain only in the negative control. Oocytes were stained with 10 µg/mL bisbenzimide (Hoechst 33342) for 40 min using an epifluorescence microscope (Zeiss Axiostar) 400× magnification for observation. Oocytes with a germinal vesicle (GV) or in metaphase I (MI) were considered immature; and those in MII with the first polar body, as matured.

Oocyte and cumulus cells viability

After treatments, COCs were denuded mechanically to remove the CCs and evaluate both cell types separately. Oocytes were transferred to a 100 µL drop of 0.5 mg/mL methyl-thiazolyl-tetrazolium (MTT) diluted in modified Tris-buffered medium for viability evaluation. After 90 min, oocytes were observed under a light microscope (Zeiss Axiostar). Oocytes with purple coloration were considered as viable (Fig. 1a) and colorless ones as non-viable (Fig. 1b). For CCs viability, another agent was used. 10 µL of maturation medium with the CCs were transferred to a 10 µL drop of trypan blue. This 20 µL drop was settled in a Neubauer chamber for observation under a light microscope. Colorless cells were considered as viable and those with blue coloration as non-viable (Fig. 1c, d).

Cryoprotectants exposure

After in vitro maturation (IVM), groups of eight to ten COCs were exposed to the highest CPAs concentration solution (10 µL) containing TCM-199 with Earle´s Salts without HEPES, 16% DMSO, 16% EG and 0.4 M sucrose at 38.5 °C for 1 min (Toxicity group). Immediately, COCs were recovered and washed three times in TL-HEPES-PVA medium. Finally, the comet assay was performed only in CCs. The CPAs concentration, exposure time and temperature were selected to make them comparable to values commonly used for oocyte vitrification protocols [9].

Vitrification and warming

After IVM, COCs were exposed to the first vitrification solution in a four-well dish (500 µL) containing TCM-199, 7.5% DMSO and 7.5% EG for 3 min, and for 1 min in a second vitrification solution (10 µL) containing TCM-199, 16% DMSO, 16% EG and 0.4 M sucrose at 38.5 °C solution temperature. Groups of eight to ten COCs were loaded into the Cryolock, then immediately plunged horizontally into liquid nitrogen − 196 °C and stored for 30 min [9]. For warming, the Cryolock was submerged vertically in a four-well dish containing 800 µL of TCM-199 at 38.5 °C solution temperature supplemented with 0.13 M sucrose for 5 min. COCs were washed three times in phosphate buffer solution (PBS) and denuded

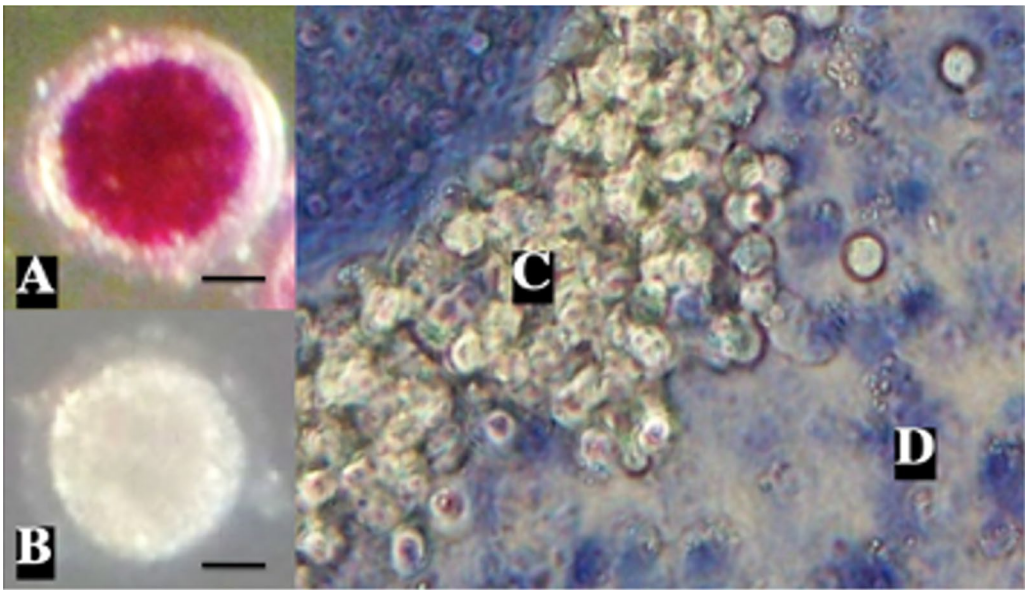

Fig. 1 Viability evaluation in oocytes and cumulus cells. Representative images from oocytes (**a**, **b**) and cumulus cells (**c**, **d**) for viability evaluation in different groups at 40×. Oocyte (n = 304) and cumulus cells (n = 400) viability was evaluated after 44 h of in vitro maturation. For oocytes and cumulus cells, different staining agents were used: methyl-thiazolyl-tetrazolium (MTT) and trypan blue, respectively. **a** Stained purple oocyte: alive; **b** unstained oocyte: dead; **c** unstained cumulus cells: alive; **d** stained cumulus cells: dead. Scale bar: 30 µm. n = number of evaluated cells

mechanically to obtain only the CCs in order to perform the comet assay.

DNA damage in cumulus cells by the comet assay

The DNA damage generated by CPAs exposure and vitrification in CCs was evaluated by the alkaline comet assay following the protocol by Einaudi et al. [36]. Results were expressed as Comet Tail Length (CTL) and Olive Tail Moment (OTM), then analyzed with the ChromaGen program (ODP, México). Low melting point (0.5%) and normal melting point agarose (0.1%) were prepared in PBS magnesium salt-free. Frosted slides were covered with normal melting point agarose until solidification at room temperature for at least 24 h. CCs were dissolved in low melting point agarose and added to a slide previously treated with normal melting point agarose in darkness for 10 min until solidification. Another layer of low melting point agarose was added, and immediately covered by a coverslip until solidification. Slides were immersed in a lysis solution containing 2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris–HCl (pH 10), 1% Triton X-100 and 10% DMSO at 4 °C for 24 h; then placed in horizontal electrophoresis and equilibrated in the buffer solution for 15 min; afterward, electrophoresis was performed at 25 V, 300 mA for 15 min. After electrophoresis, slides were placed in a neutralization solution 0.4 M Tris–HCl (pH 7.5) for 10 min. Then submerged in 70% ethanol for 5 min and, finally, dried at room temperature, for approximately 3 h. To assess DNA damage, slides were stained with 25 mL of ethidium bromide for 10 min [37] and analyzed using an epifluorescence microscope (Zeiss Axiostar) with the red filter (band-pass filter, 515–560 nm; long-pass filter, 590 nm), observing comets at 400×. Comet pictures were analyzed with the ChromaGen program, considering the CTL in micrometers. The CTL refers to the extent of the DNA damage. The percentage of DNA integrity refers to less DNA damage. Approximately < 15 µm of CTL is related to undamaged DNA, 15–30 µM medium damaged, and > 30 µm strong damaged. The OTM = (% tail DNA x tail length)/100.

In vitro fertilization and embryo development

To determine the importance of the CCs during IVF and ED after warming, oocytes were fertilized in the absence (− CCs) or presence (+ CCs) of the CCs. IVF and ED were carried out following the protocol described by Casillas et al. [9]. Briefly, in vitro matured oocytes were washed twice in 500µL of TCM-199 medium and later in 500 µL of modified Tris-buffered medium (mTBM). Groups of 30 oocytes from all groups were placed into a four-well dish with 50 µL drops of mTBM covered with mineral oil and incubated for 45 min. The semen sample was obtained from one Landrace boar using the gloved hand method at a commercial insemination center, diluted in Duragen (Magapor, México) 1:2 (v:v), then transported to the laboratory at 16 °C within 2 h after collection. Sperm

motility was evaluated; only semen samples with >80% motile spermatozoa were used. Evaluation of sperm viability was performed by observation of the sample under an optical microscope. For IVF, 5 mL of the semen sample were diluted with 5 mL of Dulbecco's phosphate buffered saline (DPBS; In Vitro, S.A., México) medium supplemented with 0.1% BSA fraction V, 75 µg/mL potassium penicillin G and 50 µg/mL streptomycin sulfate. The suspension was centrifuged (61×g for 5 min). The pellet was discarded and 5 mL of the supernatant were diluted 1:1 (v:v) with DPBS and centrifuged (1900×g for 5 min). The supernatant was discarded, and the pellet was diluted with 10 mL of DPBS and centrifuged twice under the same conditions. Later, the pellet was diluted in 100 µL of mTBM to obtain the final sperm concentration (5 X 10^5 spermatozoa/mL). After dilution, 50 µL of the suspension were added to the medium containing oocytes, and gametes were co-incubated in mTBM for 6 h. After co-incubation, 30 putative zygotes were transferred to four-well dishes containing 500 µL drops of North Carolina State University medium (NCSU-23). ED was evaluated under an inverted microscope at 48 h (2 days post-IVF) and 168 h (7 days post-IVF). To evaluate IVF, oocytes were stained with 10 µg/mL bisbenzimide (Hoechst 33342) diluted in PBS for 40 min. The oocytes were fixed with 2% glutaraldehyde and mounted in a PBS-glycerol solution (1:9). Putative zygotes were analyzed under an epifluorescence microscope (Zeiss Axiostar) at 400 X magnification. Fertilization was assessed 16 h after IVF by visualizing pronucleus (PN) formation by the Hoechst staining method. The embryo cleavage (number of zygotes cleaved per total cultivated) and blastocyst rates (number of blastocysts per total cultivated) were determined at 48 h (2 days post-IVF) and 168 h (7 days post-IVF), respectively, by morphological evaluation under an inverted microscope (Olympus-Optical).

Statistical analysis

Seven replicates were performed for all experiments. The data obtained from oocyte and CCs viability, DNA damage in CCs, and oocyte fertilization, cleavage, and blastocyst rates were treated as non-parametric and then analyzed by one-way analyses of variance (ANOVA) followed by a post-hoc multiple comparison Duncan test with a confidence level of $P<0.05$ using the NCSS[11] program. Data are presented as Mean ± SD.

Results

In the present study, all oocytes were matured in vitro. Results indicate that the percentage of control oocytes that reached the MII stage was 73 ± 8.47% (Table 1).

Table 1 In vitro maturation of porcine oocytes

Maturation (mean ± SD)			
Control	GV	MI	MII
	44/369 (12 ± 1.51)	57/369 (15 ± 8.81)	268/369 (73 ± 8.47)

Porcine oocytes were matured in vitro for 44 h and Hoechst stain was performed to evaluate oocyte maturation stages in control (n = 369 evaluated oocytes). Oocytes in GV and MI, were considered immature and oocytes in MII as matured. Data are presented as mean ± standard deviation (SD)

GV = germinal vesicle; MI = metaphase I; MII = metaphase II

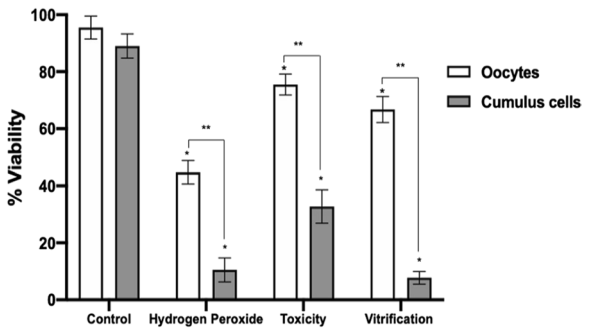

Fig. 2 Percentage of oocyte and cumulus cells viability. Hydrogen peroxide (COCs exposed to 2.2% H_2O_2), Toxicity (16% EG + DMSO), and Vitrification (EG + DMSO + Vitrification). Cumulus cells (CCs) were removed from oocytes (n = 304) for evaluation. Control (n = 242 evaluated CCs), H_2O_2 (n = 263 evaluated CCs), Toxicity (n = 204 evaluated CCs), Vitrification (n = 219 evaluated CCs). In all groups, decreased viability was observed in both cell types compared to control. Compared to oocytes, CCs viability decreased significantly in all groups. Data are presented as mean ± standard deviation (SD). Significant differences were considered when $P<0.0001$. *Indicates significant difference versus control. **Indicates significant difference between oocytes and cumulus cells. H_2O_2 = hydrogen peroxide; EG = ethylene glycol; DMSO = dimethylsulphoxide. n = number of evaluated cells

Oocyte and cumulus cells viability after CPAs exposure and vitrification

Viability was evaluated in oocytes and CCs separately after IVM by staining in all groups (Figs. 1, 2). COCs treated with H_2O_2 were used as a positive control. Results demonstrate that viability after CPAs exposure Toxicity group (75.5 ± 3.69%, oocytes; 32.7 ± 5.85%, CCs) and vitrification (66.7 ± 4.57%, oocytes; 7.7 ± 2.21%, CCs) was significantly lower (*$P<0.001$) in both cell types compared to control (95.5 ± 4.04%, oocytes; 89 ± 4.24%, CCs) (Fig. 2). CCs viability was significantly reduced (*$P<0.001$) after vitrification compared to control (7.7 ± 2.21% vs. 89 ± 4.24%, respectively). Compared to oocytes, CCs viability decreased significantly in all treatment groups (**$P<0.0001$).

Cumulus cells DNA damage after CPAs exposure and vitrification

In the comet assay, fragmented DNA shows the characteristic appearance of a comet tail, while undamaged DNA appears as an intact head. Results demonstrate that higher (*$P < 0.05$) CTL was obtained in all groups (67.1 ± 39.88 µm, H_2O_2; 61.2 ± 32.98 µm, Toxicity; 55.2 ± 27.21 µm, Vitrification) compared to control (13.1 ± 9.22 µm) (Fig. 3; filled circle). Also, results indicate that the percentage of DNA integrity (less DNA damage) was significantly reduced (*$P < 0.05$) in all groups (28.7 ± 22.09%, H_2O_2; 45.1 ± 20.29%, Toxicity; 40.1 ± 27.52%, Vitrification) compared to control (79.1 ± 21.33%) (Fig. 3; empty square).

The DNA damage in CCs was also measured using the alkaline comet assay and expressed as OTM. The OTM = (% tail DNA x tail length)/100. In terms of the OTM, results indicate that H_2O_2, Toxicity, and Vitrification groups (38.5 ± 18.30, 39 ± 17.41, 33.6 ± 16.69, respectively) were significantly higher (*$P < 0.05$) than control (7.4 ± 4.22), demonstrating that higher DNA damage is produced after CPAs exposure and vitrification (Figs. 4, 5).

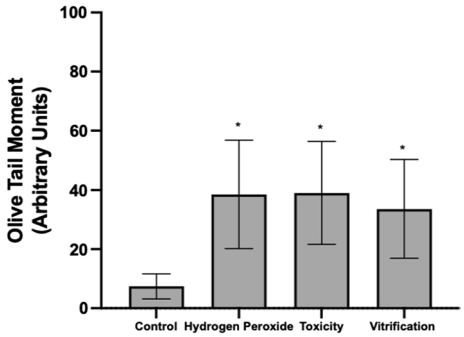

Fig. 4 Cumulus cells genotoxicity assessment by the comet assay expressed by the Olive Tail Moment (OTM). Hydrogen peroxide (COCs exposed to 2.2% H_2O_2), Toxicity (16% EG + DMSO), and Vitrification (EG + DMSO + Vitrification). Cumulus cells (CCs) were removed from oocytes for evaluation. Control (n = 242 evaluated CCs), H_2O_2 (n = 263 evaluated CCs), Toxicity (n = 204 evaluated CCs), Vitrification (n = 219 evaluated CCs). The OTM represents the product of the percentage of total DNA in the tail and the distance between the centers of the head and tail regions. High OTM value indicates DNA damage. Higher OTM values were obtained in all groups compared to control. Data are presented as arbitrary units. Significant differences were considered when $P < 0.05$. *Indicates significant difference versus control. OTM = olive tail moment; H_2O_2 = hydrogen peroxide; EG = ethylene glycol; DMSO = dimethylsulphoxide

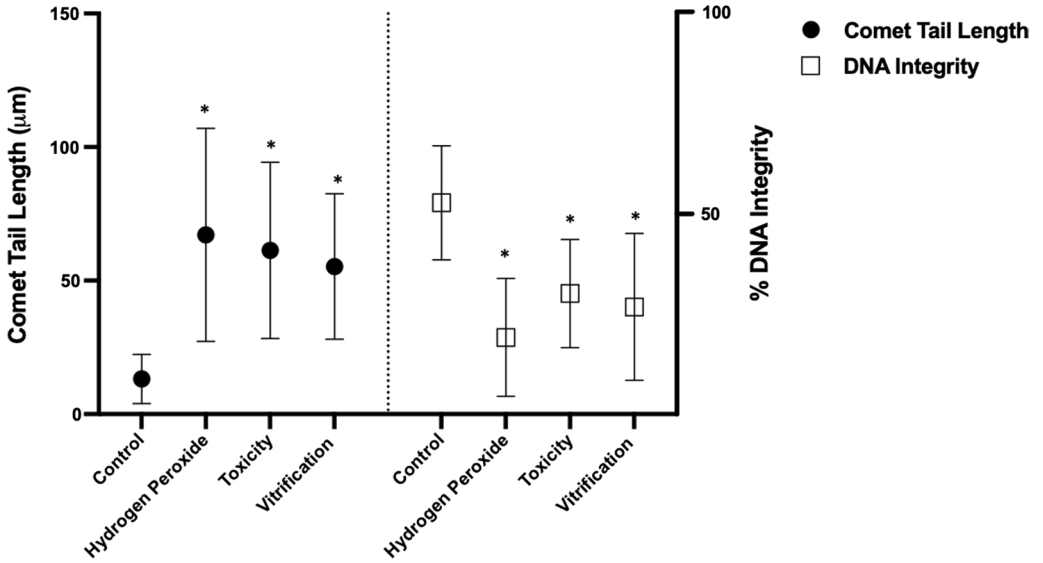

Fig. 3 Cumulus cells genotoxicity assessment by the comet assay expressed by the Comet Tail Length (CTL) and DNA Integrity. Hydrogen peroxide (COCs exposed to 2.2% H_2O_2), Toxicity (16% EG + DMSO), and Vitrification (EG + DMSO + Vitrification). Cumulus cells (CCs) were removed from oocytes for evaluation. Control (n = 242 evaluated CCs), H_2O_2 (n = 263 evaluated CCs), Toxicity (n = 204 evaluated CCs), Vitrification (n = 219 evaluated CCs). The CTL refers to the extent of DNA damage, and DNA integrity to the percentage of DNA in the comet's head (no DNA damage). Approximately < 15 µm of CTL is related to normal or undamaged DNA, and damaged > 30 µm. Higher CTL and lower DNA integrity was obtained in all groups compared to control. Data are presented as mean ± standard deviation (SD). Significant differences were considered when $P < 0.05$. *Indicates significant difference versus control. CTL = comet tail length; H_2O_2 = hydrogen peroxide; EG = ethylene glycol; DMSO = dimethylsulphoxide

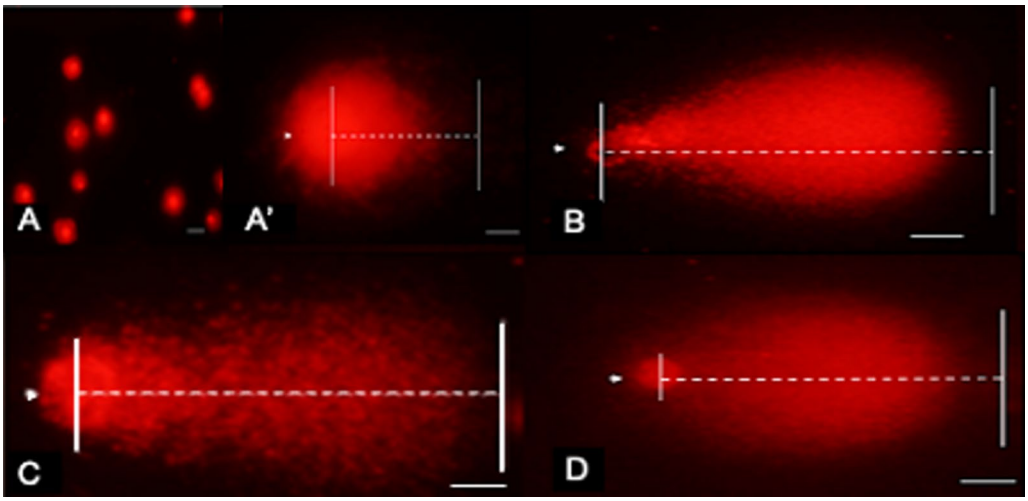

Fig. 5 Cumulus cells comet assay evaluation. Representative images of comet assay evaluation. The direction of electrophoresis was left to right, and DNA fragments are observed as a comet tail. **a** Cumulus cells control magnification at 200×; no DNA migration. Scale bar: 15 µm. **a'** Cumulus cells control: one cell magnification at 400×; no DNA migration. Scale bar: 15 µm. **b** Cumulus cells exposed to H_2O_2 magnification at 400×; DNA migration. Scale bar: 15 µm. **c** Cumulus cells exposed to EG + DMSO magnification at 400×; DNA migration. Scale bar: 15 µm. **d** Cumulus cells EG + DMSO + Vitrification group magnification at 400×; DNA migration. Dotted line indicates the CTL and the arrowhead the nucleoid of the cumulus cell. The percentage of DNA integrity is presented as less DNA damage, shown in **a** and **a'** pictures

Oocyte in vitro fertilization and embryo development after CPAs exposure and vitrification in the absence or presence of cumulus cells

Results demonstrate that oocyte fertilization (70.6 ± 2.08% vs. 82 ± 2%), cleavage (59 ± 3.60% vs. 77 ± 3%), and blastocyst rates (13.6 ± 3.21% vs. 22.33 ± 2.51%) was significantly higher in granulose-intact oocytes (+CCs) compared to denuded oocytes (− CCs) in control groups (*$P < 0.0001$). Additionally, fertilization, cleavage and blastocyst rates significantly decreased in granulose-intact oocytes in the Toxicity (35.3 ± 16.65%, 22.6 ± 3.05%, 1.6 ± 0.57%, respectively) and Vitrification (32.3 ± 9.29%, 20 ± 1%, 2 ± 1%, respectively) groups compared to control (82 ± 2%, 77 ± 3%, 22.3 ± 2.57%, respectively) (*$P < 0.0001$) (Fig. 6).

Discussion

In different mammalian species including humans and swine, the preservation of intact CCs after COCs vitrification is of great importance because these cells play important roles in the maturation and fertilization processes [38, 39]. However, most studies have been carried out to evaluate the effects caused by vitrification on the oocytes leaving aside the importance of the CCs [28–30].

Effect of vitrification on cumulus cells and oocyte viability

Our results demonstrate that CPAs exposure (toxicity group) and vitrification decreased CCs and oocyte viability. Moreover, the decrease in viability after vitrification was greater in CCs compared to oocytes (7.7 ± 2.21% vs. 66.7 ± 4.57%, respectively). In the present study, the decrease in viability in both cell types may be mainly due to the toxicity of CPAs, as these substances are used at high concentrations in vitrification protocols. Gurtovenko and Anwar [40] reported the possible mechanism of the interaction of several of the most used CPAs with the lipid bilayer. DMSO has a greater ability to diffuse across the phospholipid bilayer than EG. DMSO at high concentrations (40%) can destroy cell membranes completely [40]. The sulfinyl oxygen binds to water strongly and DMSO can surround polar head groups of cell membranes, which may help explain the compound toxicity [41]. In agreement with the results obtained in the present study, it was previously reported that the use of EG affects CCs survival after freezing. Surprisingly, even though EG is widely used for embryo cryopreservation, low survival rates in CCs are reported [32]. According to the literature, toxicity is reduced by combining CPAs [41, 42]. Therefore, in the present study, we used EG + DMSO for vitrification since it has been proven that this mixture allows high survival rates in ewe oocytes [42] and porcine embryos [43]. Another study with porcine oocytes reported that EG + DMSO and EG + PROH resulted in similar viability and IVM rates after vitrification [44]. Somfai et al. [2] reported that the mixture of EG + DMSO allows the production of viable blastocysts after immature oocyte vitrification. Also, we

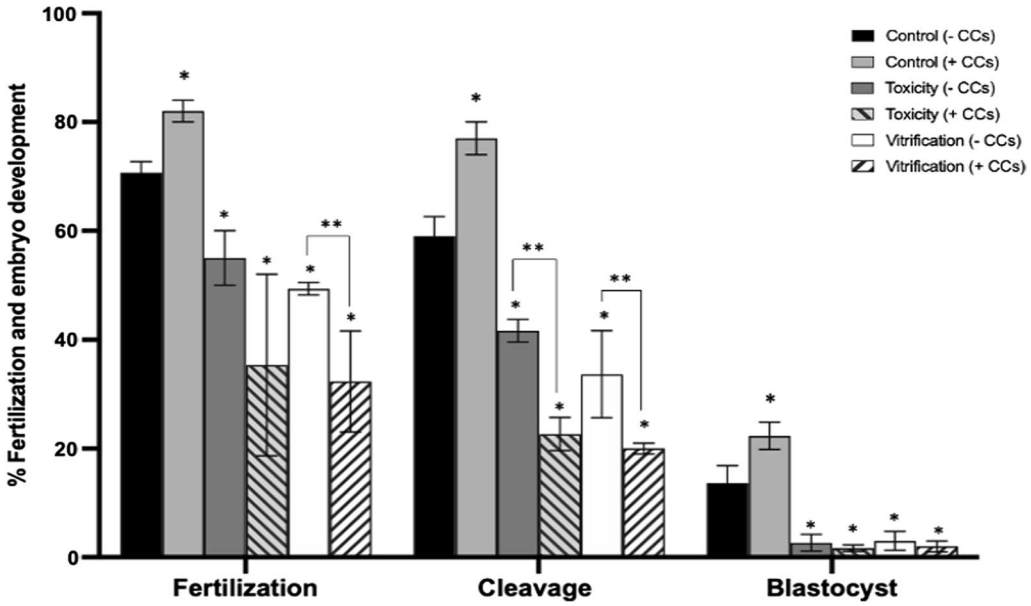

Fig. 6 In vitro fertilization, cleavage, and blastocyst rates evaluation. Toxicity (16% EG + DMSO), and Vitrification (EG + DMSO + Vitrification). After treatments, to determine the importance of the cumulus cells during fertilization and embryo development, oocytes were fertilized in the absence (− CCs) or presence (+ CCs) of the CCs. The number of evaluated oocytes/treatment was n = 214. Oocyte fertilization, cleavage, and blastocyst rates increased significantly with the presence (+ CCs) of CCs compared to denuded oocytes in control groups. However, fertilization, cleavage and blastocyst rates significantly decreased in the Toxicity and Vitrification groups. Also, the presence of CCs in the toxicity and vitrification groups did not increase fertilization, cleavage, and blastocyst rates compared to control. Data are presented as mean ± standard deviation (SD). Significant differences were considered when $P < 0.0001$. *Indicates significant difference versus the respective control. **Indicates significant difference between treatments. EG = ethylene glycol; DMSO = dimethylsulphoxide. n = number of evaluated cells

previously reported that EG + DMSO resulted in a 30% blastocyst formation [9]. However, high concentrations of CPAs are still used (16–50%), promoting detrimental effects in cells, especially DNA damage either in the CCs or the oocytes [20, 28, 31, 32, 37, 45–47]. For example, it was reported that DMSO inhibits CCs expansion in a concentration-dependent manner, resulting in cell death by apoptosis [48]. EG has been used as an effective cryoprotectant for bovine oocytes [49] and embryos [50]. El-Shanat et al. [49] reported that immature buffalo oocytes vitrified with EG or DMSO resulted in 85 and 83% of morphological normal oocyte-CCs, respectively. However, maturation rates were low (47% when vitrified with EG and 46% when vitrified with DMSO). One study evaluated the viability of ewe oocytes after vitrification with different cryoprotectants. They reported that oocyte viability was higher (88.16%) when using (17% EG + 17% DMSO mixture) compared to (70.95%) with (34% EG) or (68.76%) with (17% EG + 17% PROH mixture) [42]. However, most studies evaluate the viability of the oocytes after vitrification but not that of the CCs. In this regard, compared to oocytes, the CCs are smaller in size, and are the first in contact with the CPAs protecting the oocytes during vitrification, which implies that high CPAs concentrations are initially received by these cells, producing greater cytotoxic damage. Accordingly, the results obtained in the present study indicate that CCs do protect oocytes after vitrification; however, most of them lose their viability. In agreement, other study reported that the CCs protect and promote cumulus enclosed MII oocyte survival after vitrification in equine oocytes [20]. Tharasanit et al. [20] reported that the proportion of dead CCs after the vitrification of GV oocytes with 10% EG + 10% DMSO equilibration solution, and 20% EG + 20% DMSO and 0.5 M sucrose vitrification solution was 13.7% and CPAs exposure without vitrification was 2.7%. When evaluating the viability of the CCs in oocytes that reached maturation (MII stage) an even greater decrease in viability was observed (36%). It was also reported that CCs are more affected than oocytes after vitrification since membrane damage is produced in mouse GV oocytes [51].

In the present study, MII oocyte viability diminished up to 66.7 ± 4.57% after vitrification. In this regard, the nuclear cell stage before vitrification is another key factor to be considered. GV [52] or MII oocytes have fewer CPAs and water permeability than zygotes and later-stage embryos [53]. The vitrification of denuded MII oocytes could generate alterations in the plasma membrane, mitochondrial distribution, meiotic spindle, and

chromosomes [54]. Rojas et al. [28] reported that vitrified MII oocytes show spindle abnormalities because chromosomes are exposed directly to CPAs. When oocytes are vitrified with the CCs, these cells can prevent oocyte cryodamage [21]. In the present study, the reduction of oocyte viability up to 75.5 ± 3.69% after CPAs exposure and 66.7 ± 4.57% after vitrification could be due to the possible oocyte injuries caused by the CPAs as already mentioned above.

Effect of vitrification on cumulus cells DNA integrity

Results demonstrate that CPAs exposure and vitrification generated DNA damage in CCs. According to the literature, little is known about the DNA damage generated after vitrification in porcine CCs and most studies only evaluate the cryoinjuries produced in oocytes [55]. In this regard, it was reported that the use of 20% of EG + 20% DMSO produced DNA damage in porcine vitrified GV oocytes, where 54.8% of oocytes resulted in DNA damage compared to 5.6% in the control group [55]. The DNA damage in CCs may be generated since the concentrations of CPAs used during vitrification are very high for this cell type. Generally, in most vitrification protocols, these concentrations are calculated considering the characteristics of the oocytes but not those of the CCs. Therefore, this may cause the CCs to suffer more damage by vitrification than the oocytes. In agreement, Taghizabet et al. [21], reported that CCs create a natural protective shield around the oocyte against physico-chemical insults due to vitrification. In addition, the DNA damage generated in CCs after vitrification could also be due to the production of reactive oxygen species (ROS) [56, 57]. For example, H_2O_2 is believed to cause DNA strand breaks after conversion to the hydroxyl radical [37]. Accordingly, H_2O_2 was used in the present study as DNA damage-inducer (positive control).

For the evaluation of cell genotoxicity caused by CPAs exposure and vitrification, the comet assay has generally been used as an evaluation method. Most studies consider the CTL as an indicator of the damage extent [58] and the percentage of DNA as fragmentation; however, the OTM is considered the most reliable value [59]. The CTL is related to the percentage of DNA integrity as high CTL values indicate less DNA integrity. In the present study, an alkaline comet assay was performed to detect different types of DNA lesions including single (SSBs) and double-strand breaks (DSBs). The SSBs represent the most common type of DNA damage and unrepaired SSBs can alter DNA replication and transcription, resulting in diseases [60]. In contrast, DSBs are one of the most severe forms of DNA damage, and can cause cell death, chromosome aberrations or loss of genetic material. However, one of the limitations of this study is that the alkaline version does not allow simultaneous discrimination between SSBs and DSBs.

Effect of vitrification on fertilization and embryo development in the presence or absence of cumulus cells

In porcine oocytes, more studies are needed since ED rates after vitrification are still reported to be low [9, 61, 62]. In the present study, the CCs were not removed from vitrified oocytes to evaluate their importance during fertilization, cleavage and ED. Results demonstrate that oocyte fertilization, cleavage, and blastocyst rates increased with the presence of the CCs compared to denuded oocytes in control groups. In vitro studies reported that CCs removal decreases fertilization rates in humans [63], and pigs [64]. Also, another study in porcine oocytes reported that the presence of CCs during IVF has a positive influence on ED [65]. However, in CPAs exposed and vitrified oocytes, fertilization, cleavage, and ED rates significantly decreased compared to control. This fact could be explained by the results obtained in the present study, in which the decreased CCs viability and the generation of DNA damage after vitrification, could affect CCs-sperm recognition prior to fertilization. In this regard, Dos Santos-Neto et al. [66] suggested to avoid CCs removal before IVF in sheep MII oocytes and the addition of a fresh CCs co-culture system for improving blastocyst production. This suggests that during vitrification the CCs protect the oocyte but for the subsequent processes it is necessary to replace damage cells with intact ones to ensure better ED rates. They reported that vitrification of MII oocytes, fertilized with CCs resulted in 22% cleavage rate, and 9.2% blastocyst rate. In matured oocytes without CCs, cleavage resulted in 15.1% and blastocyst rate in 4.6%. In the present study with porcine oocytes, we obtained 33.6 ± 8.02% cleavage (− CCs) and 3 ± 1.73% blastocyst rate (− CCs) compared to 20 ± 1% cleavage (+ CCs) and 2 ± 1% blastocyst (+ CCs) [66]. Results obtained in the present study were similar to those reported in sheep oocytes regarding cleavage and blastocyst rates when fertilized with or without CCs [66]; however, differences between species should be considered. Therefore, we suggest that the vitrification of porcine mature oocytes should be carried out without removing the CCs since a higher oocyte viability is obtained. However, since viability in the CCs is significantly reduced, the use of a co-culture system with fresh intact CCs after vitrification could increase IVF and ED rates. In agreement, Dos Santos-Neto et al. [66] reported that the addition of a co-culture system with CCs increases blastocyst rates up to 10.7% in sheep. Also, it was previously reported that, in the case of

vitrified porcine immature oocytes, these cells can be used in co-culture systems improving IVM [23], cleavage and blastocyst rates [22, 62].

CCs are important in all processes of oocyte development from maturation [39] to embryo development. The CCs can prevent premature exocytosis of cortical granules as well as the hardening of the zona pellucida to avoid failure of sperm-oocyte recognition, allowing fertilization [15]. It has also been reported that *HAS2*, *VCAN* and progesterone receptor mRNA expression is increased in CCs associated with oocytes that have reached the blastocyst stage [18]. Therefore, the results obtained in the present study strongly suggest that CCs integrity after CPAs exposure and vitrification is an important factor to be considered for further oocyte developmental competence.

Conclusions

This study demonstrates that oocyte exposure to CPAs or vitrification reduced viability in oocytes and CCs, and generated DNA damage in the CCs, affecting fertilization and ED rates. The decline in oocyte fertilization, cleavage, and blastocyst rates after CPAs exposure or vitrification can be attributed to the reduction in both cell types viability, and the generation of DNA damage in the CCs. These findings will allow to understand some of the mechanisms of oocyte damage after vitrification that compromise their developmental capacity, as well as the search for new vitrification strategies to increase fertilization and ED rates by preserving the integrity of the CCs.

Abbreviations

CC: Cumulus cells; COCs: Cumulus-oocyte complexes; CPAs: Cryoprotectant agents; CTL: Comet tail length; DMSO: Dimethylsulfoxide; ED: Embryo development; EG: Ethylene glycol; EGF: Epidermal growth factor; GV: Germinal vesicle; ICSI: Intracytoplasmic sperm injection; IVF: In vitro fertilization; IVM: In vitro maturation; MI: Metaphase I; MII: Metaphase II; MTT: Methyl-thiazolyl-tetrazolium; OTM: Olive tail moment; PG: Propylene glycol; PROH: 1,2-Propanediol; PVA: Polyvinyl alcohol; ROS: Reactive oxygen species.

Acknowledgements

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Authors' contributions

Conception and design of the study: AL, MB, YD, and FC; Direction of the experiments: MB and FC; Technical assistance: JJR; Execution of experiments: AL and FC; Data analysis: AL, MB, EC, and FC; Manuscript writing: AL and FC; Manuscript revision: JJR, EC, EB, IB, SRM, and LJR. All authors revised, discussed, read, and approved the manuscript for publication.

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Availability of data and materials

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Declarations

Ethics approval and consent to participate

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Competing interests

The authors declare no competing interests.

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Publisher's Note

Reproductive Health

REVIEW

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The need for regulation in the practice of human assisted reproduction in Mexico. An overview of the regulations in the rest of the world

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Abstract

Background: The emergence of assisted reproductive technology (ART) in humans has been an important tool for the treatment of infertility. The number of treatments performed in Latin America has been increasing, and Mexico is the third country with the most assisted reproduction cycles performed in the region. However, Mexico lacks a national regulation for assisted reproduction. Therefore, it is necessary to implement regulations that allow for a safe clinical practice based on ethics which can be available to any social group.

Main body: The aim of this review was to examine the existing legislation that regulates human assisted reproduction practices in Mexico, but also to examine the legal analysis of the policies, laws, and regulations in effect in some countries in Latin America, North America, and Europe. For this, seven databases were consulted, and 34 articles from 2004 to 2021 referring to the practice of ART within the legal framework and the anthropological analysis that this entails were analyzed. Eight documents were also consulted such as the Mexican General Health Law of the Official Journal of the Federation (February 7, 1984) with its last published reform (DOF 01-06-2021). And three official agency websites were also consulted. No specific legislation was found for human assisted reproduction practices in Mexico; however, assisted reproduction clinics are ruled under some agreements implemented by national organizations such as the Mexican Association of Reproductive Medicine and, at the Latin America level, the Latin America Network of Assisted Reproduction (abbreviated REDLARA in Spanish); in addition, the practice of ART is considered, although not explicitly, in the General Health Law.

Conclusion: In Mexico, there is no legal regulation in charge of assisted reproduction practices, which is why there is an urgent need to establish human assisted reproduction laws without incurring discriminatory and unconstitutional acts, and at the same time, be in accordance with scientific advances. This will allow a considerable reduction in the violation of human rights.

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Plain language summary

The emergence of ART in humans has been an important tool for the treatment of infertility. It is reported that one in four couples in developing countries has fertility problems. In 2009, the International Committee for Monitoring Assisted Reproductive Technology (ICMART) established ART as "*all treatments or procedures involving* in vitro *manipulation of oocytes, sperm or embryos for the purpose of establishing a pregnancy*". The number of treatments performed in Latin America has been increasing, and Mexico is the third country with the most assisted reproduction cycles performed in the region. However, Mexico lacks a national regulation for human assisted reproduction. This has caused Mexico to become a medical tourism paradise, which increases the possibility of abuses, fraud, and clinical risks. In addition, it allows each institution offering assisted reproduction services, whether public or private, to establish arbitrary requirements for inclusion. Thus, the emergence of a regulation that allows a safe clinical practice based on ethics, which will also make this reproductive tool available to any social group, is a social need. Therefore, the aim of this review was to examine the existing legislation that regulates human assisted reproduction practices in Mexico, but also to examine the legal analysis of the policies, laws, and regulations in use in some countries in Latin America, North America, and Europe, as well as highlighting the importance of working on the establishment of regulations that allow for safe and ethically based clinical practices.

Keywords: Legislation, Regulations, Reforms, Law, Human Assisted Reproduction, Mexico, Latin America, North America, Europe

Background

The World Health Organization (WHO) states that it is the decision of each individual and couple, according to their conscience, to determine whether they intend to have a pregnancy and if so, when they wish to have a child, as well as determining the size of the family unit. However, fertility problems may affect the possibility of pregnancy. The WHO states infertility as "*a disease of the reproductive system defined by the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected intercourse*" [1]. It is reported that one in four couples in developing countries has been affected by infertility [2]. In 2012, infertility in women remained within a similar range over 20 years, from 1990 to 2010 [3]. However, in 2019 infertility increased worldwide, as it was found that the age-standardized infertility prevalence rate increased by 0.37% per year for women and by 0. 29% per year for men. Furthermore, it was observed that the highest upward trend in women occurred in countries with high sociodemographic index, and conversely, the upward trend of infertility in men occurred in countries with low sociodemographic index [4]. Infertility is not a problem limited to a region or a social group. The main factors that lead to infertility are multiple, ranging from health issues some of these conditions derived from the habits and lifestyles of a modern society as well as problems relating to the advancing age of women, derived from personal decisions, such as the delay of motherhood for professional, work, or social reasons [5]. Also, it is impossible to isolate the fact that modern society has established new forms of interaction and family conformation, expanding the concept of the idea of the formation of a nuclear family [6]. The concept of family is not limited to a heterosexual couple, but also to same-sex couples or those formed by a single father or mother with children, among others [6].

The emergence of assisted reproductive technology (ART) in humans, more than 40 years ago, has been an important tool for infertility. Some of these ARTs are in vitro fertilization (IVF), embryo transfer, gamete intrafallopian transfer, zygote intrafallopian transfer, tubal embryo transfer, gamete and embryo cryopreservation, oocyte and embryo donation, and gestational surrogacy. Assisted or artificial insemination with sperm from the woman's partner or a sperm donor is not included in ART [1, 7]. Technological and scientific advances in human assisted reproduction have enabled treatments for most infertility cases. Although the number of treatments performed in Latin America is increasing, this is the world region with the fewest treatments performed, below Europe, North America, the Middle East, and Australia/New Zealand; countries in which assisted reproduction is considered part of the public health system [8]. This limitation of ART is largely due to the lack of coverage since in Latin America countries, individuals or couples must pay for most or all treatment costs [9] favoring the use of these services to some social groups. Therefore, there is a social need for legal regulation, since Mexico, being the third country with the largest number of assisted reproduction cycles performed in Latin America [8, 10], lacks this type of regulation at a national level. This will allow safe clinical practices based on ethics and will guide the discussion on the need to make this reproductive tool available to any social group. Therefore, the aim of this review was to examine the existing legislation that regulates human assisted reproduction practices

in Mexico, but also to examine the legal analysis of the policies, laws, and regulations in use in some countries of Latin America, North America, and Europe, as well as highlighting the importance of working on the establishment of regulations that allow for safe and ethically based clinical practices.

Methods

The research question was, "What is the current legislation that regulates human assisted reproduction practices in Mexico and the rest of the world?" For this, seven databases were consulted: PubMed, ScienceDirect, Redalyc, SciELO, Virtual Law Library (UNAM), Senate Information (abbreviated INFOSEN in Spanish) and Judicial Weekly of the Federation, in which 34 articles from 2004 to 2021 were selected using the keywords: legislation, regulations, reforms, law, human assisted reproduction, Mexico, Latin America, North America, and Europe. Likewise, searches were made in databases of legal organizations in Mexico (INFOSEN, of the Senate of the Republic and the Judicial Weekly of the Federation), in the electronic version of the General Health Law of the Official Journal of the Federation (February 7, 1984), consulted with its last published reform (DOF 01-06-2021), as well as those in countries such as Colombia, Peru, Costa Rica, Canada, Spain, and the United Kingdom. Also, three official agency websites were consulted, with the term "Assisted Reproduction" used as a search criterion: Elected Reproduction Information Group, (abbreviated GIRE in Spanish), Latin American Network of Assisted Reproduction (known as REDLARA in Spanish), and the Secretary of Foreign Relations. The dates of consultation of all search resources were February, June, and August of 2021. Articles that pointed out the ARTs allowed and practiced within the legal framework of each of the countries were included, as well as articles that highlighted an anthropological and social analysis of the advantages and disadvantages of the regulation corresponding to the area of assisted reproduction; conversely, articles referring to clinical cases and which evaluated the efficacy of assisted reproduction techniques were excluded. From legal organization databases in Mexico, documents mentioning the current regulations of assisted reproduction were included; since those are the databases that compile the laws, regulations, and decrees in place, it was sufficient to shorten the search to "Assisted Reproduction". Consulted websites were from official pages updated at least 6 months ago, which provided data on practices in assisted reproduction centers (ARCs) in Mexico (GIRE), as well as from a scientific and educational institution in charge of compiling information from more than 200 ARCs in Latin America. The data obtained was divided in four topics based on geographic regions: (1) Regulation of Human Assisted Reproduction in Mexico, (2) Regulation of Human Assisted Reproduction in Latin America, (3) Regulation of Human Assisted Reproduction in North America, (4) Regulation of Human Assisted Reproduction in Europe (Fig. 1).

Results

Structure of the Mexican government

Mexico is a Federal Republic and the Constitution currently in effect was approved by the Congress in 1917. The Supreme Power of the Federation is divided, for its exercise, into the Legislative, Executive and Judicial Powers. The Executive Power is headed by the Constitutional President of the United Mexican States, who is in charge of governing as established by law. The Legislative Power is deposited in the Congress of the Union, which is divided into the Chambers of Deputies and Senators. The Congress is in charge of issuing laws that regulate the internal structure and operation of the Mexican Republic. Finally, the Judicial Power of the Federation is formed by the Supreme Court of Justice of the Nation, which is in charge of overseeing compliance with the Constitution (the Supreme Law) and the laws [11].

Regulation of human assisted reproduction in Mexico

Since the birth of the first girl born by IVF more than 40 years ago and the growing use of these techniques at the present, there are many countries in the world that lack regulations pertaining to this subject. Mexico is no exception, since it does not have a law that supports, protects, or regulates the operation of ARCs at a national level, consistent with the advances in science and human rights. In the absence of a legal regulation on the practice of ART, the ARCs are governed by some agreements implemented by national organizations such as the Mexican Association of Reproductive Medicine and, at the Latin American level, by the REDLARA; also, the practice of ART is also considered, although not explicitly, in the General Health Law (Table 1) [9, 10, 12].

Derived from the need to regulate and control the ART performed, as well as to report the results obtained, the REDLARA emerged in Latin America in 1990. This is a scientific and educational institution responsible for compiling results and information, and establishing the standards of good practice in ARCs. This institution has more than 90% of the Latin American ARCs in its registry, being a joint endeavor of more than 15 Latin American countries, including Mexico; the second country with the most registered ARCs, only below Brazil [10, 12]. Also, Mexico is the third country with the highest number of assisted reproduction procedures performed, and babies born due to the same from 2002 to 2017 [8,

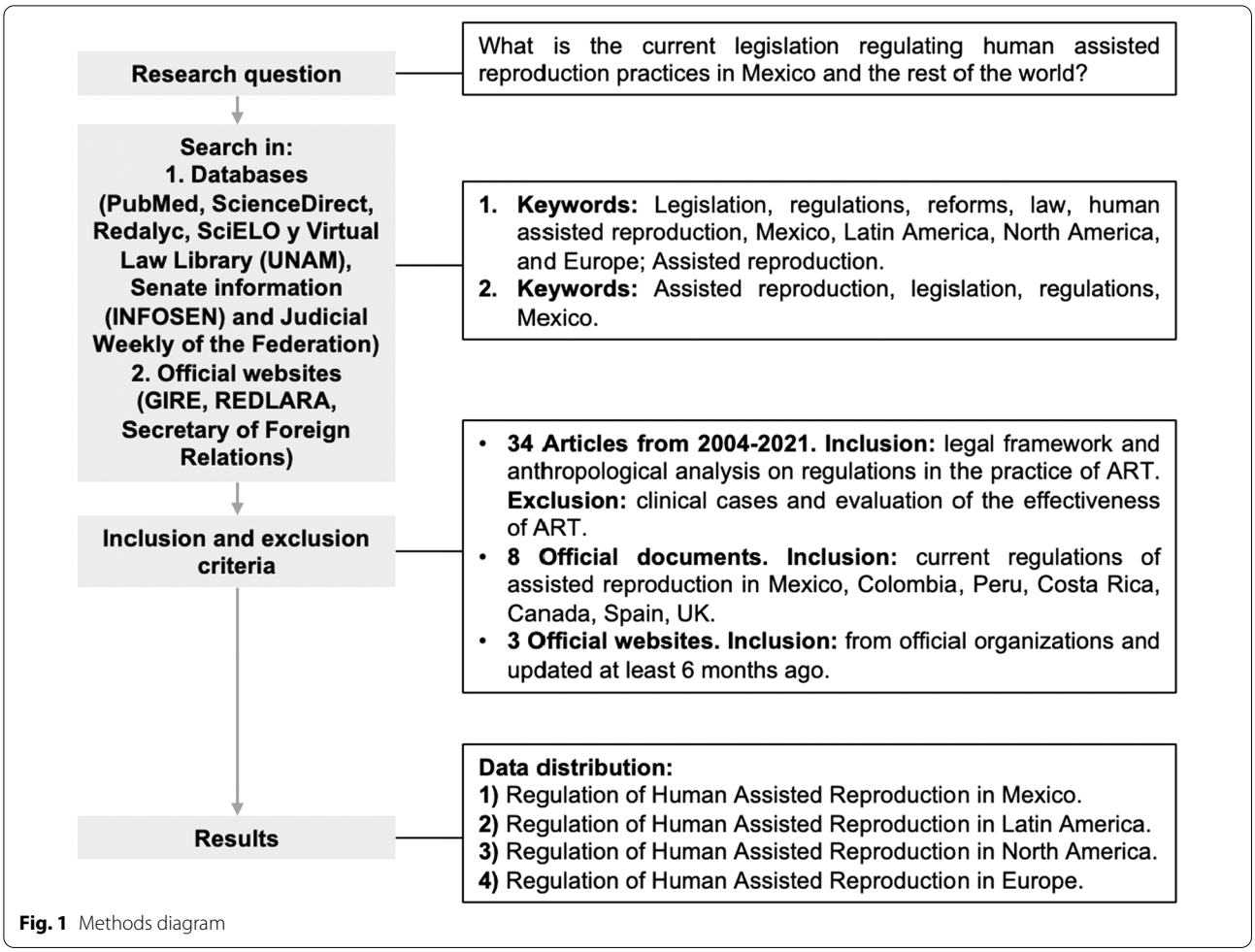

Fig. 1 Methods diagram

10, 12]. Therefore, the emergence of regulations for this matter has been proposed [13, 14], based on the General Health Law and Article 4 of the Political Constitution of the United Mexican States reformed in 1984, in which the right to health protection and to free reproductive decision is considered an individual guarantee [15]. This opens the possibility of making access to the necessary mechanisms to exercise the right to procreation of Mexicans, including the services of human assisted reproduction, without restriction due to sexual preferences and/or marital status [15], obligatory for the government. Article 3 of the General Health Law establishes that the sanitary control of the disposal of organs, tissues and cells is an exclusive federal matter of general health, so the possible issuance of a regulation applicable to assisted reproduction services would be based on this article [15].

ART practices and regulations in Mexico

The ARTs performed in Mexico, such as IVF, ICSI (Intracytoplasmic Sperm Injection), gamete cryopreservation, embryo transfer, gamete donation, mitochondrial replacement therapy (by altering the genome), etc., are all allowed without any restrictions. However, the only regulated practice is gestational surrogacy. Only 4 of the 32 states of the Mexican Republic have a regulation on this matter. Tabasco (Article 92 and Chapter 6 of the Civil Code "Surrogacy and Surrogate Pregnancy") and Sinaloa (Chapter 5 of the Family Code) recognize and regulate surrogacy in their Civil Codes. On the other hand, Coahuila (Article 491 of the Civil Code) and Queretaro (Article 400 of the Civil Code) explicitly disregard any surrogacy agreement. Likewise, the Legislative Assembly of the Federal District, now Mexico City (CDMX), presented the Surrogacy Law of the Federal District on November 30, 2010. Said regulation was never published so it is not in effect. The rest of the Mexican states have not included surrogacy within their legislation in the corresponding matters (Table 2) [16].

Assisted reproduction practices in Mexico

Despite the absence of legal regulation, the Mexican government has provided human assisted reproduction

Table 1 Some countries in America and their ART regulations

Art regulations			
Country	**Current legislation/regulations**	**Year**	**Specifications**
Mexico	None available	–	ARCs are governed under agreements established by the Mexican Association of Reproductive Medicine and the REDLARA Assisted reproduction is considered, although not specifically, within the General Health Law
Latin America			
Argentina	*Law 286.862/13*	2013	Allows national access to ART
Uruguay	*Law 19.167/2013*	2013	Inclusion of ART within the public health system Surrogacy for altruistic purposes
Colombia	*Law of 1953*	2019	Public policy for infertility prevention and treatment within the parameters of reproductive health
Peru	*Article 7, of Law 26842 (General Health Law)*	1997	Everyone has the right to access ART treatments, but the genetic mother and the gestational carrier must be the same person
Brazil	None available	–	It is governed by an administrative agreement issued by the Federal Council of Medicine, emphasizing the ethical rules governing the use of ART
Costa Rica	None available	–	In 2000, Executive Decree 24,029-S was declared unconstitutional
Bolivia	None available	–	In 2001, draft PL 185–2001/2002 was proposed
Chile	None available	–	In 2008, Bill 6306–07 was proposed, which would penalize participants in surrogacy with imprisonment
North America			
United States	*Fertility Clinic Success Rate and Certification Act*	1992	Regulations vary by state
Canada	*The Canadian Act Respecting Assisted Human Reproduction and Related Research; AHR Act*	2004	The creation of chimeras or hybrids, germline alterations, commercialization of gametes and surrogate motherhood are prohibited

Table 2 ART practices and regulations in Mexico

ART practices and regulations in Mexico		
ART	**Current legislation/regulations**	**Specifications**
In Vitro Fertilization (IVF) (Including Intracytoplasmic Sperm Injection, ICSI)	None available	None available
Embryo transfer	None available	None available
Gamete intrafallopian transfer	None available	None available
Zygote intrafallopian transfer	None available	None available
Tubal embryo transfer	None available	None available
Gamete and embryo cryopreservation	None available	None available
Oocyte and embryo donation	None available	None available
Mitochondrial replacement technique (MRT)	None available	None available
Gestational surrogacy	Civil and Family codes of the states of Tabasco, Sinaloa, Coahuila, and Queretaro	Tabasco and Sinaloa recognize and regulate surrogacy Coahuila and Queretaro explicitly disregard any surrogacy agreement

services in public institutions of its National Health System, such as the Mexican Institute of Social Security (abbreviated IMSS in Spanish), the Institute of Security and Social Services for State Workers (known as ISSSTE in Spanish) and the Isidro Espinosa de Los Reyes National Institute of Perinatology. At the same time, private clinics of human assisted reproduction offer a wide variety of treatments which are not subject to the same restrictions as they are in the public sector although they employ management and marketing schemes at higher costs [13, 16].

The wide variety of treatments offered has allowed even foreign professionals to perform therapies that are not allowed in other countries, as was the case of a Jordanian

couple who resorted to the mitochondrial replacement technique (MRT; specifically, maternal spindle transfer) and embryo transfer in Mexico, which resulted in the birth of a healthy child [17]. In this regard, one of the scientists involved in the procedure indicated that the manipulation-derived embryo transfer treatment was performed in Mexico due to a lack of rules. Palacios-González and Medina-Arellano [18] claim that, under their interpretation of the law and with the information available on the case, the research team that performed this practice violated Article 56 of the Regulations of the General Health Law, which establishes that research on assisted fertilization is only permitted when it is intended to solve infertility problems. Since this practice is not explicitly permitted or prohibited legally, it may fall into an interpretation gap, where the practice performed was not legal, since the woman treated in the study was not infertile (two naturally conceived children died at the age of 6 years and 8 months, respectively, due to Leigh syndrome) [18].

Requirements for the access to public assisted reproduction practices in Mexico

Of the 42 centers registered in the REDLARA, more than half of them belong to the private sector [12]. However, access to reproduction treatments performed in these institutions continues to have major limitations, as the lack of regulations in the country leaves open the possibility of incurring in abuses and human rights violations, and there is a lack of legal protection for the medical workers involved. This also lends itself to each institution, whether public or private, determining its criteria for inclusion, which in many cases can be discriminatory and arbitrary. Such is the case of allowing access to ART only to married couples (Women's Specialties Clinic, ISSSFAM and ISSSTE's 20 de Noviembre National Health Center, although the latter also allows for cases of cohabitation) and in some cases only to a man and a woman (Isidro Espinosa de los Reyes National Institute of Perinatology) or with a maximum of previous children (Women's Specialties Clinic, ISSSFAM and ISSSTE's 20 de Noviembre National Health Center) [16]. Another condition is the age limit. In women, the age range varies from 40 years old or younger (Women's Specialties Clinic, ISSSFAM), 36–35 years old or younger (ISSSTE's 20 de Noviembre National Health Center and Isidro Espinosa de los Reyes National Institute of Perinatology, respectively) and between 19 and 37 years old (Mónica Pretellini Maternal Perinatal Hospital, in the State of Mexico). In men, the age range varies from 50–55 years or younger (Women's Specialties Clinic, ISSSTE's 20 de Noviembre National Health Center and Isidro Espinosa de los Reyes National Institute of Perinatology, respectively) [16]. In addition, it is important to guarantee the health of the beneficiaries of public programs. Couples must be emotionally stable persons without life-threatening diseases during the pregnancy, or who suffer from diseases that may be transmissible, as well as the presence of infertility or the loss of two or more consecutive pregnancies, and in heterosexual marriages, having had unprotected sex for at least 1 year previously without having achieved pregnancy (Table 3) [16].

The incurrence of discriminatory acts

Several cases in Mexico have set a precedent for discriminatory acts committed by some institutions. Some of these cases are related to discriminatory acts based on the woman's age. Due to the legal system established in

Table 3 Requirements for the access to public Assisted Reproduction Practices in Mexico (modified from GIRE)

Requirements for the access to public assisted reproduction practices in Mexico				
Requirements	**Women's Specialty Clinic (ISSSFAM)**	**20 de Noviembre National Health Center (ISSSTE)**	**Isidro Espinosa de Los Reyes National Institute of Perinatology**	**Monica Pretellini Maternal Perinatal Hospital (State of Mexico)**
Women's age	< 40 years	< 36 years	< 35 years	Between 19 and 37 years
Men's age	< 50 years	< 55 years	< 55 years	Between 19 y 55 years
Health	Emotionally stable persons, without diseases that can be transmitted, or which can be life-threatening during the pregnancy	People without transmissible diseases	With infertility	With infertility or the loss of two or more consecutive pregnancies
Marital status	Legally constituted couples	Married or cohabiting couples	Heterosexual couples under any type of cohabitation	"A female and her male partner", without specifying marital status
Children	Couples with less than two living children with their current partner	Maximum one previous child	No requirements	No requirements

Mexico, a judge determines the facts for each case and, with them, the application of provisions related to it, giving a final resolution [19, 20]. In 2017, the GIRE reported several cases registered, documented, and litigated from 2015 to 2017. These included the documentation of cases of pregnant women who were defrauded by intended parents due to a lack of legal contracts drawn by some surrogacy agencies, as well as by intended parents, and litigation of international parents who were denied registration of their children due to the failure of the Civil Registry system to adapt to the new types of affiliations demanded by modern society [16]. Some judges have ruled the age limit imposed by some institutions as a discriminatory act, arguing that the success of ART is not associated exclusively with the age of the patients, but also by their reproductive capacity. Other judges have decided not to pronounce discrimination in the requirements for admission to assisted reproduction practices, thus requesting the intervention of the Supreme Court of Justice of the Nation (abbreviated SCJN in Spanish) [16]. This shows that, in the absence of regulations, resolutions are based on the interpretations of judges and public officials. Even though these processes are quite slow and open to interpretation, institutions such as the Judicial Power of the Nation, the National Council to Prevent Discrimination (known as CONAPRED in Spanish) and the National Human Rights Commission (abbreviated CNDH in Spanish), can help regulate the requirements for the access to the ARTs that may incur in discriminatory acts.

Current reforms in the field of human assisted reproduction

According to some experts, the 2016 approval of a reform to the Civil Code of the State of Tabasco rushes in some respects such as invasion of competencies, discrimination, and legal insecurity. For example, pregnant women must be between 25 to 35 years old, and the gestation contract must be signed by the contracting mother and father with the pregnant woman (Article 380 BIS 2), the contract will be annulled for intervening agencies, offices and third parties (Article 380 BIS 4). Access is only for Mexican citizens, the implantation has a limit of up to two embryos, and any contract must be approved by a competent judge (Article 380 BIS 5) [16, 20–22]. In 2013, agreements in the matter of human assisted reproduction were introduced in the Civil Code of the State of Sinaloa. These considered restrictions which had not previously been taken into account in the State of Tabasco and resulted in achieving that the State of Sinaloa did not become a destination for reproductive tourism as is the case of Tabasco [16, 20, 22].

In 2016, the Chamber of Deputies issued a regulation on human assisted reproduction which was based on an initiative previously presented initiative. It considers the requirement of a medical diagnosis of infertility to permit access to ART, prohibits the use of sperm donation (not applicable for egg donation), the restriction for gamete donation and the approval of spouses of married women who wish to undergo any assisted reproduction procedure [16]. In 2018, a new initiative to the General Health Law regarding human assisted reproduction was presented before the same relevant commissions, and it establishes reproduction achieved through ovulation induction, controlled ovarian stimulation, ovulation triggering, and techniques such as intrauterine, intracervical, or intravaginal insemination with semen from the husband, partner, or donor. In addition to all treatments or procedures that include manipulation, both of oocytes and sperm or embryos, for the establishment of a pregnancy (Art. 71 bis, I and II). However, up to date, it has not been approved by the incumbent authorities in force [23].

Regulation of human assisted reproduction in Latin America

Most Latin American countries do not have regulations that specifically control ART. Some rules with relevance in the matter consist of general principles based on Civil and Criminal Codes or which are mentioned in their Constitution [24]. Although there are differences between countries, economic inequality and the high influence of Catholicism have a significant impact on ART regulations in Latin American countries [7]. In 2014, countries such as Chile, Colombia, Ecuador, Peru, Uruguay, Venezuela, Argentina, and Brazil, reported the most ARTs used, and the latter two countries reported a higher number of cycles performed, with fertilizations being the most recurrent techniques with IVF/ICSI [12].

Latin America countries with ART regulations

Only Argentina (Law 286.862/13, issued by the Chamber of Deputies) and Uruguay (Law 19.167/2013, issued by the Senate), have specific regulations on the subject which were issued in 2013. These laws accredit ARTs and stipulate the requirements to be met by public and private institutions for the practice of such procedures [24]. Argentina regulated ARTs for the first time in 2010. It recognizes the right of a person to procreation, and categorizes infertility as a disease. In addition, the Law that arose in 2013, broadens access to ARTs to any adult person, regardless of age, marital status and whether they present pathological infertility, thus allowing national access to ARTs [19, 24]. In 2013, Uruguay approved Law 19.167/2003, which addresses the inclusion of ARTs

within the Uruguayan public health system (Art. 3). Likewise, it mentions that surrogacy should only be for altruistic purposes [19, 24].

In Colombia, according to Article 42-6 of the Colombian Constitution, children born naturally or through ART have the same rights and obligations. In 2009, a legal precedent (T-968/2009) emerged concerning surrogate motherhood, to protect the rights of newborns and surrogate women. In 2014, it was ruled that same-sex couples can adopt a child when one of them is the biological parent of said child [19]. Finally, in 2019, Law 1953 was established, which is the agreement "whereby the guidelines for the development of public policy for the prevention of fertility and its treatment within the parameters of reproductive health" [25]. In Peru, Article 7 states that everyone has the right to access ART treatments, but the genetic and gestational mother must be the same person. There is no specific legislation on surrogacy, and the Health Law (Law 26,842) partially addresses the issue (Table 1) [26].

Latin America countries without ART regulations

Brazil does not have a specific law for ARTs; however, it is regulated under an administrative agreement issued by the Federal Council of Medicine, which highlights the ethical norms governing the use of ARTs are accentuated. Costa Rica is the only country in the world in which IVF was concretely prohibited, through an appeal of unconstitutionality appeal against Executive Decree (24029-S), resolved in 2000, in which the right to life was challenged, due to the argumentation which considers embryos as human beings [18, 27]. Bolivia is one of the countries that does not have specific legislation on surrogacy. In 2001, Bill PL 185-2001/2002 was proposed in the Bolivian National Congress. The law was not clear on whether the commercialization of surrogacy was allowed or not; however, it was intended to address the issue of infertility and it raised the written consent of all parties involved before initiating any fertilization procedure [19]. In 2008, Chile presented Bill 6306-07 which contained a single article (Article 23), stating it would penalize the participants in surrogacy with jail; this bill has not been approved yet. Because of this, judges must intervene according to a test that defines the person who has given birth to the baby as the biological mother (Article 183 of the Chilean Civil Code) (Table 1) [19, 24]. Surrogacy is the practice that is most regulated in Latin American countries, unlike other ARTs. In countries such as Chile and Colombia, it has been attributed the slow progress of a normative regulation has been attributed to the strong social influence of the Catholic Church, which extends to public policies and national legislation [7, 19, 24].

Regulation of human assisted reproduction in North America

ART regulations in the United States of America

In 1992, the Fertility Clinic Success Rate and Certification Act was created in USA; its aim was to standardize the reporting of ART success rates throughout the country. This was to be done through the joint work of various organizations such as the Society for Assisted Reproductive Technology (SART), American Society of Reproductive Medicine (ASRM), Centers for Disease Control (CDC), and the National Institutes of Health (NIH), among others, which are responsible for reporting data on assisted reproductive treatment cycles from ART clinics in the USA each year [28–31].

Among the practices they regulate are those already established such as IVF and experimental techniques that have been able to transcend to clinically accepted treatments due to the promising results obtained, such as cryopreservation of oocytes, embryos, and ovarian tissue [24, 29, 30]. In addition to the collection of clinical outcomes, the regulation of ART practices achieved by the Fertility Clinic Success Rate and Certification Act, some aspects were also considered, such as the barriers that limited access to ART procedures in certain social groups, in addition to the ethical and legal implications regarding processes such as preimplantation genetic test (PGT), as well as gamete and embryo donation [24, 30, 31]. Regarding the latter, the National Organ Transplant Act of 1984 allows the commercialization of sperm and eggs for specific purposes, so financial compensation to egg donors is accepted [29, 30]. Also, it considers issues such as the use of egg donation from family members, oocyte donation to women of advanced reproductive age, recovery and posthumous use of oocytes, information to offspring about their conception, the establishment of paternity/maternity (considering surrogacy), as well as disclosure of medical errors made, informed consent even for donation for research purposes and on the rights and obligations in gamete donation [30]. It is important to note that these regulations vary at the state level. Seven states prohibit human cloning for reproductive and research purposes, eight more prohibit only reproductive cloning. Some states prohibit commercial surrogacy or regulate surrogacy arrangements, as well as sperm, egg, and embryo donation (Table 1) [30, 31].

ART regulations in Canada

In Canada, the Canadian Act Respecting Assisted Human Reproduction and Related Research (AHR Act) came into effect in 2004. This law is based on ethical and social considerations, which prohibits the practice of a variety of technologies including the creation of chimeras

or hybrids, alterations of human germlines, as well as the commercialization of gametes (eggs and sperm) and surrogate motherhood, in which, contrary to the regulation in the USA, participation for profit in these acts would be considered criminal offenses (Table 1) [31–33]. The development of this law took into account bioethical interests, medicine, women's health, feminist activism (which achieved an anti-commercialization stance, notable in the AHR law), the rights of people with disabilities, services for immigrant women, theology, political advocacy, law, and human rights. The support and objection of health professionals to the proposals for these regulations were also considered. In addition, it is mentioned that a strong influence of the policies of nations close to Canada, such as the United Kingdom and the USA, played an important role in the structuring of the AHR law [32].

In March 2013, with the passage of federal budget Bill C-38, Canada's Human Assisted Reproduction Program was closed. That program was responsible for administering and enforcing the AHR Act. Among the most significant changes made by Bill C-38 was the inclusion of the importation, distribution and clinical use of donated oocytes and sperm into the criminal framework of the AHR Act. This moved the right to regulate donors from the Food and Drugs Act to the AHR Act, resulting in the attribution of criminal liability to health professionals, who, if not subject to the regulations, could be sentenced to up to 5 years in prison and/or a fine of $250,000 [34]. However, this also meant an advance in the inclusion of social groups that had been segregated under the old regulations, such as lesbian, gay, bisexual, trans and queer Canadians. For example, gay men were excluded from donating their sperm unless they were in a sexual relationship with the recipient or if they received special permission from the Minister of Health. The use of donor sperm for procreation was more difficult for lesbians and single women, as they were subject to strict regulatory requirements, while women who used the sperm of their sexual partners had an easier process [34, 35]. Before Bill C-38, there were no regulations for the regulation of importation and distribution of oocytes [36], which set the tone for the implementation of rigorous testing for their handling.

In 2018, a workshop entitled Consensus Statement: gene editing, genetic testing, and reproductive medicine in Canada was held in Ottawa, Canada (Consensus Statement: Gene Editing, Genetic Testing, and Reproductive Medicine in Canada). It aimed to propose a restructuring of the AHR Act (amended in 2013) [35, 37], in order to take into account the interests of physicians and researchers for the promotion of medical and scientific innovation through the adaptation of in vitro and in vivo research that is prohibited, such as gene editing research for the correction of genetic mutations, somatic cell nuclear transfer (SCNT), the use of embryos produced in vitro that will be discarded and that could later be used for research to expand the knowledge of processes such as early embryonic development and developmental disorders, as well as research on mitochondrial replacement therapy (through genome alteration), research on the development of human organs, the origin of human diseases and the study of human primordial germ cells (through the creation of chimeras) [35, 37].

Regulation of Human Assisted Reproduction in Europe

Common ART regulations in the European Union

In the European Union (EU), 43 countries have a legal framework regarding human assisted reproduction. Almost all these countries, except for Albania, Bosnia and Herzegovina, Ireland, Romania, and Ukraine [38], have specific legislation and public health legislation in this area. Even though most of these legislations converge in similar characteristics between countries, each country governs and stipulates its specific conditions for the use of ARTs. At present, there have been important reforms in the legislation, which considers the needs of today's society have been considered, including social groups that a few years ago were still excluded from the use of ART, such as single women, lesbians, and same-sex couples. However, some countries, such as the Czech Republic, France, Italy, Poland, Slovakia, Slovenia, Switzerland, and Turkey, continue to limit access to ART exclusively to heterosexual couples with a verifiable pathological diagnosis of infertility, thus excluding single women and lesbians [38–43]. In most countries, the minimum age for sperm donors is 18, with a maximum of 40. Likewise, 30 countries have established the condition of conceiving a maximum of five infants from the same donor. In the case of women egg donors, a minimum age of 18 and a maximum of 35 have been established in most countries for egg donors [38]. Sex selection of the embryo by PGT-A testing is not allowed in any country, except for the detection of sex chromosome-related diseases, in which case, some countries allow the test to be performed [43]. The freezing of gametes, particularly oocytes, is permitted in all countries under medical considerations; for example, for the preservation of fertility before beginning chemotherapeutic treatment [38, 39].

In countries like Denmark, Sweden, Netherlands, France, Belgium, Czech Republic, and Slovenia, patients are provided public financial assistance by their respective governments [38]. Funding is conditioned on the maximum age of the woman, on whether she has had children previously, and on having received public support for previous treatments. Only four EU countries,

including Ireland, do not have such financial assistance. This brings ease of access to AMR treatment for most people in these countries, regardless of socioeconomic status. However, this has also led to longer waiting times (between 12 and 24 months) in public centers, as compared to private centers, resulting in cross-border reproductive tourism. For example, French and Italian citizens travel to other countries such as Greece, Spain, and Belgium. Sperm and oocyte donation are the practices most in demand by these citizens, most of whom are same-sex couples, single women, or heterosexual couples who did not qualify for the procedures in their country, since access to AMR in France and Italy was allowed only for the resolution of sterility or infertility problems in adult heterosexual couples of potentially fertile age, which also proved to be married or living together, in addition to both partners being alive [38, 40, 42–44].

Specific ART regulations in the European Union

In Spain, the first law for ART was approved in 2006 (Law 14/2006) [44]. In 2007, the approval of Biomedical Law 14/2007, led to the creation of the National Commission for Assistance to Human Reproduction, a committee that regulates ART in that country (Table 4) [30, 38]. In Spain, the "menopausal age" is considered as a limit, and surrogacy is not recognized [38]. Some specifications of the laws referred to in each country are described in Table 4.

The laws governing assisted reproductive practices in the UK date back to the 1985 *Surrogacy Arrangements Act*, and to the *Human Embryology and Fertilization Act* and the *Human Reproductive Cloning Act of 1990*. Nowadays, with its suggestion in 2005 and its last regulation in 2015, the *Human Fertilization and Embryology (Mitochondrial Donation) Regulations 2015, No. 572*, has established the Human Fertilization and Embryology Authority (HFEA) as being responsible for licensing ARCs (Table 4) [45]. HFEA limits the transfer of 1–2 embryos per reproductive cycle in women under 40 years of age and a maximum of 3 embryos in women over 40 years of age [30, 45]. Although anonymity is also applicable for donation recipients, when the children born through donation exceed a defined age, they can

Table 4 Some countries in Europe and their ART regulations

Art regulations			
Country	**Current legislation/regulations**	**Year**	**Specifications**
Spain	*Human Assisted Reproduction Technique Law 14/2006* *Biomedical Law 14/2007*	2006 2007	It prohibits reproductive cloning, transfer of more than three embryos per reproductive cycle, germline modification, non-medical sex selection and the use of PGT for non-medical purposes Surrogacy is not recognized
United Kingdom	*Surrogacy Arrangement Act* *The Human Embryology and Fertilization Act* *Human Reproductive Cloning Act* *Human Fertilization and Embryology (Mitochondrial Donation) Regulations 2015, Nº 572*	1985 1990 1990 2005	Prohibit reproductive cloning, germline modification, non-medical sex selection, commercial egg and sperm donation, and commercial surrogacy. Regulates the use of donor gametes, assisted fertilization, PGT, gamete and reproductive tissue banking, and human embryo research
Italy	*Law No. 40. Medically Assisted Procreation Law*	2004	2009: the Constitutional Court declared as unconstitutional the maximum limit of embryos to be produced and transferred for each cycle (three, according to the original version) 2014: the Constitutional Court allowed heterologous assisted reproduction 2015: the Constitutional Court granted the right to access ART to couples who are fertile but carriers of genetic diseases
France	*Law on the Donation and Use of Elements and Products of the Human Body, Medically Assisted Procreation, and Prenatal Diagnosis, No. 94–654* *Bioethics Law No. 2004–800*	1994 2004	The Bioethics Law prohibits reproductive and research cloning, germline modification, non-medical sex selection and surrogacy PGT is only allowed when a parent or close relative has a serious genetic disease
Germany	*Federal Embryo Protection Law* *Adoption Brokerage Law* *Guideline of the German Federal Medical Chamber*	1990 2006 2006	Reproductive and research cloning, gamete donation, creation of hybrid embryos, cryopreservation of fertilized eggs, sex selection (except sperm selection for the prevention of certain sex-related genetic disorders), PGT and all forms of surrogacy are prohibited
Switzerland	*Federal Law on Medically Assisted Reproduction* *Federal Act on Research Involving Embryonic Stem Cells* *Federal Law on Medically Assisted Reproduction*	1998 2003 2004	Reproductive and research cloning, egg and embryo donation, creation of an embryo for research purposes, creation of a hybrid embryo, germline modification, PGT, non-medical sex selection and surrogacy are prohibited. The destruction of cryopreserved gametes and embryos is mandated after 5 years

have access to the donors' identity [38]. The legal practice of MRT has only been explicitly allowed in the UK since 2015 after both houses of parliament accepted proposals made by the Department of Health [18].

In Italy, the *Medically Assisted Procreation Law No. 40* was approved in 2004. However, there have been restructurings to its system, as it was in 2009 when the Constitutional Court declared as unconstitutional a maximum limit of embryos to be produced and transferred for each cycle (three, according to the original version) [30]. In 2014, the prohibition on donor insemination and gamete donation was declared unconstitutional, making heterologous assisted reproduction legitimate again, although with the limitation of being applied exclusively in different-sex couples, married or cohabiting, and only in women of childbearing age [42, 43]. In 2015, the right of access to AMR was granted to fertile couples who were carriers of genetic diseases, thus allowing the use of PGT-A in these couples (Table 4) [46].

In France, the *Law on the Donation and Use of Elements and Products of the Human Body, Medically Assisted Procreation and Prenatal Diagnosis, No. 94–654* was approved in 1994 and, in 2004, the *Bioethics Law No. 2004-800* was also approved (Table 4). An important reform to the legislation considers the elimination of anonymity of sperm and oocyte donors. Since the laws of 1994, the law of 2004, and the current law enacted in 2011, is considered the voluntary nature of the donation and the anonymity of the donor, in addition to respect for human dignity and the non-commercialization of such practice are taken into account, but with the possibility of retribution for expenses that may be generated by the donor, as well as any activity that generates a profit is legally banned for all involved [32, 38, 39, 41, 42]. Also, egg cryopreservation for non-medical purposes is not allowed [30, 32]. The *Federal Embryo Protection Law (1990),* the *Adoption Brokerage Law (2006)* and the *Guideline of the German Federal Medical Chamber (2006)* are the regulations governing ART practices in Germany (Table 4). There are no legal limits for egg donation. It is legal to donate sperm for IVF and intrauterine insemination; however, egg donation is prohibited [30, 32]. Also, egg cryopreservation for non-medical purposes is allowed [30]. The *Federal Law on Medically Assisted Reproduction (1998),* the *Federal Act on Research Involving Embryonic Stem Cells (2003)* and *Federal Law on Medically Assisted Reproduction (2004)* are the treaties under which assisted reproductive practices are governed in Switzerland (Table 4) [30, 32]. In most EU countries it is legal to donate sperm for IVF and intrauterine insemination; however, egg donation is prohibited in Switzerland [30]. Also, egg cryopreservation for non-medical purposes is allowed [30, 32].

Discussion

No specific legislation was found for human assisted reproduction practices in Mexico, but it was found that ARCs in Mexico are governed by some agreements implemented by national organizations (Mexican Association of Reproductive Medicine), and at the Latin America level by the REDLARA. In addition, it was found that reproductive health is considered within the General Health Law and in Article 4 of the Political Constitution of the United Mexican States, which mentions the free reproductive decision-making of all Mexicans. However, Mexico does not have a law that supports, protects, and regulates the practices in ARCs at the national level. The ART practices carried out are standardized through the parameters created by organizations such as REDLARA, which establish good practice standards in ARCs in Mexico and Latin America. ART practices performed in Mexico include IVF, ICSI, gamete freezing, embryo transfer, and gamete donation, among others. Some practices performed in Mexico are not allowed in most countries of the world, such as MRT and surrogacy, the latter being the only practice explicitly included in the Civil Code of four Mexican States. The application of regulations for the use of ART in Mexico, based on the ethical principles of science and social responsibility, could ensure secure access to ARTs for the entire socioeconomic and cultural spectrum, making it possible to protect the public's health without limiting the scientific progress that these practices bring with them. It has been considered that social, cultural, and religious factors established in different countries, including Mexico, limit the possible regulations and their application, especially those concerning treatments related to gamete donation and surrogacy. The latter practice being the one that is regulated to a greater extent in most countries, due to the risk of human rights violations.

It is necessary to consider the problems related to human assisted reproduction from a transnational perspective because they arise as a result of technological and cultural progress, and from need for the laws that regulate them to adapt to these advances. It is also essential to consider the new family structures in order not to incur in discriminatory and unconstitutional acts that restrict access to ARTs only to a certain group of people, since they are true family structures that participate, collaborate, and interact in all personal, social, cultural, and political spaces [21, 47]. In addition to being an essential human right, the formation of a family and access to the benefits that scientific advances bring, regardless of marital status, sexual orientation, gender, or age should be preserved [47].

It is important to emphasize that a lack of regulations can cause countries to become an assisted reproduction

destinations, and even medical tourism paradises, as in the case of Mexico, which also increases the possibility of abuses, frauds, and clinical risks, since procedures are cheaper than in other countries. Also, it allows each institution offering assisted reproduction services, whether public or private, to establish its requirements for inclusion, which can be arbitrary, in addition to establishing its costs for each of the ARTs offered.

Specialists in the field have put forward some recommendations of elements for a model legislation on human assisted reproduction. Some of these recommendations are to avoid the criminalization of the parties involved in the agreements, as well as to avoid discrimination based on arbitrary criteria such as nationality, age, sexual orientation, and marital status in the access to practices [20, 21, 47, 48], in addition to assuring quality and confidential health services, as well as having independent legal representation that guarantees the protection of the persons requesting the services and of the health professionals involved [15, 21]. The structuring of legislation with a gender perspective that protects the interests of the women involved, particularly in cases of surrogacy, is also recommended [48].

Although this article points out the need that exists for the creation of specific and explicit legal regulations in the area of assisted reproduction in Mexico, there are still limitations to a deeper investigation into the subject, since it is still not clear which treatments are allowed in practice due to the lack of a source that compiles this information promptly, as each ARCs, both public and private, manages its catalog of ARTs offered and its criteria for inclusion and exclusion for access to these ARTs.

Conclusion

In Mexico there is an urgent need to regulate and establish human assisted reproduction laws without incurring in discriminatory and unconstitutional acts and, at the same time, being in accordance with scientific advances. This will allow a considerable reduction in the violation of human rights. Because of this, there is a need to establish regulations that help to homogenize the procedures allowed in public and private ARCs, as well as the criteria for inclusion and exclusion of the population that can make use of these ARTs, all within a legal framework that does not violate human rights and does not incur in acts of arbitrariness, thus seeking the common good of both patients and health professionals, and allowing scientific progress in the same way. For this reason, it is recommended that more multidisciplinary studies be carried out in which not only legal specialists are involved, but also health professionals and social specialists who have the necessary perspective to guide the conversation towards the emergence of these areas of opportunity.

Abbreviations

IVF: In vitro Fertilization; ICSI: Intracytoplasmic Sperm Injection; ART: Assisted Reproductive Technology; ARCs: Assisted Reproduction Centers; SCNT: Somatic Cell Nuclear Transfer; PGT-A: Preimplantation Genetic Testing for Aneuploidy; REDLARA*: Latin American Network of Assisted Reproduction; IMSS*: Mexican Institute of Social Security; ISSSTE*: Institute of Security and Social Services for State Workers; MRT: Mitochondrial Replacement Technique; CONAPRED*: Judicial Power of the Nation, the National Council to Prevent Discrimination; CNDH: National Human Rights Commission; CDMX: Mexico City; EU: European Union; SART: Society for Assisted Reproductive Technology; ASRM: American Society of Reproductive Medicine; CDC: Centers for Disease Control; NIH: National Institutes of Health; AHR Act: Canadian Act Respecting Assisted Human Reproduction and Related Research; Bill C-38: Federal Budget Bill C-38; *: Abbreviated in Spanish.

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Authors' contributions

AL developed the methodology, conducted the investigation, and prepared and wrote the original draft. EC, SR-M, and LJR reviewed and edited the manuscript. MB and FC conceptualized the study, prepared, and wrote the original draft, conducted the visualization, investigation, supervision, validation of the study, reviewed, edited, and wrote the final manuscript. All authors contributed to the article and approved the submitted version. All authors read and approved the final manuscript.

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Availability of data and materials

The databases used during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Vitrificación de ovocitos porcinos y su efecto a nivel estructural en embriones y en el ADN de las células del cúmulo.

ALMA GUADALUPE LOPEZ LOPEZ
ALUMNA

En la Ciudad de México, se presentaron a las 10:00 horas del día 10 del mes de agosto del año 2022 en la Unidad Iztapalapa de la Universidad Autónoma Metropolitana, los suscritos miembros del jurado:

DR. JOSE MIGUEL BETANCOURT RULE
DRA. CRISTINA CUELLO MEDINA
DR. JUAN JOSE RODRIGUEZ MERCADO
DR. FILIBERTO FERNANDEZ REYES
DRA. MIRIAM FAHIEL CASILLAS AVALOS

Bajo la Presidencia del primero y con carácter de Secretaria la última, se reunieron a la presentación de la Disertación Pública cuya denominación aparece al margen, para la obtención del grado de:

DOCTORA EN CIENCIAS BIOLOGICAS Y DE LA SALUD

DE: ALMA GUADALUPE LOPEZ LOPEZ

y de acuerdo con el artículo 78 fracción IV del Reglamento de Estudios Superiores de la Universidad Autónoma Metropolitana, los miembros del jurado resolvieron:

APROBAR

Acto continuo, el presidente del jurado comunicó a la interesada el resultado de la evaluación y, en caso aprobatorio, le fue tomada la protesta.

REVISÓ

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