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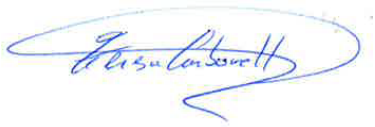
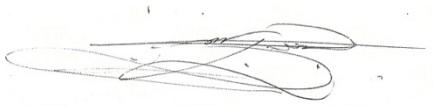
Programa de Doctorado en Biomedicina

Universitat de Barcelona

Tesis Doctoral para optar al título de Doctora por la Univeristat de Barcelona presentada por:

Raquel Gómez Bardallo





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Resumen

La lesión por isquemia-reperfusión (IR) es la mayor causa de pérdida y disfunción de tejidos y órganos en trasplantes clínicos, como es el trasplante hepático. Durante la isquemia, la carencia de oxígeno provoca acumulación de especies reactivas de oxígeno (ROS), que causan daños a los hepatocitos a través de la peroxidación lipídica, la oxidación de proteínas, la disfunción mitocondrial y el daño al ADN. Posteriormente, las células de Kupffer y los neutrófilos acumulados se activan en respuesta a la muerte de los hepatocitos y causan inflamación hepática. Además, la acumulación de succinato y la depleción de trifosfato de adenosina (ATP) se han considerado eventos claves para prever los daños moleculares producidos en la reperfusión. El incremento de la demanda de hígados para el trasplante así como los hábitos actuales de la población hacen que se considere la utilización de hígados esteaóticos, menos óptimos y con mayores riesgos post-trasplantes, de cara a aumentar el *pool* y reducir las listas de espera. La preservación de los injertos hepáticos ha resultado ser una de las terapias más efectivas para conservar el tejido y minimizar el daño por IR, como es la preservación estática en frío y la utilización de los medios de preservación. Las soluciones de preservación clásicas, como son la solución University of Wisconsin (UW) y la solución HTK (histidina-triptófano-cetoglutarato), han sido las más utilizadas hasta la actualidad. Sin embargo, en los últimos años se han desarrollado nuevas estrategias de preservación, como es la solución Institute George Lopez (IGL-1), que contiene Polietilenglicol 35 (PEG35) como agente oncótico y glutatión (GSH) como antioxidante. El objetivo de esta tesis será evaluar la eficacia de los medios de preservación disponibles actualmente así como el papel del PEG35 y el GSH en la preservación estática en frío de hígados grasos. Para ello, se preservaron los injertos hepáticos grasos (24 h, 4 ºC) en las soluciones de preservación IGL-1 y sus modificaciones (IGL-0, sin PEG35; IGL-2, PEG35 y GSH aumentado) y las soluciones clásicas UW y HTK. Los resultados obtenidos demuestran que el PEG35 mejora el estado hepático y confiere protección mitocondrial durante la isquemia, a través de la activación de la enzima mitocondrial Aldehído Deshidrogenasa 2 (ALDH2), reduciendo la acumulación de succinato y manteniendo los niveles de ATP respecto a la preservación sin PEG35. Además, activa el factor de transcripción Nrf2, promoviendo la respuesta redox y reduciendo el estrés oxidativo (TBARS, 4-HNE y AOPP). Por otro lado, en los hígados la preservados en las soluciones con presencia de GSH y PEG35 se presentan niveles menores de estrés oxidativo, inflamación, apoptosis y piroptosis; así como IGL-2 previene la secreción de citoquinas inflamatorias respecto las soluciones UW y HTK. Finalmente, dado que la solución IGL-2 muestra una mejora de la calidad del injerto, se propone su utilización en la preservación dinámica hipotérmica oxigenada (HOPE). En conclusión, la adición de PEG35 y GSH a los medios de preservación ha demostrado ser protectora en la preservación estática en frío de los hígados esteatósicos.

Abstract

Ischemia-reperfusion (I/R) injury is the main cause of loss and dysfunction of tissues and organs in clinical transplants, such as liver transplants. During ischemia, oxygen deprivation causes accumulation of reactive oxygen species (ROS), which cause damage to hepatocytes through lipid peroxidation, protein oxidation, mitochondrial dysfunction, and DNA damage. Subsequently, the accumulated Kupffer cells and neutrophils become activated in response to hepatocyte death and cause hepatic inflammation. In addition, the accumulation of succinate and the depletion of adenosine triphosphate (ATP) have been considered key events to prevent the molecular damage produced in reperfusion. The increased demand for livers for transplantation, as well as the current habits of the population, make the use of steaotic livers, less optimal and with higher post-transplant risks, be considered in order to increase the pool and reduce waiting lists. . Ischemic preconditioning has proven to be one of the most effective therapies for preserving tissue and minimizing I/R damage, such as cold static dehydration and the use of dehydration media. Classical solutions, such as the University of Wisconsin (UW) solution and the HTK (histidine-tryptophan-ketoglutarate) solution, have been the most widely used to date. However, in recent years new development strategies have been developed, such as the Institute George Lopez (IGL-1) solution, which contains Polyethylene Glycol 35 (PEG35) as an oncotic agent and glutathione as an antioxidant. The objective of this thesis will be to evaluate the efficacy of currently available methods of stabilization as well as the role of PEG35 and glutathione in cold static stabilization of fatty livers. For this, the fatty liver grafts were preserved (24 h, 4 ºC) in the solutions of IGL-1 and its modifications (IGL-0, without PEG35; IGL-2, PEG35 and warm glutathione) and the classic UW and HTK solutions. The results obtained show that PEG35 improves liver status and protects mitochondrial protection during ischemia, through the activation of the mitochondrial enzyme Aldehyde Dehydrogenase 2 (ALDH2), reduces the accumulation of succinate and maintains ATP levels with respect to achieved sin PEG35. Furthermore, it activates the transcription factor Nrf2, promotes the redox response and reduces oxidative stress (TBARS, 4-HNE and AOPP). On the other hand, livers preserved in UW and HTK solutions show higher levels of oxidative stress, inflammation, apoptosis and pyroptosis; while IGL-2 prevents oxidation and inflammation. Finally, given that the IGL-2 solution shows an improvement in graft quality, its use in hypothermic oxygenated dynamics (HOPE) is proposed. In conclusion, enhancement of PEG35 and glutathione to media proved to be protective in cold static due to steatotic livers.

Agraïments

Abans de tancar aquesta etapa, tant dura com enriquidora, voldria agrair la seva dedicació als meus directors, la Dra. Teresa Carbonell i el Dr. Arnau Panisello Rosselló, i al Dr. Joan Roselló.

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ABREVIATURAS

4-HNE: 4-hydroxy-2-nonenal

ALDH2: Aldehido deshidrogenasa 2

ALT: alanina Transaminasa

AMPK: quinasa activada por AMP

AOPP: advanced oxidation protein products

ARE: Antioxidant Response Elements

AST: aspartato transaminasa

ATF-6: factor de activación de transcripción-6

ATP: trifosfato de adenosina

BI-1: Inhibidora de Bax 1

CAT: catalasa

CD: células dendríticas

COX-2: ciclooxigenasa-2

Cul3: cullin-3

DAMPS: patrones moleculares asociados al daño, del inglés, Damage-Associated Molecular Pattern

EC: Euro-Collins

eNOS: óxido nítrico sintasa endotelial

GLDH: glutamato deshidrogenasa

GRP78: proteína regulada por glucosa de 78 kDa

GSH: glutatión

GSH-Px: GSH peroxidasa

GSH-T: GSH transferasa

GSSG: glutatión oxidado

HES: hidroxietilalmidón

HMGB1: caja de grupo 1 de alta movilidad

HO-1: hemoxigenasaa-1

HOPE: preservación dinámica hipotérmica oxigenada

HSP: proteína de choque térmico

HTK: histidina-triptófano-cetoglutarato

IGL: Institute George Lopez

ABREVIATURAS

IL-1β: Interleuquina 1β

IR: isquemia-reperfusión

IRE1: enzima que requiere inositol 1

Keap1: Kelch ECH asociado a la proteína 1

LT: Linfocitos T

MDA: malondialdehido

NAFLD: Non Alcoholic Fatty Liver Disease

NASH: Non Alcoholic Steatohepatitis

NF-κB: factor nuclear-κB

NLR: receptores similares a NOD

NMP: máquina de perfusión normotérmica

NO: Nitric oxide

NQO1: NADPH quinona reductasa 1

Nrf2: factor nuclear eritroide similar al factor 2

OXPHOS: fosforilación oxidativa

PEG35: Polietilenglicol 35

PERK: cinasa similar a la PKR

PINK1: quinasa 1 inducida por PTEN putativa

PRR: receptores de reconocimiento de patrones

RE: Retículo Endoplasmático

RAGE: receptores para los compuestos de glicosilación avanzada

ROS: especies reactivas de oxígeno

SCS: preservación estática en frio, del inglés, Static Cold Storage

SOD: superóxido dismutasa

TLR: receptores tipo Toll

TNF-α: factor de necrosis tumoral-α

UCP: proteína desacopladora

UPR: respuesta a proteínas mal plegadas, del inglés, Unfolded Protein Response

UW: University of Wisconsin

XBP1: proteína de unión a la caja X 1

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INTRODUCCIÓN

El hígado graso y el trasplante hepático

El hígado representa un 2% del peso corporal, siendo el segundo órgano más voluminoso. Se ubica en el cuadrante superior izquierdo de la cavidad abdominal, justo encima del diafragma. El hígado cumple con más de 1500 funciones químicas, entre ellas: funciones metabólicas, en que destaca la síntesis de proteínas plasmáticas, pero también el almacenamiento de glucógeno y la gluconeogénesis; detoxificación de sustancias exógenas y tóxicas y excreción en forma de bilis; filtración y almacenamiento de la sangre, así como procesamiento de la hemoglobina para el almacenamiento del hierro [1].

Existen diferentes procesos fisiopatológicos que degeneran en el malfuncionamiento del hígado. Este malfuncionamiento puede ser hereditario, como la hemocromatosis y la enfermedad de Wilson; originarse a nivel vírico, como la hepatitis vírica, o bien deberse a malos hábitos, como el consumo de alcohol y malos hábitos alimentarios. Sea cual sea el origen, esta insuficiencia hepática crónica puede llegar a provocar una cirrosis [2].

En aquellos casos en que estas patologías deriven a una evidente insuficiencia hepática fulminante, se considerará el trasplante hepático, siempre y cuando no exista ninguna alternativa terapéutica [3]. Por otro lado, el trasplante hepático podría estar indicado como tratamiento oncológico de tumores hepáticos que no pueden ser extirpados quirúrgicamente, y con bajo riesgo de recurrencia después del trasplante.

La enfermedad del hígado graso no alcohólico (NAFLD por sus siglas en inglés, Non Alcoholic Fatty Liver Disease) es una afección en la cual se acumula exceso de grasa en el hígado, con más de un 5% de grasa infiltrada, proceso conocido como esteatosis, así como un estado inflamatorio crónico [4]. El estilo de vida sedentario sumado a una mala alimentación son las causas principales del aumento de la prevalencia de esta patología. Actualmente en España se estima que un 25% de la población sufre esta enfermedad [5].

La progresión de la enfermedad puede derivar a diferentes estadios de fibrosis y esteatohepatitis no alcohólica (NASH por sus siglas en inglés, Non Alcoholic Steatohepatitis), cirrosis y carcinoma hepatocelular (Figura 1). La falta de tratamiento terapéutico así como el aumento progresivo de la prevalencia e incidencia de los pacientes con NAFLD, con el consecuente desarrollo de cirrosis y hepatocarcinoma, provoca que se incremente la indicación de trasplante hepático por esta patología [6,7]. Además, el aumento de la prevalencia de esteatosis en la población general también afecta al número de hígados potenciales donantes, dado que actualmente no se utilizan los injertos esteatósicos con más de un 30% de grasa infiltrada por su mala tolerancia a la isquemia. De hecho, se estima que en 2030 el uso de los hígados de los donantes descienda drásticamente del 78% al 44%, debido al aumento de prevalencia de esta enfermedad [8].

El hígado es el segundo órgano más trasplantado, después del riñón. En Cataluña, se realizaron 182 trasplantes hepáticos, según datos del 2019 previos a la pandemia, del informe de la OCATT (Organización Catalana del Trasplante) . En 2019, un paciente no urgente esperaba de media 121 días para ser trasplantado. Durante ese año se obtuvieron 377 donantes válidos, de manera que un 60% de ellos fueron descartados

por diversos motivos, como la edad elevada del donante y la alteración en la analítica o la imagen del tejido [9].

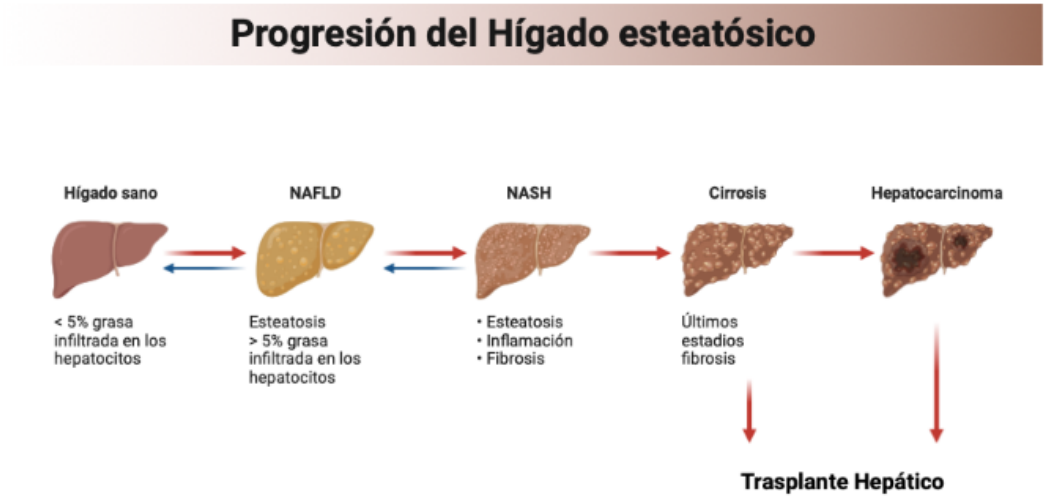


Figura 1. Progresión de la enfermedad del hígado graso no alcohólico (NAFLD). Las primeras fases de la enfermedad pueden ser reversibles con una alimentación y estilo de vida saludable. Su progresión puede ser irreversible, acabando en la necesidad del trasplante hepático como único tratamiento. Adaptado de EHGNA. Enfermedad de hígado graso no alcohólico: un estudio integral [10].

De cara a reducir la lista de espera de trasplante así como ampliar los criterios para entrar en la lista, es necesario aumentar la viabilidad y la disponibilidad de órganos para el trasplante. Para ello, los expertos consideran diferentes estrategias. Desde inicio de los 2000 se la mejorado la técnica del trasplante de porción o técnica *Split*, en que la unidad de trasplantes del Hospital Vall d'Hebrón fue pionera [11]. Este tipo de trasplante se basa en la funcionalidad independiente de los lóbulos del hígado, de manera que a partir de un hígado sano pueden ser trasplantados dos pacientes.

Por otro lado, existen estrategias de preservación hepática en frío para mejorar la viabilidad del injerto hepático, basadas en los medios de preservación. La suplementación de estos medios sumada a la hipotermia ha demostrado ser una buena estrategia para contrarrestar el daño por isquemia-reperfusión [12], así como el rescate de los hígados esteatósicos, de manera que se reduciría el porcentaje de hígados descartados inicialmente. Además de la preservación estática, surgen nuevas estrategia de preservación dinámica mediante máquinas de perfusión [13,14]. La sinergia de estas dos estrategias podría ser clave para reducir la lista de espera así como para ampliar los criterios para recibir un hígado sano.

Modelos experimentales de hígado graso en rata

Los modelos animales para el estudio de enfermedades son esenciales para establecer mecanismos así como para el desarrollo de tratamiento. La mayoría de modelos experimentales se desarrollan en roedores, tanto en ratas como en ratones, debido a su similitud con la especie humana y la facilidad de trabajar con estas especies respecto a otras más complejas con ciclos de vida más largos. El desarrollo de NAFLD en modelos de animales se basa en el acúmulo de grasa en el hígado, ya sea por ingesta o bien por la modificación para procesarla, como un aumento de la síntesis o bien una reducción

de su eliminación. Oligschlaeger y colaboradores clasificaron los modelos preclínicos de NAFLD en los siguientes: factores dietéticos, entre los cuales desatacan la dieta alta en grasa (HFD) y alta en grasa y colesterol (HFC); factores genéticos, como la deficiencia en leptina (ob/ob), resistente a leptina (*db/db*) y deficiente en ApoE (ApoE$^{-/-}$); inducido por factores químicos, como la tiocetamida (TAA); así como modelos híbridos, como es el caso de las ratas Zucker obesas (*fa/fa*) [15].

Las ratas Zucker obesas son el modelo más utilizado y mejor conocido de obesidad genética. Presentan una mutación en el receptor de leptina, lo que en consecuencia provoca mayor concentración de esta hormona en circulación, provocando un aumento de la ingesta, lo cual provoca el desarrollo de obesidad y las consecuentes alteraciones metabólicas, como hiperinsulinemia e hiperfagia. En el caso del hígado, la infiltración de grasa macrovesicular y microvesicular en los hepatocitos se encuentra entorno al 60-70%, y muestran signos de esteatosis respecto las Zucker lean [16].

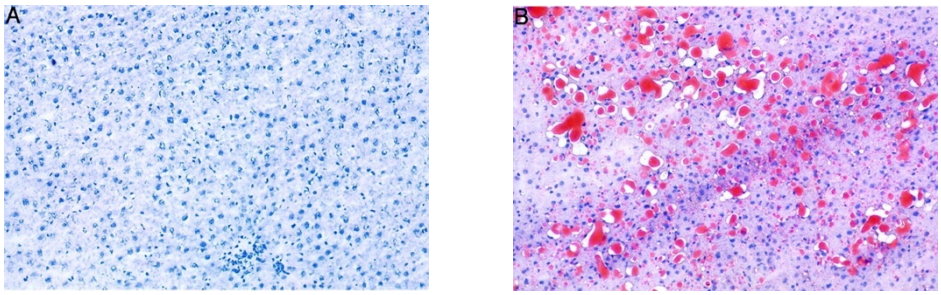


Figura 2. Evidencia de esteatosis en ratas Zucker obesas respecto Lean utilizando la tinción Oil Red. (A) Las ratas Zucker Lean no muestran evidencias de esteatosis, (B) las ratas Zucker obesas muestran infiltración de grasa en los hepatocitos. Secciones congeladas de 3 ; aumento x 214). Imágenes cedidas por la Dra. Anna Serafín.

Mecanismos moleculares implicados en el daño por isquemia hepática

Daño por isquemia-reperfusión y estrés oxidativo

En el trasplante hepático, la interrupción de la sangre a un órgano – isquemia – y su posterior restauración – reperfusión – conducen a un daño agudo en el tejido. Este daño propio del trasplante se conoce como lesión por isquemia-reperfusión (IR), y se produce en diferentes fases [17]. En primer lugar, la isquemia caliente, desde el momento en que se interrumpe la circulación sanguínea del órgano, en el caso del hígado por pinzamiento de la vena porta y a continuación de la cava supra e infrahepática, hasta su resección del paciente. En segundo lugar, la fase de isquemia fría, en que el órgano se almacena en frio (4ºC) en la solución de preservación. Finalmente, la reperfusión caliente, en que el órgano sano se implanta en el paciente. En esta última fase, además del desorden metabólico por la falta de oxígeno y nutrientes, habrá una gran respuesta inflamatoria con el consecuente estrés oxidativo [18].

El daño por IR es multifactorial, y la extensión del daño dependerá de diferentes factores como la prolongación de la isquemia, el estado del hígado y su preservación. Debido a

su estado inflamatorio crónico, el hígado graso no alcohólico es más vulnerable al daño por isquemia-reperfusión [19,20]. Por tanto, es necesario conocer los procesos fisiológicos y moleculares que se dan durante la fase de isquemia y que agravarán el estado inflamatorio en la reperfusión, de cara a encontrar mecanismos preventivos.

Durante la fase de isquemia, los tejidos carecen de aporte de oxígeno y nutrientes, hecho que conlleva a la acumulación de residuos metabólicos y la consecuente alteración de la homeóstasis iónica; se induce la glucólisis anaeróbica, que genera lactato, con la consecuente acidosis metabólica, hasta llegar a la necrosis [21]. Para reducir la lesión por IR, la preservación del injerto hepático en frío es el método más efectivo y consigue reducir el daño [22]. Así mismo, la hipotermia provoca un cambio de fase en los lípidos que genera cambios profundos en la estabilidad de la membrana, aumentando la permeabilidad. Este hecho contribuye a la inflamación celular, reduciendo la actividad de las enzimas mitocondriales y dificultando la obtención de energía por parte de las células hepáticas, con la consecuente disminución de los niveles de ATP [23]. Este conjunto de alteraciones multifactoriales recibe el nombre de lesión fría por IR y conducen, en definitiva, a la muerte celular y, finalmente, a un fallo orgánico [24].

En la fase inicial de la isquemia se observa el estrés oxidativo inducido por las células de Kupffer, que producirán especies reactivas de oxígeno (ROS, del inglés Reactive Oxygen Species), provocando daño a los hepatocitos. A continuación, se produce una respuesta inflamatoria activada también por las células de Kupffer, dando lugar al reclutamiento de neutrófilos. Esta respuesta inflamatoria agravará el estrés oxidativo y por tanto el daño al tejido hepático [19,23].

El estrés oxidativo es la condición que se produce cuando el equilibrio de los prooxidantes y antioxidantes se desplaza en dirección al primero, creando el potencial de daños orgánicos. Los prooxidantes son, por definición, radicales libres, átomos o grupos de átomos con un único electrón desapareado [25]. Las especies reactivas de oxígeno, como el anión superóxido (O^{2-}), el peróxido de hidrógeno (H_2O_2) o los radicales hidroxilo (OH^-) se producen como subproductos de las actividades metabólicas normales [26]. Cuando la producción de ROS supera la capacidad de desintoxicación de las células, se produce el estrés oxidativo, lo cual provocará daño a las diferentes estructuras celulares, como la oxidación de lípidos, proteínas y ADN, así como una modificación de las vías de señalización redox [27]. La respuesta de los neutrófilos así como la inflamación, son la fuente principal de ROS durante la isquemia, pero también lo será la mitocondria, como se expondrá en el siguiente apartado.

La inflamación crónica del hígado graso no alcohólico agrava la vulnerabilidad al daño por isquemia [19,28]. Además, el hígado esteaótico, debido a sus infiltraciones de grasa, presenta mayores niveles de ROS basales, con la consecuente lipoperoxidación [29]. Como consecuencia, la defensas antioxidantes, como las enzimas antioxidantes y el sistema de GSH, se ven lastrados. Por otro lado, se encuentra una reducción de la microcirculación debido a la vasoconstricción sinusoidal, por lo que el flujo sanguíneo se ve disminuido. Esto provoca una hipoxia celular crónica, que será clave en la fase de reperfusión, motivo principal por el cual el hígado graso es descartado para el trasplante [19]. De esta manera, la respuesta al daño por isquemia de los hígados esteatósicos será, no solo más susceptible, sino menos aplacable.

No obstante, el hígado tiene una gran capacidad detoxificadora gracias a la presencia de glutatión (GSH), un potente antioxidante (Figura 3). El hígado es el órgano con más concentración de GSH, en parte gracias a su papel en la detoxificación de compuestos xenobióticos [30]. Estos compuestos forman conjugados con el GSH en reacciones espontaneas gracias a la enzima GSH-S-transferasa (GSH-S-T), y serán excretados [31]. Además, el GSH es imprescindible para mantener el balance redox, ya que es capaz de oxidarse (GSSG) para reducir el peróxido de hidrógeno (H_2O_2). Esta reacción está mediada por la GSH peroxidasa (GSH-Px), mientras que su reacción adversa de reducción es a través de la GSH reductasa (GSH-R), usando NADPH como receptor de electrones [32].

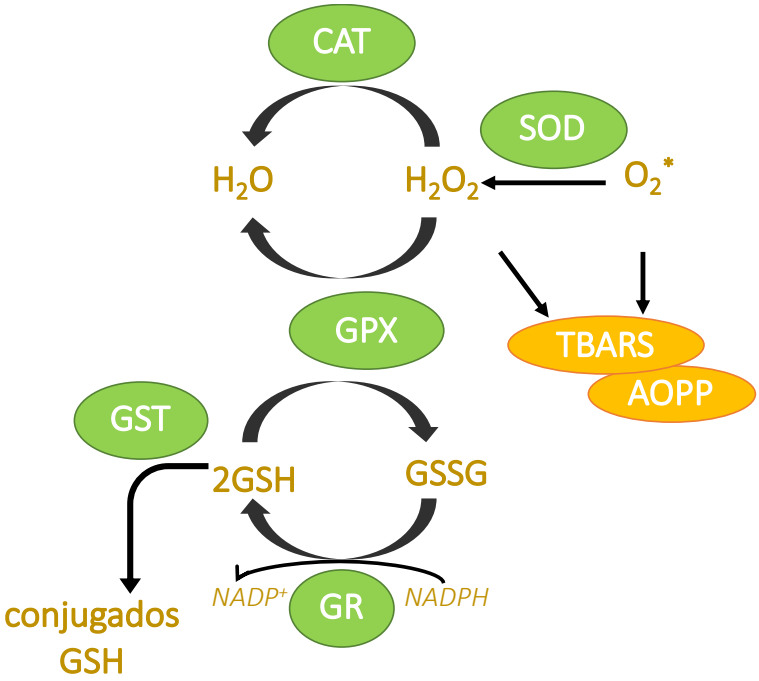


Figura 3. Mecanismos enzimáticos y sistema del GSH en el estrés oxidativo.

En el caso de los hígados esteatósicos, se ha evidenciado un aumento de los niveles de GSH, justificados por el aumento del estrés oxidativo en éstos [33], aunque en muchos casos se agota más rápidamente [34]. La utilización de GSH así como de sus precursores en el tratamiento de NAFLD ha resultado ser muy positiva ya que reestablece los niveles de alanina aminotransferasa, disminuye los niveles de estrés oxidativo (MDA) y reduce la actividad de GPx, SOD y CAT, atenuando así el progreso de la enfermedad [33]. Así mismo, la vía del GSH ha sido utilizada también en el daño por IR. Diversos estudios realizados en modelos de IR concluyen que la administración de N-acetilcisteina, un precursor de GSH, tiene efectos protectores incrementando los niveles de GSH, reduciendo las ROS y preservando el metabolismo energético mitocondrial [35,36].

El papel de la mitocondria en el daño por Isquemia

El hígado es un órgano con una alta demanda metabólica, por lo que necesita un gran aporte de oxígeno para generar energía (ATP). Por tanto, la mitocondria tiene un papel esencial en su funcionalidad.

La isquemia, debido a la falta de oxígeno, provocará la disrupción de la cadena de transporte de electrones mitocondrial, y por tanto de la formación de ATP, cayendo sus reservas de forma rápida. Esta es una de las primeras señales de daño celular, siendo la vía de inicio de la formación de edema celular, desorganización del citoesqueleto y, en caso de no restituirse los niveles de ATP, muerte celular [37–39]. Además, el deterioro de la producción de ATP induce un incremento del ADP (del inglés, adenosine diphosphate) intracelular, y por consiguiente de AMP (del inglés, adenosine monophosphate). El incremento de ambos activará la quinasa activada por AMP, AMPK (del inglés, AMP kinase), el principal responsable del balance energético celular, asociado también a mecanismos citoprotectores.

Por otro lado, la falta de ATP conlleva a la utilización del metabolismo anaeróbico y una acumulación de metabolitos, como es el ácido láctico, con la consecuente acidosis metabólica. El aumento de protones H^+ estimula el intercambio de Na^+. Además, la depleción del ATP provoca la acumulación de sus productos de degradación, como la hipoxantina y la xantina. La interacción del oxígeno con estas moléculas durante la reperfusión dará lugar a la formación de superóxido y otras especies reactivas de oxígeno, y en consecuencia al estrés oxidativo [40,41].

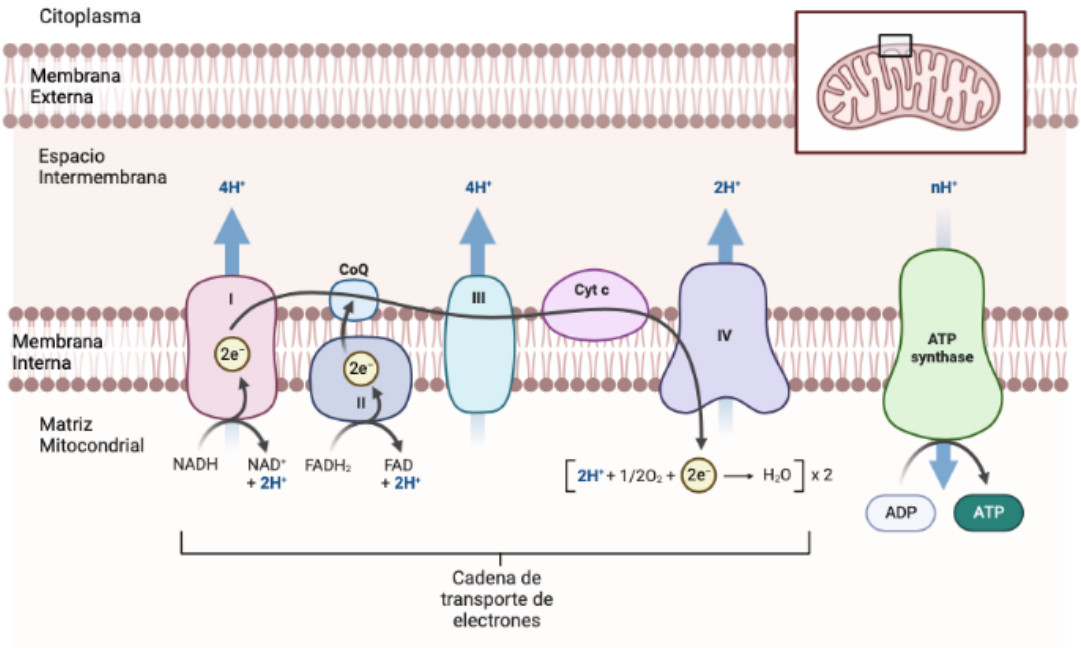


Figura 4. Cadena de transporte de electrones y fosforilación oxidativa.

La fase de isquemia provocará también un aumento de ROS que se formarán a nivel mitocondrial. Los responsables principales son los complejos I y II de la cadena de transporte de electrones [42] (Figura 4). El Complejo I, también conocido como NADH deshidrogenasa, es responsable de captar dos electrones del NADH y trasportarlos a la ubiquinona, a la vez que transloca cuatro H^+ a través de la membrana. Por su parte, el Complejo II, también conocido como succinato deshidrogenasa, es uno de los componentes del ciclo de Krebs, y por tanto el puente entre el metabolismo y la cadena de trasporte de electrones. Como parte del ciclo de Krebs, el Complejo II cataliza la oxidación de succinato a fumarato, mientras que reduce FAD a FADH2 en el complejo de flavoproteína asociado [42]. Durante la hipoxia, se ha visto que el bloqueo del

Complejo I de la cadena de transporte de electrones provoca el aumento de ROS en la matriz mitocondrial, durante la transferencia de electrones de NADH a la ubiquinona [43]. En el complejo II, se asocia la producción de ROS a la enzima succinato deshidrogenasa. Se relaciona su capacidad de producción de ROS con la cantidad de flavoproteína reducida, ya que el FAD es un sitio potente de fuga de electrones para generar ROS, que serán producidas en la matriz [44].

El bloqueo del mismo Complejo II dará paso a la acumulación de succinato. El grupo de Chouchani demostró los diferentes mecanismos por los cuales la acumulación de succinato provoca estrés oxidativo durante la reperfusión, así como la señalización inflamatoria de este metabolito [45]. Tras la reperfusión del injerto hepático, el succinato acumulado se reoxida rápidamente por la enzima succinato deshidrogenasa, lo cual genera una gran cantidad de superóxido y el consecuente peróxido de hidrógeno, debido al gran flujo de traslocación de protones a la membrana en el Complejo I. Este proceso se asocia a la necrosis celular producida después del trasplante hepático, y por tanto a la disfuncionalidad y pérdida del tejido [46].

En el caso del hígado graso, debemos tomar en cuenta la disfuncionalidad crónica de la mitocondria debido a la acumulación de grasa. La mitocondria es el centro del metabolismo de los ácidos grasos, donde son transportados a la matriz mitocondrial y convertidos a acetil-CoA a través de la β-oxidación. El acetil-CoA será una fuente de energía para el resto de tejidos a través de su oxidación completa en el ciclo de Krebs o bien por formación de cuerpos cetónicos [47,48]. Además, debido al alto contenido en ácidos grasos, tras la liberación de ROS, los hígados esteatósicos estarán expuestos a la lipoperoxidación, tal y como se ha expuesto anteriormente. Como consecuencia de esta lipoperoxidación, se formarán diversos productos secundarios, entre los cuales encontramos el malondialdheido (MDA) y el 4-hidroxinonenal (4-HNE), que son los más utilizados para medir el daño por estrés en tejidos [49]. En la mitocondria, estos subproductos, con una vida media más larga que las propias ROS, son potencialmente más nocivos en las células, siendo capaces de difundirse, amplificado así los efectos del estrés oxidativo. Concretamente, en la mitocondria, son capaces de formar aductos que bloquean la citocromo c oxidasa, dando lugar a la disfuncionalidad de estas [49].

En el caso que la célula no pueda revertir los efectos de la hipoxia en la mitocondria, se dará lugar a la mitofagia [50,51]. La mitofagia es un proceso de regulación homeostático, la forma de autofagia especializada en mitocondrias, que dará lugar a la degradación y reciclaje de este orgánulo. Este proceso se regula en respuesta a falta de nutrientes, así como de oxígeno, para reciclar las mitocondrias disfuncionales y evitar la sobreproducción de ROS [52].

Existen dos vías de degradación mitocondrial: dependiente de PINK1 (Quinasa 1 inducida por PTEN putativa) / Parkin e independiente de Parkin. En la vía de degradación mitocondrial dependiente de PINK/Parkin, las mitocondrias dañadas estabilizan PINK1, que reclutará Parkin. Una vez activa en la mitocondria, Parkin promueve la ubiquitinización de proteínas de la membrana mitocondrial externa, como p62. A su vez, estas proteínas serán fosforiladas por PINK1 y dará lugar al reconocimiento por adaptadores de LC3, secuestrando la mitocondria en un autofagosoma para su degradación [53,54]. Se ha descrito esta vía como la principal protectora de la mitocondria frente al daño por IR, considerándose gran una diana terapéutica [55].

A nivel mitocondrial se encuentra la enzima Aldehído Deshidrogenasa-2 (ALDH2), que recientemente se ha relacionado con la protección por IR. Inicialmente su mecanismo de acción se asociaba a la detoxificación de alcohol en el hígado. Actualmente se conoce que es responsable de la protección en el daño por IR en diversos órganos, incluyendo el hígado [56]. La ALDH2 está clasificada como una enzima oxidorreductasa, que utiliza NAD o NADH como aceptor final de electrones, y es responsable de eliminar aldehídos tóxicos catalizando su oxidación en productos no reactivos [57]. Algunos de estos Aldehídos son productos de la peroxidación lipídica, como el 4-Hidroxinonenal y los lipoperoxidos (Malondialdehído, MDA) formados en la célula en condiciones de estrés y exceso de ROS, tal y como se ha descrito anteriormente. Este mecanismo de detoxificación coloca a la enzima ALDH2 como diana terapéutica en diferentes procesos fisiopatológicos relacionados con el estrés oxidativo, como es el daño por IR en hígado. De hecho, algunos autores defienden que la activación de ALDH2 mediante Alda1 protege del daño por IR a través de la inhibición del estrés oxidativo [58,59].

El Estrés Oxidativo del Retículo Endoplasmático en la isquemia hepática

Los hepatocitos, debido a la gran actividad del hígado, son excepcionalmente activos en la síntesis de proteínas para su exportación. El Retículo Endoplasmático (RE) es un orgánulo intracelular responsable principalmente de la síntesis, las modificaciones postraduccionales, el plegamiento y el tráfico de una amplia variedad de proteínas, que incluyen desde hormonas, receptores de membrana, canales iónicos, etc. [60]. En condiciones fisiológicas, existe un equilibrio homeostático entre las proteínas mal plegadas o desplegadas y la capacidad máxima de plegado del RE. Sin embargo, cuando se rompe este equilibrio homeostático, como es el caso en el daño por isquemia-reperfusión, entramos en la fase de estrés del retículo endoplasmático [61]. Estas circunstancias estresantes estimulan una sobrecarga de Ca^{+2} intracelular del lumen del ER [62], lo que, a su vez, modula el calcio mitocondrial y favorece la vulnerabilidad de los hepatocitos a la apoptosis, seguida de la liberación del citocromo C y la activación de caspasas [62]. La serie de respuestas adaptativas del RE a esta situación se denomina respuesta a proteínas mal plegadas (UPR, del inglés: Unfolded Protein Response), que consta de 3 fases: adaptativa, de alarma y, por último, muerte celular. La finalidad de la UPR es reestablecer la función normal del RE, ya sea aumentando la capacidad de plegamiento de las proteínas o promoviendo su degradación [61,62].

La fase adaptativa consiste en la degradación de las proteínas por el proteasoma y la inhibición de la transducción de proteínas, lo que conduce al aumento de chaperonas y enzimas como el factor de transcripción activador-4 (ATF-4) [63]. Esta fase adaptativa contribuirá a reducir la cantidad de proteínas mal plegadas. Si esta vía citoprotectora falla, se inducirá el proceso inflamatorio en la fase de alarma. Finalmente, si ninguno de los pasos anteriores consigue revertir el estrés del retículo, se promueve la entrada a la última fase o la activación de la muerte celular [62].

La respuesta UPR está mediada principalmente por tres proteínas: la enzima que requiere inositol 1 (IRE1), el factor de activación de transcripción-6 (ATF-6) y la cinasa similar a la PKR (PERK). En condiciones fisiológicas homeostáticas, la proteína regulada por glucosa de 78 kDa (GRP78) se une al dominio del lumen de estas proteínas, manteniéndolas inactivas. Sin embargo, en una situación de estrés, este dominio se libera, activándose la UPR [64,65]. Durante la isquemia, la reducción del flujo sanguíneo

provoca el mal plegamiento de las proteínas, así como una acumulación de Ca^{2+} intracelular, lo cual liberará GRP78, activando la UPR [66,67].

La enzima IRE1 está altamente conservada en la UPR de los mamíferos, y su dominio citoplasmático controla positivamente la transcripción de los genes implicados en la lipogénesis a través de la activación de la proteína de unión a la caja X 1 (XBP1) [68]. El tándem IRE1 y XBP1 controla el mecanismo de adaptación que combina la capacidad de plegado ER con las demandas de plegado de proteínas [69,70]. Otra proteína transmembrana encargada de regular la UPR es PERK, que a través de su activación disminuye la cantidad de traducción global de proteínas, lo que a su vez disminuye la carga de plegado de proteínas del RE [71]. Por último, el último regulador transmembrana es ATF6, que en presencia de proteínas mal plegadas se disocia de GRP78 y transloca al núcleo para inducir la transcripción de genes diana del UPR [72,73]. En general, estas proteínas inducen una cascada de señales que regula al alza la expresión de las chaperonas y la exportación retrógrada de proteínas al citosol del RE así como su degradación por el UPS, disminuyendo, en definitiva, la acumulación de proteínas mal plegadas en el RE [74].

El estrés del retículo, así como la respuesta UPR, tienen un papel clave en el daño por IR durante la preservación en frio [75]. La lesión por IR induce la expresión de la proteína citoprotectora Inhibidora de Bax 1 (BI-1), la cual tiene una alta expresión en hígado. La pérdida de esta proteína mejora la activación de IRE1 y ATF6, estimulando la respuesta UPR. Será, por tanto, de gran interés encontrar mecanismos que prevengan la activación de UPR en condiciones de isquemia.

Inflamación, piroptosis y apoptosis en el daño isquémico

Después de un daño hepático, se activan las señales inflamatorias, lo que podría convertirse en una respuesta inflamatoria hepática. El equilibrio entre los mediadores proinflamatorios y antiinflamatorios establece la magnitud de esta respuesta inflamatoria. La respuesta inflamatoria es un mecanismo de defensa frente al ataque de patógenos, y es indispensable para evitar la sepsis, en que la respuesta a la infección puede llegar a ser mortal. Sin embargo, esta respuesta rápida puede ser perjudicial en otros casos, como es el daño por isquemia, donde se produce una inflamación estéril [76].

La inflamación estéril es un componente del daño por IR y se basa en el autoconocimiento oculto de los "patrones moleculares asociados al daño" (DAMPS, del inglés Damage-Associated Molecular Pattern). Los DAMPS son moléculas que pueden causar inflamación y se pueden producir y encontrar a niveles fisiológicos en la célula. La necrosis durante la isquemia provoca la pérdida de la integridad de la membrana celular, liberando sus componentes intracelulares, entre ellos los DAMPS. La liberación de DAMPS al torrente sanguíneo o bien al medio desencadenará en diferentes procesos inflamatorios. En primer lugar, serán reconocido por los receptores de reconocimiento de patrones (PRRs), que dará lugar a la activación de múltiples vías de señalización, como el factor nuclear-κB (NF-κB), que regulará la expresión de genes inflamatorios con la consecuente respuesta amplificada y dirigida [76].

En el hígado, las células de Kupffer, tal y como se ha comentado anteriormente, desempeñan un papel en el estrés oxidativo, juntamente con las células dendríticas

(CDs) y los linfocitos T (LT), y todas ellas serán las responsables del reconocimiento de los PRRs. Los PRR se clasifican en múltiples familias, entre los cuales se destacan los siguientes en el reconocimiento del daño por isquemia: los receptores tipo Toll (TLR), como TLR4 y TLR9; los receptores similares a NOD (NLR), entre los cuales destacan los inflamosoma NLRP3 y AIM2, los receptores ausentes en el melanoma 2 de AIM2 (ALR), todos ellos citoplasmáticos [77]; los receptores para los compuestos de glicosilación avanzada (RAGE) [78]. En el daño por IR, los DAMPS mejor caracterizados son la caja de grupo 1 de alta movilidad (HMGB1), el Ca^{2+} procedente del estrés del retículo y el complejo histona/ADN, que activan TLR4, TLR9, RAGE y los inflamasomas NLRP3 y AIM2, pero también se ha descrito el papel de Hsp70 como DAMPS [79,80], que es responsable de la activación de RAGE [78].

La activación de los PRR dará lugar al inicio de una cascada de repuesta inflamatoria a través de una vía canónica que dará lugar a la piroptosis celular, un proceso de muerte programada inflamatoria no apoptótica. Durante el proceso de piroptosis, la activación de TLR-4 inducirá NF-κB, que en el núcleo promueve la transcripción de las interleucinas 1b y 18 (pro-IL-1β y pro-IL-18) y de la Caspasa 1. Paralelamente, se dará la activación y ensamblaje del inflamasoma NLRP3, el cual promueve la maduración de pro-IL1β y la pro-caspasa 1 a sus formas activas, promoviendo la inflamación. La caspasa-1, además, promueve la escisión de la gasdermina D (GSDMD), que provocará la formación de poros en la membrana. En conjunto, estas señales provocarán una segunda ola de ROS y DAMPS, amplificando la respuesta inflamatoria [81–83].

Por otro lado, la inflamación afectará a la estabilidad de la membrana celular y al glicocálix. El Glicocálix se conoce como el exudado que rodea la membrana celular, compuesto por proteoglicanos, glucosaminoglicanos y glicoproteínas, que contribuye a la comunicación intercelular y mantiene la integridad de la membrana. En condiciones normales, el glicocálix regula el tono vascular y la permeabilidad de la célula, e inhibe la coagulación de la sangre. Mientras tanto, durante el proceso inflamatorio, el glicocálix regula la adhesión y transmigración de los leucocitos, y retiene las citoquinas para evitar su migración [84].

En diferentes procesos fisiopatológicos, como es el caso de la isquemia, se producirá la degradación del glicocálix por parte de las ROS, las metaloproteasas de la matriz, la elastasa derivada de neutrófilos y la trombina. De esta manera, el glicocálix pierde su integridad y su función, exacerbando la lesión por isquemia al inducir vasoconstricción y edema [85].

La apoptosis, al contrario de la piroptosis, es una vía de muerte celular programada no inflamatoria y que no libera DAMPS. En el caso de NAFLD, la deposición de grasa en los hepatocitos, así como la hiperinsulinemia asociada y el aumento de ROS basal son señales claves para mantener la apoptosis activada, en comparación a los hígados sanos, a través de la señalización del factor de necrosis tumoral-α (TNF-α) [86]. Por otro lado, se ha comprobado que la isquemia aumenta también la expresión de TNF-α y por tanto promueve la apoptosis . Ambas vías son responsables de la activación de TNF-α, que activa la Caspasa 8, iniciadora de apoptosis, la cual activará la Caspasa 3, caspasa efectora. Paralelamente, el estrés mitocondrial liberará la Citocromo C, responsable de la activación de la Caspasa 9, también iniciadora y precursora de la activación de la

Caspasa 3. Finalmente, la Caspasa 3 que será responsable de la fragmentación del DNA y la muerte celular.

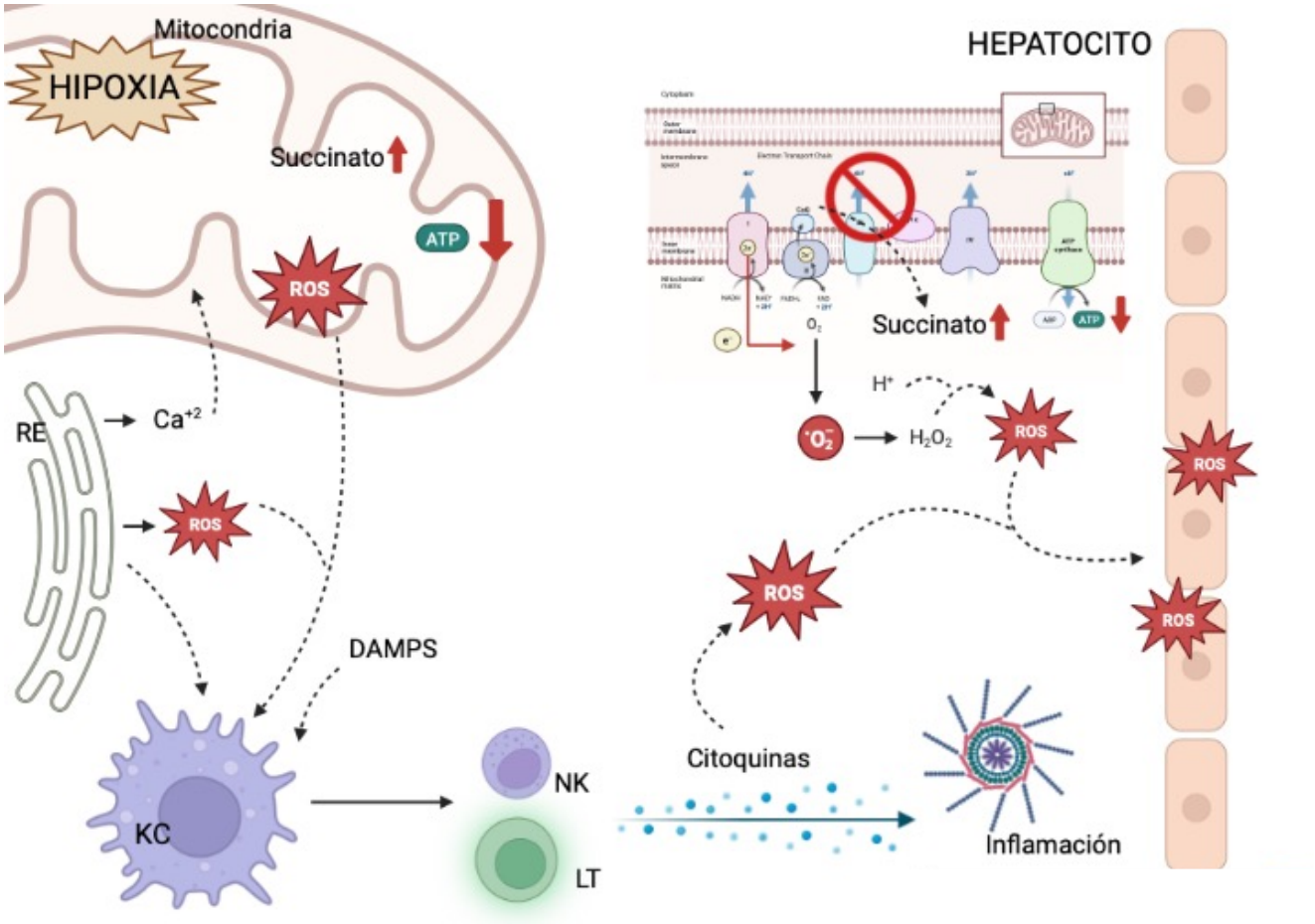


Figura 5. Mecanismos moleculares implicados en el daño por isquemia en hígado. La falta de oxígeno provocará el bloqueo de la cadena de transporte de electrones, acumulando succinato y deteniendo la producción de ATP por vía aeróbica, a la vez que se produce una acumulación de ROS. El estrés del Retículo Endoplasmático liberará Ca^{2+} intracelular y aumentará la producción de ROS. Las ROS producidas por la mitocondria y el RE junto con los DAMPS activarán las Células de Kupffer, provocando la respuesta inflamatoria, con el consecuente daño al tejido que acabará necrosado.

El potencial terapéutico de Nrf2 en la prevención del daño por IR

En los últimos años, se ha considerado Nrf2 (del inglés, Nuclear factor-erythroid 2 related factor 2) la pieza clave en la regulación de la homeóstasis redox [87–89]. Nrf2 es una proteína miembro de la familia Cap “n” Collar (CNC) de factores de transcripción básicos en cremallera de leucina. En mamíferos, su distribución normal se encuentra en el citoplasma, unido a su supresor citosólico Kelch ECH asociado a la proteína 1 (Keap1). La unión Nrf2-Keap1 promueve la ubiquitinización por el componente adaptador Cul3 (cullin-3) basado en la proteína ligasa E3, para su posterior degradación por parte del proteasoma 26S [26].

En condiciones de estrés, existen diferentes mecanismos que disocian la unión Nrf2-Keap1, a través de modificaciones de residuos de cisteína de Keap1, como son la exposición a electrófilos, las ROS y el óxido nítrico [90]. Estas modificaciones permiten

cambios conformacionales en Keap1 e inhiben la ubiquitinación de Nrf2, que se fosforila y se transloca al núcleo activando sus genes diana ARE (del inglés, Antioxidant Response Elements). Los genes ARE incluyen una batería de genes para hacer frente al estrés celular: enzimas antioxidantes como la superóxido dismutasa (SOD), la GSH-S transferasa (GSH-T), la GSH peroxidasa (GSH-Px), la catalasa (CAT); proteínas ubiquitinantes y proteasómicas, como las proteínas contra el choque térmico (HSP); proteínas antinflamatorias; y proteínas metabólicas como la hemoxigenasa-1 (HO-1) y la cicloxigenasa-2 (COX-2) [91,92].

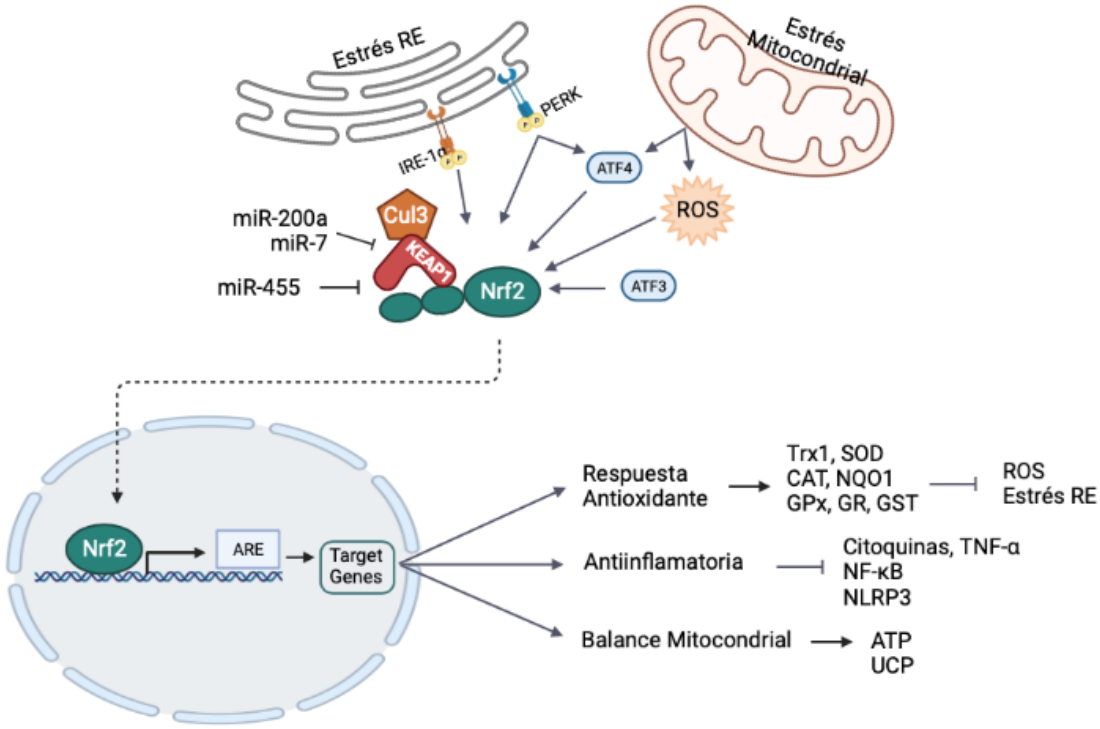


Figura 6. Activación de Nrf2 en respuesta al estrés mitocondrial y del RE. En respuesta al estrés, Nrf2 se libera de KEAP1 y Cul3, se transloca al núcleo y se une a las secuencias ARE, activando la transcripción de genes antioxidantes y antiinflamatorios.

Se ha establecido Nrf2 como diana terapéutica para múltiples enfermedades, como la enfermedad del Alzheimer, la enfermedad de Parkinson, epilepsia y, recientemente, también como estrategia contra el síndrome de dificultad respiratoria aguda y neumonía, causados por la COVID-19, así como otras enfermedades inflamatorias y relacionadas con el estrés oxidativo [93–95]. El daño por IR no es una excepción, y su función protectora en hígado fue descrita por Ke y colaboradores en un modelo de trasplante hepático con preservación fría en ratones [96]. En su estudio, en que preservan el hígado de ratones (20 h a 4 ºC) y realizan un trasplante ortotópico, muestran que los ratones *knockout* para Keap1 específicamente en hepatocitos mejoran la recuperación por IR después del trasplante, obteniendo mejores resultados de función celular respecto al grupo control.

Otro ejemplo del papel protector de Nrf2 en el daño por IR en hígado fue mostrado por el grupo de Kudoh y colaboradores mediante un experimento con ratones deficientes en Nrf2 [97]. Este grupo exhibió un mayor daño tisular, alteraciones en la inducción de las enzimas GSH-T y NADPH quinona reductasa 1 (NQO1); aumento de la expresión de TNF-α en comparación con el grupo control.

Recientemente, un estudio liderado por Ahmed defiende que la expresión hepática de Nrf2 podría utilizarse para descartar injertos hepáticos antes de su trasplante [98]. En este estudio, hígados humanos descartados para el trasplante fueron divididos en dos grupos según su nivel de expresión de Nrf2 y se expusieron a una máquina de perfusión normotérmica (NMP). Después de 6 h de NMP, los hígados con mayor nivel de Nrf2 presentaron mejoras significativas en cuanto a la alteración de las enzimas hepáticas, así como mejoraron la depuración del lactato. Aplicar esta técnica a hígados grasos descartados podría ser una gran estrategia para ampliar el pool de órganos disponibles para el trasplante.

La compleja relación entre las vías moleculares activadas por Nrf2 y el daño por IR hepática así como su potencial terapéutico se expone detalladamente en el Capítulo 1.

Mecanismos de preservación del hígado para el trasplante

La preservación estática en frío en el trasplante hepático

Los hígados esteatósicos, tal y como se ha descrito anteriormente, son más vulnerables al daño por IR, por lo que son descartados inicialmente para el trasplante hepático. Tras la extracción del órgano del donante, es necesario mantenerlo en condiciones óptimas hasta su trasplante en el paciente receptor. La preservación estática en frío (SCS, del inglés, Static Cold Storage) del tejido así como la optimización de los medios de preservación son la estrategia principal para la optimización de estos hígados y extender el criterio de donación. La SCS consiste en perfundir o lavar internamente el órgano con una solución de preservación fría inmediatamente después de su extracción. A continuación, se utiliza esa misma solución de preservación (o una alternativa de composición similar) para bañarlo y mantenerlo almacenado en una nevera a 4 ºC hasta el momento del trasplante. La SCS es el método más habitual de preservación, gracias a su disponibilidad y la facilidad del transporte [99]. Las ventajas de la SCS se centran, por un lado, en la hipotermia, y que conduce a una reducción del metabolismo celular y de las consiguientes demandas de oxígeno, disminuyendo el posible daño oxidativo; por otro lado, en establecer una solución de preservación que prevenga los efectos negativos de la propia hipotermia en el órgano, que provoca alteraciones del citoesqueleto y edema celular [100,101].

La primera solución comercial desarrollada fue la solución Euro-Collins (EC), en 1970, que no contenía ningún agente oncótico y sí glucosa, lo que conlleva efectos osmóticos que conducen a la formación de edema e inducen su metabolismo anaeróbico [102,103].

Más tarde, en los años 90, surgió la solución de la Universidad de Wisconsin (UW), en la que la glucosa se sustituye por lactobionato y rafinosa [104], dos azúcares que previenen los eventos osmóticos provocados la glucosa. Además, esta solución contiene un soporte oncótico, el hidroxietilalmidón (HES), así como otros aditivos como la adenosina, como precursor de ATP, y el GSH y el alopurinol como antioxidantes. No obstante, se ha demostrado que esta solución de preservación presenta varias desventajas. Por ejemplo, se ha demostrado que la presencia de HES reduce el flujo sanguíneo [105], y el alto contenido en K^+ desencadena otras complicaciones en el tejido

debidas a la osmolaridad [106]. Por estas razones, se desarrollaron otras soluciones de conservación, como la solución Histidina-triptófano-cetoglutarato (HTK) y la solución del Instituto Georges Lopez (IGL-1) [107–109].

La solución de preservación HTK fue originalmente establecida para la su uso en corazón, donde demostró ser un medio muy efectivo, y más tarde se aplicó para la preservación de riñón e hígado [110]. Esta solución se caracteriza por una baja concentración de iones Na^+, K^+ y Mg^{2+}, siendo su base la histidina, el manitol como barrera osmótica y aminoácidos de baja permeabilidad (ácido alfa-cetoglutárico y triptófano), que ayuda a las membranas celulares a estabilizarse y posiblemente ser un sustrato para el metabolismo anaeróbico. Además, la baja viscosidad de la solución HTK proporciona un rápido enfriamiento de los órganos. La ventaja de usar HTK se basa en las propiedades básicas de la histidina, que compensan la acidosis y parecen permitir una glucólisis prolongada.

La solución del Instituto Georges Lopez (IGL) contiene Polietilenglicol (PEG) con un peso molecular de 35 KDa (PEG35) en lugar de HES y, paralelamente, incluye una mayor concentración de Na+ en relación al K+. Este hecho conlleva varios efectos beneficiosos en comparación con otras soluciones, ya que la apoptosis, el estrés del RE y la inflamación se ven reducidos tanto en hígados sanos como esteatósicos [75,111,112]. Por lo tanto, algunos de los beneficios que conlleva la solución de IGL-1, a través de la estimulación de la producción de óxido nítrico (NO), son la prevención del estrés oxidativo, el daño mitocondrial y la lesión hepática [113]. Los mecanismos protectores mediante la preservación en el medio IGL-1 se asocian a la presencia del PEG35. De hecho, para probar su efectividad, se desarrollaron dos soluciones basadas en IGL-1: IGL-0, sin presencia de PEG35; IGL-2, con una mayor concentración de PEG35 y GSH [114], cuya composición de detalla en la Tabla 1 junto con las soluciones clásicas.

Componentes	**UW**	**HTK**	**IGL-0**	**IGL-1**	**IGL-2**
K^+ (mmol/L)	125	10	25	25	25
Na^+ (mmol/L)	27	15	125	125	125
Mg_2^+ (mmol/L)	5	4	5	5	5
SO_4^{2-} (mmol/L)	4	-	5	5	5
Ca^{2+} (mmol/L)	-	0.015	0.5	0.5	0.5
Cl^- (mmol/L)	-	50	-	-	-
Difosfato (mmol/L)	25	-	25	25	25
Histidina (mmol/L)	-	198	-	-	-
Histidina-HCl (mmol/L)	-	18	-	-	-
Triptófano (mmol/L)	-	2	-	-	-
Rafinosa	-	-	30	30	30
Ácido lactobiónico (mmol/L)	105	-	100	100	100
Manitol (mmol/L)	-	30	-	-	-
Almidón Hidroxietil (g/L)	50	-	-	-	-
Polietilenglicol 35 (g/L)	-	-	0	1	5
GSHn (mmol/L)	3	-	3	3	9
Alopurinol	-	-	1	1	1
Adenosina (mmol/L)	5	-	5	5	5
Ácido glutámico(mmol/L)	-	-	-	-	-
Ketoglutarato (mmol/L)	-	1	-	-	-
pH	7.4	7.4	7.4	7.4	7.4
Osmolaridad (mosmol/L)	320	310	320	320	320

Tabla 1. Composición de las soluciones de preservación clásicas (UW y HTK) y de la solución IGL-1 y sus modificaciones, IGL-0 e IGL-2.

Efectos fisiológicos de los Polietilenglicoles

Los polietilenglicoles son polímeros neutros y de peso variable con alta solubilidad debido a su hidrofilia, lo cual confiere biocompatibilidad y procesabilidad [115]. Aunque

no se han demostrado en detalle los mecanismos moleculares que intervienen en la protección por parte de los polietilenglicoles, se relacionan con su estructura así como la interacción de estos con las membranas celulares. Además, se caracterizan por su baja toxicidad, por lo que no desencadena una respuesta inmunológica ya que las moléculas de PEG, a lo largo de sus cadenas, se adsorben en la superficie celular creando una capa estructurada [116,117]. En 1977 el grupo de Davis propuso que los PEGs presentan la capacidad de establecer enlaces covalentes con biomacromoléculas, lo cual se conoce como PEGilación [118]. Además, establecieron que esta unión confiere mayor estabilidad física y térmica, un aumento de la solubilidad, reducción del aclaramiento plasmático, reducción de la inmunogenicidad y de la antigenicidad, así como mejora del perfil de toxicidad.

Recientemente, se ha estudiado el uso de los PEGs y su potencial terapéutico en diferentes patologías, principalmente en la inflamación. Durante un proceso inflamatorio se liberan citoquinas que afectan a la estructura del glicocálix, tal y como se ha comentado anteriormente, provocando un aumento de la permeabilidad de la membrana dañada. Esto provocará un desequilibrio osmótico y por tanto la formación de edema [118]. El papel protector de los PEGs en este caso será gracias a su capacidad de formar coloides [119]. Estas estructuras coloidales aumentan la presión oncótica y evitan la desestabilización del glicocálix, de manera que disminuye el edema. Además, el PEG se adhiere a los glicerofosfolípidos de las membranas celulares, de forma que esta interacción ayuda a preservar su integridad [120]. Este hecho será muy importante en el daño por isquemia reperfusión en el trasplante hepático, puesto que el edema es una de las complicaciones a erradicar durante la preservación en frío [111,121,122].

El uso de los PEGs es muy variado, desde la aplicación en medicina como hidrogeles para tratamiento de heridas [123], en cultivos celulares para dar soporte a las células y la formación de matriz [124], en tratamientos farmacológicos, en que los PEGs se utilizan como vehículo para el transporte de fármacos a través de las membranas [125]. A nivel clínico, en los últimos años se ha potenciado su uso como aditivo a las soluciones de preservación durante la isquemia, así como tratamiento antiinflamatorio en páncreas [126]. Además, durante la preservación hepática, se ha demostrado que el PEG35 reduce el daño mitocondrial y el estrés oxidativo debido a su capacidad antioxidante indirecta y su contribución a la generación de óxido nítrico. En este sentido, protege los lípidos de los radicales libres mediante la inhibición de la peroxidación lipídica, reduciendo los niveles intracelulares de MDA [115,121].

La preservación dinámica oxigenada

En los últimos años, Schlegel y colaboradores han desarrollado una nueva técnica de preservación dinámica y oxigenada en frio para combatir los efectos de la hipoxia durante la preservación estática. La máquina de preservación hipotérmica oxigenada (HOPE, del inglés Hypothermic oxygenated perfusión) [122]. Su protección se basa en dos mecanismos. Por un lado, la oxigenación del tejido hepático en condiciones hipotérmicas protege de la lesión mitocondrial y nuclear, reduciendo la actividad mitocondrial antes de la reperfusión. En segundo lugar, la perfusión en frio, en condiciones de baja presión (≤ 3 mmHg), mantiene el glicocálix y el endotelio sin residuos, evitando el daño endotelial. De esta manera, una hora de HOPE en el centro de trasplante tras el traslado en frio es suficiente para recuperar la integridad hepática

y mejorar el éxito del trasplante [127]. Actualmente, en la clínica se utiliza HOPE con el medio de perfusión Belzer MP, una modificación de la solución de preservación en frio UW. No obstante, mantiene el HES como agente oncótico, por lo que no evita la agregación de glóbulos rojos. Los beneficios de la preservación en HOPE como nueva estrategia en el trasplante hepático, así como nuestra propuesta de utilizar la solución IGL-2 se detallan en los capítulos V y VI.

OBJETIVOS

El pretratamiento de órganos marginales es una oportunidad prometedora para aumentar la disponibilidad de órganos para el trasplante. Estudios previos han evidenciado que las propiedades del PEG35 contribuyen a la integridad del injerto durante la conservación en frío. El objetivo general de este proyecto de tesis es investigar los mecanismos celulares y moleculares del daño isquémico durante la conservación estática en frío (SCS) en diferentes soluciones de preservación de hígado graso utilizando un modelo de rata obesa, con el fin proponer nuevos enfoques terapéuticos y herramientas de diagnóstico para trasplante hepático de donante con esteatosis. El objetivo final es aumentar el pool de órganos aptos y así acortar las listas de espera de los pacientes, un problema crucial en el trasplante hepático.

Los objetivos específicos son los siguientes:

- Investigar los mecanismos moleculares asociados a la funcionalidad y al daño mitocondrial en la preservación hepática del hígado graso en frio (SCS) en el medio IGL-1, así como el papel del PEG35 y del GSH mediante el uso de las soluciones modificadas IGL-0 e IGL-2, utilizando injertos de ratas Zucker obesas.

- Analizar el daño por estrés oxidativo y la respuesta antioxidante en la preservación hepática del hígado graso de rata en frio en el medio IGL-1, así como el papel del PEG35 y el GSH mediante el uso de las soluciones modificadas IGL-0 e IGL-2.

- Estudiar el estrés del retículo endoplasmático y el estrés oxidativo en la preservación estática de injerto hepático graso comparando la solución que contiene PEG35 y GSH, IGL-2, con las soluciones comerciales UW y HTK utilizando injertos de ratas Zucker obesas.

- Evaluar el daño inducido por inflamación estéril y la respuesta inflamatoria durante la preservación estática en frio en las soluciones IGL-2, UW y HTK de hígados de rata con esteatosis.

- Explorar el papel del PEG35 y el GSH del medio IGL-2 en la preservación dinámica hipotérmica oxigenada (HOPE) mediante un posible efecto protector de la integridad y funcionalidad mitocondrial.

INFORME DE LOS DIRECTORES

La Dra. Teresa Carbonell i Camós i el Dr. Arnau Panisello-Rosello com a directors d'aquesta tesi doctoral, fem constar que:

La memòria presentada per la Sra. Raquel Gómez Bardallo i titulada: "Mecanismos moleculares en el daño por isquemia en la preservación fría del hígado graso" és una tesi doctoral elaborada en format d'articles. Recull els resultats originals obtinguts per la doctoranda i articles de revisió. Els seus resultats han estat publicats o preparats per a ser sotmesos per a la seva publicació a revistes internacionals amb revisió per parells.

La Sra. Raquel Gómez Bardallo ha participat activament en l'elaboració dels articles presentats en aquesta tesi, ha dut a terme el disseny dels experiments i el treball experimental i ha contribuït a l'anàlisi crítica de les dades i resultats. A més, també ha participat en la redacció dels articles.

Els articles presents a la tesi són:

1- **Review:** Nrf2 and oxidative stress in liver ischemia/reperfusion injury. **Bardallo, R.G**., Panisello-Roselló, A., Sanchez-Nuno, S., Alva, N., Roselló-Catafau, J. and Carbonell, T. (2022), FEBS J, 289: 5463-5479.

https://doi.org/10.1111/febs.16336

Factor d'impacte: 5.622. Q2, BIOCHEMISTRY & MOLECULAR BIOLOGY (índex SJR)

Nº de cites: 20 (Web of Science)

La doctoranda ha col·laborat en la recerca bibliogràfica, escriptura del manuscrit i disseny de les figures. La Figura 2 de l'article ha estat portada de la revista (Volum 289, num18, setembre 2022).

2- **Article original:** Role of PEG35, Mitochondrial ALDH2, and Glutathione in Cold Fatty Liver Graft Preservation: An IGL-2 Approach. **Bardallo, R.G**.; da Silva, R.T.; Carbonell, T.; Folch-Puy, E.; Palmeira, C.; Roselló-Catafau, J.; Pirenne, J.; Adam, R.; Panisello-Roselló, A. Int. J. Mol. Sci. 2021, 22, 5332.

https://doi.org/10.3390/ijms22105332

Factor d'impacte: 6.208. Q1, BIOCHEMISTRY & MOLECULAR BIOLOGY (índex SJR)

Nº de cites: 12 (Web of Science)

La doctoranda va realitzat els procediments quirúrgics i el treball experimental.

3- **Article original:** PEG35 and Glutathione Improve Mitochondrial Function and Reduce Oxidative Stress in Cold Fatty Liver Graft Preservation. **Bardallo, R.G**.; Company-Marin, I.; Folch-Puy, E.; Roselló-Catafau, J.; Panisello-Rosello, A.; Carbonell, T. Antioxidants 2022, 11, 158.

https://doi.org/10.3390/antiox11010158

Factor d'impacte: 7.675. Q1, BIOCHEMISTRY & MOLECULAR BIOLOGY (índex SJR)

Nº de cites: 5 (Web of Science)

La doctoranda ha col·laborat en la conceptualització, ha dut a terme la posta a punt metodològica, la realització dels experiments, l'anàlisi formal i la redacció de l'esborrany inicial.

4- **Article original:** DAMPS induce inflammatory injury in cold-static fatty liver graft preservation. The key role of redox and ER stress. **Bardallo, R.G**.; Alva,N; Roselló-Catafau, J.; Panisello-Rosello, A.; Carbonell, T.

Article preparat per ser sotmès a Redox Biology. Els resultats d'aquest treball varen ser presentats al Congrés de la Societat Internacional de Trasplantaments (ILTS) del 2022 (Gomez Bardallo R, et al. Relevance of the oxidative and endoplasmic reticulum stress against ischemia insult during cold fatty liver graft preservation: a comparison between three preservation solutions. Transplantation. 2022;106(Suppl 8):94)i ha estat citat com una de les principals recerques presentades sobre perfusió hepática (Bhat M,et al. What Is Hot and New in Basic and Translational Science in Liver Transplantation in 2022? Report of the Basic and Translational Research Committee of the International Liver Transplantation Society. Transplantation. 2022 Dec 23. doi: 10.1097/TP.0000000000004476). La doctoranda ha col·laborat en la conceptualització, ha dut a terme la posta a punt metodològica, la realització dels experiments, l'anàlisi formal i la redacció de l'esborrany inicial

5- **Review:** IGL-2 as a Unique Solution for Cold Static Preservation and Machine Perfusion in Liver and Mitochondrial Protection. Da Silva, R.T.; **Bardallo, R.G.;** Folch-Puy, E.; Carbonell, T.; Palmeira, C.M.; Fondevila, C.; Adam, R.; Rosello-Catafau, J.; Panisello-Roselló, A. Transplant. Proc. 2022, 54, 73–76.

http://dx.doi.org/10.1016/j.transproceed.2021.10.008

Factor d'impacte: 1.014. Q4, TRANSPLANTATION (índex SJR)

Nº de cites: 4 (Web of Science)

Aquest treball també s'ha utilitzat en una altra tesi doctoral. La doctoranda ha participat de la contextualització i ha col·laborat en la recerca bibliogràfica i escriptura del manuscrit.

6- **Review:** Liver Graft Hypothermic Static and Oxygenated Perfusion (HOPE) Strategies: A Mitochondrial Crossroads. **Bardallo, R.G**.; Da Silva, R.T.; Carbonell, T.; Palmeira, C.; Folch-Puy, E.; Roselló-Catafau, J.; Adam, R.; Panisello-Rosello, A.. Int. J. Mol. Sci. 2022, 23, 5742.

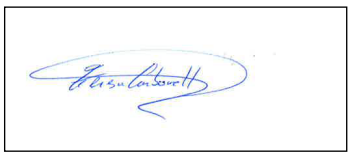
https://doi.org/10.3390/ijms23105742

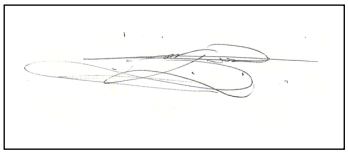
Factor d'impacte: 6.208. Q1, BIOCHEMISTRY & MOLECULAR BIOLOGY (índex SJR)

Nº de cites: 3 (Croossref)

La doctoranda ha realitzat els experiments i analitzat els resultats que es mostren. També ha participat en la redacció del manuscrit.

Barcelona, 29 de setembre 2022





Dra. Teresa Carbonell I Camós

Dr. Arnau Panisello- Rosello

RESULTADOS

CAPÍTULO I. Nrf2 and oxidative stress in liver ischemia/reperfusion injury

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STATE-OF-THE-ART REVIEW

Nrf2 and oxidative stress in liver ischemia/reperfusion injury

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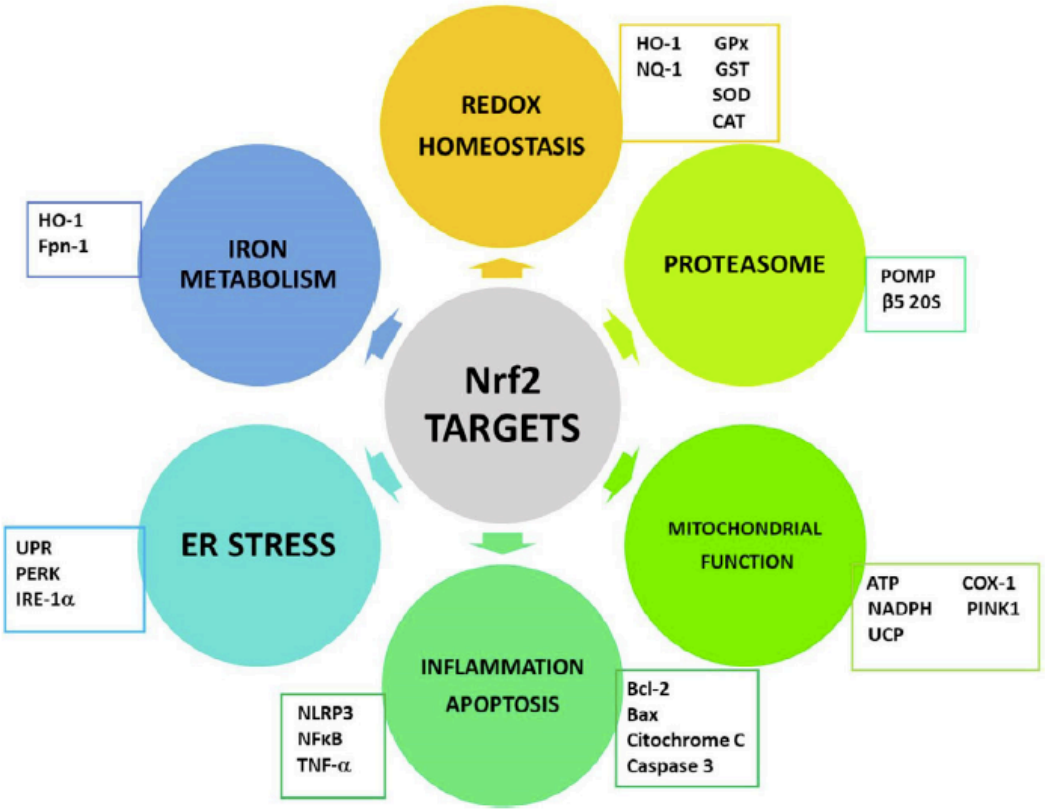
Graphical Abstract



Resumen:

El grupo de investigación en el que he realizado la tesis doctoral, que forma parte del grupo consolidado por AGAUR en el "Grupo de Fisiología Adaptativa: ejercicio e hipoxia", tiene como objetivo principal de estudio el estrés oxidativo. En los inicios de la fase experimental, en que evaluamos los diferentes marcadores redox así como la respuesta antioxidante en hígados preservados en distintas soluciones de preservación, se observó el papel clave del factor Nrf2 en la fisiología y fisiopatología de la respuesta

antioxidante. Con la finalidad de integrar conceptos así como establecer su papel en el actual diseño experimental de daño por IR, se observó la finalidad de hacer una revisión de sobre tema.

Por tanto, esta revisión se centra, por una parte, en describir los mecanismos de activación de Nrf2 por parte de Keap1 en respuesta a condiciones de estrés. Por otra parte, su respuesta tras la activación de los genes ARE y la respuesta al estrés oxidativo, así como el papel que tiene Nrf2 en la regulación del metabolismo del hierro, el proteasoma, el estrés del retículo endoplasmático, la inflamación, la apoptosis y, muy especialmente, en la regulación de la homeóstasis mitocondrial y el metabolismo energético. Finalmente, se detallan las estrategias de tratamiento de Nrf2 en daño por IR, incluyendo inductores de Nrf2 como antioxidantes, tratamientos farmacológicos, anestésicos y tratamientos de precondicionamiento isquémico.

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STATE-OF-THE-ART REVIEW

Nrf2 and oxidative stress in liver ischemia/reperfusion injury

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Keywords
antioxidant; inflammation; ischemia-reperfusion injury; liver; Nrf2; redox

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In response to stress signal, nuclear factor-erythroid 2-related factor 2 (Nrf2) induces the expression of target genes involved in antioxidant defense and detoxification. Nrf2 activity is strictly regulated through a variety of mechanisms, including regulation of Keap1-Nrf2 stability, transcriptional regulation (NF-κB, ATF3, ATF4), and post-transcriptional regulation (miRNA), evidencing that transcriptional responses of Nrf2 are critical for the maintenance of homeostasis. Ischemia-reperfusion (IR) injury is a major cause of graft loss and dysfunction in clinical transplantation and organ resection. During the IR process, the generation of reactive oxygen species (ROS) leads to damage from oxidative stress, oxidation of biomolecules, and mitochondrial dysfunction. Oxidative stress can trigger apoptotic and necrotic cell death. Stress factors also result in the assembly of the inflammasome protein complex and the subsequent activation and secretion of proinflammatory cytokines. After Nrf2 activation, the downstream antioxidant upregulation can act as a primary cellular defense against the cytotoxic effects of oxidative stress and help to promote hepatic recovery during IR. The complex crosstalk between Nrf2 and cellular pathways in liver IR injury and the potential therapeutic target of the Nrf2 inducers will be discussed in the present review.

Abbreviations
4-HNE, 4-Hydroxynonenal; ADP, adenosine diphosphate; ALT, alanine transaminase; ARE, antioxidant response elements; ATF3, activating transcription factor 3; ATF4, activating transcription factor 4; BHA, butylated hydroxyanisole; CAT, catalase; CDDO-Im, imidazol derivative; CDDO-Me, bardoxolone-methyl; COX2, cyclooxygenase-2; Cul3, cullin 3; D3T, 3H-1,2-dithiole-3-thione; DMF, dimethyl fumarate; eNOS, endothelial nitric oxide synthase; ER, endoplasmic reticulum; FGF10, fibroblast growth factor 10; Fpn1, ferroportin-1; G6PD, glucose-6-phosphate 1-dehydrogenase; GOH, geraniol; GPx, glutathione peroxidase; GSH, reduced glutathione; GSSG, oxidized glutathione; GST, glutathione-s transferase; HO-1, heme oxygenase-1; HSP, heat shock proteins; IGL-1, institute george lopez solution; IL, interleukin; iNOS, inducible nitric oxide synthase; IR, ischemia-reperfusion; IRE1, inositol requiring enzyme 1; Keap1, kelch ECH associating protein 1; LC3, microtubule-associated proteins 1A/1B light chain 3B; LPS, lipopolysaccharide; MEI, microsomal enzyme inducers; miRNA, micro RNA; mtDNA, mitochondrial deoxyribonucleic acid; NASH, non-alcoholic steatohepatitis; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NLRP3, NLR family pyrin domain containing 3; NMP, normothermic machine perfusion; NQO, NADPH quinone reductase; NRF1, nuclear respiratory factor-1; Nrf2, nuclear factor-erythroid 2-related factor; OLT, orthotopic liver transplantation; PEG-35, polyethylene glycol 35; PERK, PKR-like endoplasmic reticulum kinase; PERK, PKR-like endoplasmic reticulum kinase; PGD, 6-phosphogluconate dehydrogenase; PINK1, PTEN-induced putative kinase 1; POMP, proteasome maturation protein; ROS, reactive oxygen species; SOD, superoxide dismutase; TLR4, toll-like receptor 4; TNF- α, tumor necrosis factor α; UCP, uncoupling proteins; UPR, unfolded protein response; YAP, yes-associated protein.

Introduction

Ischemia-reperfusion (IR) injury is an important cause of organ damage that occurs in different systems, as well as in human diseases and clinical conditions [1–3]. During liver surgery, the unavoidable interruption of blood flow causes ischemia followed by a subsequent tissue reperfusion. This results in a massive injury to the hepatocytes. Several studies have demonstrated the association between hepatic IR injury and reactive oxygen species (ROS) generation. During the initial phase of IR, ROS can also cause considerable harm to hepatocytes through lipid peroxidation, protein oxidation, mitochondrial dysfunction, and DNA damage. Subsequently, Kupffer cells and neutrophils are recruited in response to the death of hepatocytes, leading to liver inflammation. Under the IR process, the damage caused to the liver tissue causes the nonviability of the organ [4].

Since ROS regulation is suggested as a new therapeutic strategy for liver IR injury [5,6], a large number of antioxidant agents have been tested both clinically and experimentally. Vitamins, such as α-tocopherol [7,8] and resveratrol [9], have shown hepatoprotective effects against IR injury. Melatonin prevents oxidative stress and inflammatory cytokine release when added to storage solutions [10] and the glutathione precursor N-acetyl cysteine improves microcirculation in the treatment of liver IR [11].

Induction of several highly protective endogenous genes occurs early in ischemic injury. They are controlled at the transcriptional level by the nuclear factor-erythroid 2-related factor 2 (Nrf2) [12,13]. Nrf2 plays a protective role against many pathological conditions, such Alzheimer's disease, Parkinson's disease, epilepsy, inflammation, aging, and ischemia, all of them being closely related to oxidative stress [14–18]. Recently, it has been proposed that Nrf2 activation may induce clinical benefits against acute respiratory distress syndrome and pneumonia due to COVID-19 [19,20]. Therefore, advances in understanding the regulation of antioxidant systems by Nrf2 may provide exciting new potential therapeutic targets.

Upon detection of various stress factors, including ROS [21], the assembly of the inflammasome protein complex NLR pyrin domain containing protein 3 (NLRP3) results in activation and secretion of pro-inflammatory cytokines. The NLRP3 inflammasome has been involved in the pathogenesis of liver injury [22,23]. Recent evidence showed an inverse relationship between the Nrf2 pathways and the NLRP3 inflammasome at different levels [24–26].

In recent years, research on Nrf2 has described its role as the backbone of multifaceted cellular defense mechanisms. Oxidative stress activates the Nrf2 transcription factor, which in turn induces the expression of a series of cytoprotective gene products. This review addresses the role of Nrf2 in hepatic IR with the aim of therapeutically targeting Nrf2 for IR protection.

The Keap1-Nrf2 signal transduction pathway

Nrf2 is a basic leucine zipper protein, with a Cap "*n*" collar structure (CNC). Nrf2 is normally distributed in the cytoplasm of mammalian cells, where it is bound to its cytosolic repressor Kelch ECH associating protein 1 (Keap1) and E3 ubiquitin ligase. Under physiological conditions, Keap1 binds to Nrf2 in the cytoplasm and promotes ubiquitination by the E3 ubiquitin ligase. This process removes Nrf2 from the cytoplasm, where it is constantly degraded, preventing the translocation of Nrf2 to the nucleus, where it would have signaling effects (Fig. 1).

Under exposure to electrophiles, ROS and nitric oxide modification of the cysteine residues of Keap1 occurs [27] leading to conformational changes in Keap1 and thus inhibition of Nrf2 ubiquitination [28–30]. As a response, Nrf2 undergoes phosphorylation and translocates to the nucleus [31] where it attaches to Antioxidant Response Elements (AREs) [32].

Activation of Nrf2 pathways has been studied in different cellular compartments. Altered redox homeostasis can cause stress in the endoplasmic reticulum (ER) which, in turn, could induce ROS production in the ER. In order to limit oxidative damage, the ER-spanning transmembrane proteins PKR-like endoplasmic reticulum kinase (PERK) and inositol requiring enzyme 1 (IRE1) phosphorylate and activate Nrf2 [33]. The mechanisms of Nrf2 activation by ER stress will be discussed in detail below. Mitochondria also activate Nrf2, as recently discussed by Tsushima et al. [34], in response to multiple redox signals with the aim of protecting cells against oxidative damage derived from inflammation. Mitochondrial or ER stress stimulates activating transcription factor 4 (ATF4) and regulates redox homeostasis in the cells [35,36]. Nrf2 and ATF4 cooperatively upregulate cytoprotective genes [34,37].

In addition to this classical pathway, it has also been described that p62 is capable of enhancing the transcriptional activity of Nrf2 [38]. The p62 protein is a selective substrate for autophagy and regulates the formation of protein aggregates. Accumulation of inclusions positive for p62 and/or p62 has been reported in human liver diseases where its overproduction competes with the interaction between Nrf2 and Keap1, resulting in Nrf2 stabilization [38,39].

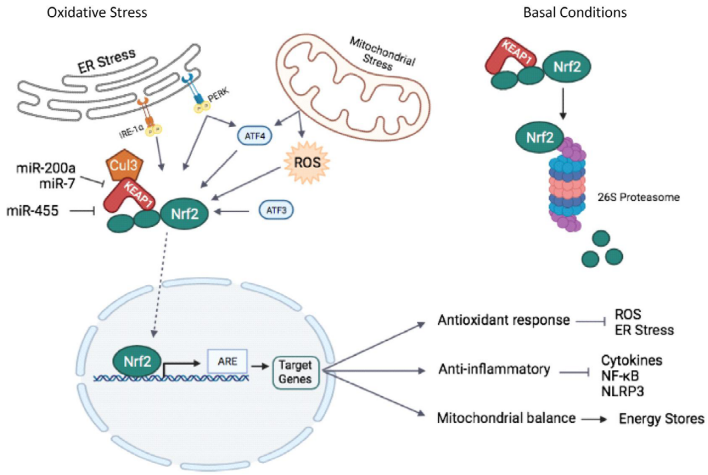

Fig. 1. The Keap1-Nrf2 signal transduction pathway. Under basal conditions, Nrf2 is polyubiquitinated by the ubiquitin ligase complex Keap1, becoming a substrate for the 26S proteasome. Under conditions of oxidative stress, Keap1 undergoes oxidation of its reactive cysteine residues, resulting in the inhibition of Nrf2 ubiquitination and thus in the cytosolic and nuclear accumulation of Nrf2. Nrf2 can also be regulated at the post-transcriptional levels. Activation of the Nrf2-ARE pathway triggers the transcription of multiple genes. This involves the activation and modulation of antioxidant and radical scavenging functions, anti-inflammatory and metabolic functions.

Activity of Nrf2 can also be modulated at the post-transcriptional level. miRNAs can target genes that indirectly modulate Nrf2 activation, mostly by targeting KEAP1 [40,41] and cullin 3 (Cul3) [42]. These studies suggest that miRNAs can constitute a novel strategy to provoke the activation of the antioxidant response through Nrf2 signaling [43].

Nrf2 as a transcription factor: cellular and molecular functions in the liver

The protective function of Nrf2 is due to the regulated expression of a battery of genes associated with oxidative stress, chronic inflammation, and cellular detoxification [13,44]. In fact, Nrf2 was originally identified for its cancer prevention function, through induction of Phase II enzymes. The search for Nrf2 targets throughout the genome has been validated by using knockout models. Furthermore, microarray technologies have been productively used to profile all genes under the control of Nrf2. This allowed the identification of many ARE regions that contain genes beyond the conventional antioxidant stress response. As shown in Fig. 2, Nrf2 affects multiple aspects of cellular functions through gene regulation or by transcription factors crosstalking.

During the last decade, several researchers reported that the activation of the Nrf2 transcription factor has a great impact on improving hepatic IR injury [45–50] (Table 1).

The hepatoprotective function of Nrf2 against liver IR injury was first described by Ke et al. [47]. They showed that the Keap1-Nrf2 complex alleviates oxidative injury in a mouse model of hepatic cold storage (20 h at 4 °C) followed by orthotopic liver transplantation (OLT). Ablation of Keap1 signaling reduced macrophage and neutrophil trafficking, pro-inflammatory cytokine programs, and hepatocellular necrosis and apoptosis, while simultaneously promoting anti-apoptotic functions in mouse orthotopic liver transplantation. The role of Nrf2 was also identified in Nrf2-deficient livers from mice after 60 min of ischemia followed by reperfusion [46]. Nrf2-deficient livers exhibited enhanced tissue damage; impaired glutathione-S transferase (GSTm1), and NADPH quinone reductase (NQO1) inductions; aggravated Tumor Necrosis Factor α (TNF-α) expression, when compared to wild-type mice. Recently, a crucial role for YAP (Yes-associated protein) effector in IR-mediated hepatocellular damage and liver fibrogenesis has been described [49]. Notably, YAP activation failed to protect Nrf2-deficient livers against IR-mediated damage, leading to extensive fibrosis.

The hepatic expression of Nrf2 could also be used as a criterion to rule out marginal liver allografts. In a recent work [50], discarded human donor livers were stratified into a high NRF2 and low NRF2 group by quantifying NRF2 expression and were then exposed to a normothermic perfusion machine (NMP). After 6 h NMP, high NRF2 livers had significantly reduced liver enzyme alterations and improved lactate clearance.

Nrf2-mediated response to oxidative stress and detoxification

It is well known that Nrf2, Keap1, and ARE genes are implicated in Phase II of the detoxification after the

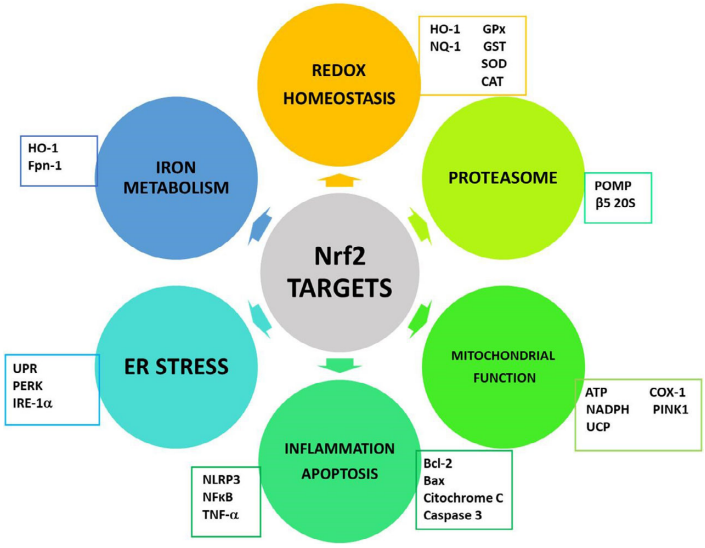

Fig. 2. Schematic representation of the cellular functions driven by Nrf2 target genes. Nrf2 targets are involved in a multiple of cellular processes, including: redox regulation, metabolism and mitochondrial function, inflammation and apoptosis, iron homeostasis, and proteasome assembly. As antioxidant response, Nrf2 promotes enzymatic activity of HO-1, GPx, GST, SOD, and CAT and attenuates inflammation targeting NLRP3 and NF-κB. Regarding energy storage, Nrf2 increases NADPH and ATP. Nrf2 also activates uncoupled proteins and COX-1, affecting mitochondrial function.

inflammatory process [46]. Most of the Nrf2 target genes are involved in providing cellular protection against damage by oxidizing molecules. ARE genes include antioxidant enzymes, like superoxide dismutase (SOD), glutathione-S transferase (GST), glutathione peroxidase (GPx), catalase (CAT), but also proteasomal proteins, as heat shock proteins (HSP), and anti-inflammatory proteins, heme oxygenase-1 (HO-1), and cyclooxygenase-2 (COX-2) [51,52]. One of the first antioxidant enzymes to be related to ARE genes was HO-1, an inducible enzyme that catalyzes the reaction of the heme group to biliverdin, which is reduced to the potent antioxidant bilirubin [53].

The experimental confirmation that phase II enzymes were under transcriptional control of AREs came from the research of Itoh et al. [54]. They used the phenolic antioxidant butylated hydroxyanisole, an inductor of phase II antioxidants, in the *nrf2* heterozygous and homozygous mutant mice. While the expression of phase II enzymes (e.g., GST, NQO) was markedly induced in wild-type and heterozygous mutant mice, the induction was eliminated in the liver and gut of homozygous $nrf2^{-/-}$ mutant mice [54]. After these experiments, they reported that Nrf2 regulates ARE.

Several subsequent studies have contributed to expanding the number of genes involved in detoxification after Nrf2 activation in the liver. In liver from $nrf2^{-/-}$ mutant mice, reduced transcription levels of UDP-glucuronyl-transferase 1A6, HO-1, and manganese superoxide dismutase (MnSOD) were obtained after induction of a cancer chemopreventive agent (D3T) confirming that these inducible detoxifying enzymes are regulated by Nrf2 [55].

Regarding the glutathione system, which is the main nonenzymatic cellular antioxidant, the $nrf2^{-/-}$ mouse model confirmed that Nrf2 is essential for constitutive and inducible expression of ARE, which mediates GST (Gsta1, Gsta2, Gstm1, Gstm2, Gstm3, and Gstm4) genes [56]. GST catalyzes the conjugation of reactive electrophilic species with glutathione (GSH) to reduce potential xenobiotics and to detoxify. Furthermore, higher levels of GSH were found in response to fisetin, a plant polyphenol from the flavonoid group, compared to the control group. This increase is granted by the transcription factor Nrf2 [57]. Thus, the increased sensitivity of Nrf2 knockout mice to xenobiotics can be partly attributed to the loss in constitutive expression of multiple GSH-dependent enzymes, which causes a reduction in intrinsic detoxification capacity in knockout animals. The attenuated induction of GSH-dependent enzymes found in $nrf2^{-/-}$ homozygous mice explains their lack of adaptation to the chronic exposure to chemical and oxidative stress.

Besides the liver, these Nrf2-mediated antioxidant mechanisms have also been described during IR in the brain [58–60], heart [61–63], and kidney [64–66].

Nrf2 regulates iron metabolism

Nrf2 play a critical role in mediating iron metabolism [67,68]. Tissue damage promotes hemolysis, and the resulting cytosolic heme can be catabolized into ferrous iron and biliverdin by HO-1, an Nrf2 target gene [53].

Table 1. Impact of Nrf2 transcription factor activation on liver IR injury.

Model	Nrf2 pathway	Mediators	Effects and outcome	References
Nrf2-deficient treated with 15d-PGJ2 subjected to hepatic IR (mice)	Amplification of Nrf2 activation	15d-PGJ2 protected livers from I/R injury via activation of Nrf2	Nrf2-deficient mice showed: Enhanced tissue damage, impaired GSTm1, NQO1, and GCLc inductions, disturbed redox state, and aggravated TNF-α	[46]
Brg1 overexpression in hepatic IR (mice)	Activation of Nrf2/HO-1 pathway	Increased antioxidants HO-1 and NQO1	Alleviate hepatic IRI Decrease 8-isoprostane levels	[48]
Liver IRI cold storage (20 h at 4 °C) followed by OLT in KEAP1 KO in the donor liver (mice)	Keap1-Nrf2 signaling pathway	Ablation of Keap1 signaling	Reduced macrophage/neutrophil trafficking, pro-inflammatory cytokine, and hepatocellular necrosis/apoptosis. Anti-apoptotic functions in OLTs	[47]
IR in Nrf2 deficient livers (human biopsies and mice)	Nrf2 required for hepatocellular protection	Intrinsic hepatic YAP expression	Reduction of Oxidative stress Reduction in apoptosis/necrosis Reduced inflammatory response Attenuates fibrosis	[49]
Mouse model of warm IRI	Nrf2 activation by FGF10 overexpression	Phosphatidylinositol-3-kinase/AKT pathway	Protective role of FGF10 in liver IRI and the role of Nrf2 in preventing oxidative stress	[45]
Human liver allografts with high Nrf2 expression exposed to 6-h of NMP	Nrf2 expression	Are not considered	Nrf2 improves liver function after NMP	[50]

Nrf2 also controls the heavy and light chains of the iron storage protein ferritin, as well as the protein responsible for the flux of iron out of the cell, ferroportin [67]. Harada et al. observed that Nrf2 activation by electrophilic compounds resulted in upregulation of ferroportin-1 (Fpn1) gene transcription in macrophages obtained from wild-type mice, but not from Nrf2 knockout mice [69]. Recently, it has been shown that the synthesis of hepcidin, the peptide hormone that maintains systemic iron homeostasis, is altered in Nrf2 knockout mice, increasing iron accumulation, and hepatic damage [70].

Moreover, Okada et al. proved the role of Nrf2 regulating iron metabolism in steatohepatitis. Using wild-type mice, Nrf2 gene-null mice and Keap1 gene-knockdown mice (sustained activation of Nrf2) they demonstrated that Nrf2 inhibits hepatic iron accumulation and counteracts against oxidative stress-induced liver injury in fatty livers [71]. It should be noted that dysregulation of iron metabolism can lead to cell damage and increased inflammation, as reactive ferrous iron can participate in Fenton reactions that result in the hydroxyl radical formation. Therefore, the role of Nrf2 in the regulation of iron metabolism could be considered as an antioxidant cytoprotective mechanism.

Proteasome regulation by Nrf2

The proteasome system is responsible for the removal of oxidized, misfolded and damaged proteins. The main proteasome activity is performed by 26S proteasome, which consist of a 20S core and two 19S regulatory subunits. Most of the genes encoding proteasome subunits and the proteasome maturation protein (POMP) contain ARE. Li et al. described that Nrf2 binds and activates the POMP promoter [72]. Furthermore, proteasome expression has been shown to be regulated by Nrf2 through ARE genes located on the proximal promoter region of several proteasome subunits in mouse cell lines [73]. Pickering et al. [74] found that Nrf2 translocated to the nucleus and bound to the ARE sequences of the proteasome β5 subunit gene, leading to increased expression of the 20S proteasome after the addition of H_2O_2.

Additionally, Nrf2 inducers also upregulate proteasome subunits and activity [30]. In senescent fibroblasts, treatment with an Nrf2 inducer results in increased transcription of proteasome subunits and increased levels of assembled proteasome. This results in elevated proteasome activities and functions, accompanied by reduced ROS levels and oxidized proteins, as well as lifespan extension [75].

Induction of proteasome subunits by the Nrf2 pathway could be relevant in liver IR, since the increase in proteasome activity has been observed [76,77], and is likely to be an important way, for a cell undergoing oxidative stress, to increase its capacity to remove damaged and oxidized proteins.

Nrf2 activation by ER stress

The production of ROS and the consequent oxidation of proteins is one of the main initiators of endoplasmic

reticulum (ER) stress. ER is an essential organelle for the synthesis, modification, and folding of proteins. Due to the system complexity, errors can occur and cause the accumulation of misfolded proteins, leading to ER stress. It arises in response to different physiological processes such as oxidative stress, Ca^{2+} imbalance, as well as associated diseases such as ischemia, atherosclerosis, and diabetes [78]. In response to ER stress, the GRP78 protein will lead to the unfolded protein response (UPR) by activating specific transmembrane receptors, associated with the initiation of the UPR signaling pathway, by binding to the lumen domains of the ER. Activation of the UPR system allows non-native polypeptides to fold or be removed; otherwise, as a last resort, apoptosis occurs [79].

Nrf2 can also be activated by UPR machinery [80]. Nrf2 has been proposed to be a direct PERK substrate and an indirect substrate for IRE1α kinase activity [33]. Following exposure to ER stress, the Nrf2 activation by PERK signaling promoted cell survival. The regulation includes phase II detoxifying enzymes induction, which maintains cellular redox homeostasis as well as the repression of genes that promote apoptosis. An alternative mechanism that drives Nrf2 activation after PERK activation has recently been described. This activation of PERK triggers ATF4-dependent control of Nrf2 mRNA abundance that enhances the cytoprotective function of Nrf2 [36]. Hence, the PERK pathway may involve different mechanisms to activate Nrf2 during ER stress.

Moreover, Nrf2 has been tested as an ER stress controller. In a recent study, the ability of the PERK/Nrf2/HO-1 axis to protect against IR damage in mice myocytes has been demonstrated [81]. Although HO-1 is only one of the Nrf2 response factors, it is essential in the response of ER. The same work showed that inhibition of HO-1 expression invalidated the roles of PERK overexpression in restricting signal transduction of the ER stress-mediated apoptotic cascade. A connection between UPR and Nrf2 was reported in the liver. Thus, Nrf2 has been observed to play a cytoprotective role in response to ER stress in animal models of nonalcoholic steatohepatitis (NASH) [71] and in hepatocellular carcinoma cells [82].

Regarding IR models, it has been described in rat brain that Nrf2 improves ER stress due to its antioxidant response and prevention of ROS formation. This protection is considered to be afforded by creating a positive feedback loop between p62/ZIP and Nrf2. p62/ZIP is an autophagy receptor and can transport misfolded proteins to a macroautophagy-lysosome system for degradation [83]. Although this mechanism is not entirely clear, it is associated with a dual role. On the one hand promoting the interaction between Keap1 and microtubule-associated proteins 1A/1B light chain 3B (LC3), thus activating autophagy and accelerating the elimination of protein aggregates. On the other hand, by the connection of a positive feedback loop between p62/ZIP and Nrf2, that can play a role in relieving ER stress in cerebral IR injury.

Nrf2 role in energy stores and mitochondrial balance

Nrf2 target genes affect multiple aspects of metabolism and mitochondrial function, such as glycolysis, pentose phosphate pathway, fatty acid metabolism [84], as well as genes for nucleotide synthesis [85], and NADPH synthesis [86].

Mitochondrion is an essential organelle for the energy and metabolic maintenance of cells. However, in stressful circumstances such as ischemia, mitochondria is known to be the main ROS generator, activating Nrf2 and leading to ARE genes transcription. Some of these genes prevents directly or through crosstalking, metabolic disruption, and consequently autophagy [84].

The first alterations during cellular ischemia occur in the mitochondria. The lack of oxygen as the final electron acceptor in the mitochondrial electron transport chain increases the reduced state of the organelle because of the increase in the $NADPH/NADP^{+}$ ratio and a decrease in the generation of ATP from ADP. During cellular anoxia, and independently of the decrease in the oxidative generation of ATP, there are intracellular mechanisms that lead to the inhibition of ATP synthase and the maintenance of the ionic gradient that preserve mitochondrial potential and integrity. This adaptive cellular response to anoxia is a consequence of the release of mitochondrial calcium in cultured hepatocytes [87–89]. When blood flow is restored, accumulated metabolites such as calcium and succinate will cause ROS overproduction and the consequent damage to liver tissue. Thus, avoiding the accumulation of ROS appears as a main therapeutic strategy, and in this sense it has been shown in recent years that Nrf2 can play an important role [64,90,91].

NADPH provides the reducing equivalents for biosynthetic reactions of oxido-reduction and protects against ROS, but also through the glutathione and thioredoxin systems. Nrf2 regulates 4 genes which are responsible for NADPH generation (G6PD, PGD, MEI, IDH) [86]. In a "gene dose-response" study, performed a different control test for Nrf2 in mice liver, testing the knockout or overexpression of Nrf2 and KEAP1. They showed that Nrf2 increased the

antioxidant response when overexpressed, as well as an increase in NADPH. Furthermore, they concluded that Nrf2 activation increases the availability of NADPH to counteract oxidative stress while decreasing its activity in metabolism.

Nrf2 not only increases the production of NADPH but also promotes the reduction through the activation of NADPH quinone reductase (NQO1 and NQO2), which are encoded in ARE genes [54]. NQO1 and NQO2 catalyze detoxification of quinones, which prevents the generation of reactive semiquinones, $O_2^{\cdot-}$ and H_2O_2. Both constitutive and inducible expression of protective genes is regulated by the antioxidant response element (ARE) that is present in the upstream regions of these genes [92]. In a study of hepatic IR in mice, Li et al. demonstrated that overexpression of fibroblast growth factor 10 (FGF10) decreased oxidative stress and ROS generation, presumably due to upregulation of Nrf2. Among its results, it is worth highlighting the increase in the expression of the NQO1 enzyme [45]. In another study comparing the response of mice deficient in Nrf2 with respect to WT mice against IR damage, it was shown that those deficient in Nrf2 would provide greater tissue damage and less induction of the NQO1 enzyme, as well as greater oxidative damage [46]. The aforementioned studies point to the relevance of Nrf2 in the stress response to IR injury, as well as the importance of targeting Nrf2 as a therapeutic tool.

One of the strategies to prevent IR injury of the liver is to avoid ATP depletion [76,93]. In a model of rat liver cold ischemia, our group demonstrated that the solutions which best preserve graft viability prevented ATP depletion while increased Nrf2 levels [94]. Although Nrf2 has not been reported to have a direct effect on ATP synthesis, different studies support the importance of this factor for the preservation of ATP levels [95,96].

An additional antioxidant defense mechanism regulated by Nrf2 is through uncoupling proteins. Uncoupling proteins (UCP) are mitochondrial inner membrane proteins, whose function is to decrease the mitochondrial electrochemical potential leading to heat dissipation. In this way, the major function of UCP1 in rodents is to produce heat to maintain body temperature, while UCP-2 and UCP-3 can have different functions. Current findings suggest that UCP-2 and UCP-3 likely play a role in controlling the ROS production and regulating ATP synthesis [97]. UCP-2 improves the mitochondrial NAD^+/NADH ratio by suppressing ATP and ROS generation. The levels of NAD^+ directly control the activity of SIRT3 and in turn SIRT3 could regulate Nrf2 through PGC1α, although this pathway has not been directly demonstrated [98]. Upregulation of UCP-3 expression through Nrf-2 has been demonstrated [99]. 4-Hydroxynonenal (4-HNE) treatment in cardiomyocytes induced nuclear accumulation of Nrf2 and enhanced the expression of UCP-3. It is worth highlighting the importance of this finding, since the positive regulation of UCP-3 mediated by Nrf2 in response to 4-HNE, a final product of lipid peroxidation, could be important in the protection against oxidative stress in conditions of IR. The fact that Nrf2 stimulates the expression of UCP proteins while maintaining ATP levels could be explained given that some UCP homologues do not act as true uncouplers; instead, their activity goes beyond what was initially defined [100].

In the case of a high and irreversible mitochondrial damage or elevated ROS production, mitophagy occurs. In this process of clearance of dysfunctional mitochondria, Nrf2 will also play a key role. Usually, mitophagy is produced by a process of mitochondrial homeostasis and is mediated by the PTEN-induced putative kinase 1 (PINK1)/Parkin pathway targeting mitochondria lacking of membrane potential to autophagosomes. One of the activating pathways of mitophagy is through the autophagic adapter protein sequestome-1 (SQSTM/p62), which competes with Keap1 to bind Nrf2. p62 disrupts the Nrf2-Keap1 interaction and induces mitophagy independently of PINK/PARKIN recruitment.

Some studies described the regulation of mitophagy via Nrf2-PINK1. Murata et al. demonstrated in different cell lines that Nrf2 binds directly to the promoter regions of PINK1 [101] corresponding to ARE sequences, giving cells an advantage for cell survival. This model was also shown in diabetic kidney disease [102]. In an IR model of the mouse brain, Xu et al. tested the efficacy of esculetin as a neuroprotective therapy [103]. Their results showed increased Nrf2 expression related to a reduction in oxidative stress markers, and triggered mitophagy in the hippocampus. Until now, no studies have been reported linking the Nrf2-PINK1 pathway with hepatic ischemic damage.

Besides, another recent study demonstrates the role of Nrf2 on mitochondria being a regulator of nuclear respiratory factor-1 (NRF1) [62,104]. NRF1 was identified as a major transcriptional regulator that connects the regulation of nuclear-encoded genes and mitochondrial biogenesis. Nrf2 binds to NRF-1 promoter sites increasing NRF-1 expression and activity [62]. Accumulation of nuclear NRF-1 protein leads to gene activation for mitochondrial biogenesis and respiratory function [104].

Nrf2 is also a relevant factor in the regulation of PINK1 and NRF1, demonstrating its involvement in quality control and mitochondrial biogenesis. Although further studies will be needed to corroborate the role of Nrf2 as a regulatory mechanism of mitochondrial homeostasis in IR liver injury.

Nrf2 and inflammasome signaling

Nrf2 has been studied for its potential role in inflammation. Nrf2 knockout mice were hypersensitive to neuroinflammation induced by lipopolysaccharide (LPS) [105]. Moreover, Nrf2 deletion aggravated the damage caused by neuroinflammation and oxidative stress, increasing the pathogenesis of Alzheimer's Disease in mice [106]. In the liver IR, recent works evidenced an inverse relationship between the Nrf2 pathways and the NLRP3 inflammasome at different levels [24–26]. Inflammasomes are key components of the innate immune response [107]. Inflammasomes are multiprotein complexes that activate caspase-1, leading to the maturation of the pro-inflammatory cytokines interleukin 1B (IL-1B) and IL-18 and the induction of pyroptosis [107,108]. The most studied is the NLRP3 inflammasome [109]. Activation of NLRP3 has been shown to result in severe liver inflammation, fibrosis, and hepatocyte pyroptosis [110].

NF-κB signaling is a necessary input for adequate activation of the NLRP3 inflammasome [109,111]. In primary hepatocytes, LPS-induced expression of NLRP3 is directly related to activation of the NF-κB pathway, while inhibitors of NF-κB signaling are effective in inhibiting NLRP3 expression [109]. NF-κB is also a redox-regulated transcription factor, it is a protein complex mainly involved in cytokine production and cell survival [112]. In brain IR models, Nrf2 activation attenuates inflammation and apoptosis through inhibition of the Nrf2-mediated ROS/NF-κB pathway [113].

Activating transcription factor 3 (ATF3) is a stress-induced transcription factor that suppresses the expression of inflammatory genes in multiple cell types and diseases. Extracellular signals, such as ER stress, cytokines, chemokines, and LPS, are linked to ATF3 induction [114]. In a mice liver IR injury model, deletion of ATF3 aggravated liver damage, as evidenced by increased serum Alanine transaminase (ALT) levels, intrahepatic macrophage/neutrophil trafficking, hepatocellular apoptosis, and up-regulation of pro-inflammatory mediators [115]. Recent studies demonstrated ATF3-mediated Nrf2/HO-1 signaling in the regulation of Toll-Like Receptor 4 (TLR4) driven inflammatory responses in IR livers [116].

Nrf2 regulates apoptosis

Cellular apoptosis contributes to liver damage during hepatic IR. The fact that oxidative stress can trigger apoptotic cell death was first demonstrated in primary rat hepatocytes [117]. In a mouse model, the activation of a Tollip-ASK1-JNK/p38 pathway during hepatic IR injury lead to apoptosis [118]. Additionally it has been described that the MAPK signal transduction pathway plays a key role in the regulation of inflammatory and apoptotic processes in a rat model of prolonged cold hepatic IR [119].

Nrf2 also play a key role in preventing apoptosis. In mouse hepatoma and human hepatoblastoma cells exposed to the antioxidant tert-butylhydroquinone, Nrf2 was found to mediate the upregulation of the anti-apoptotic protein Bcl-2 that led to a decrease in Bax, cytochrome c release, activation of caspases, and increased cell survival [120]. A recent study demonstrates that fibroblast growth factor 10 (FGF10) inhibits hepatocellular apoptosis during liver IR [45]. The protein levels of total and nuclear Nrf2 were both upregulated in FGF10-overexpressing mouse liver tissues after IR, as well as NQO-1. Furthermore, FGF10 failed to protect the liver at 6 h post-IRI in Nrf2 knockout mice.

Targeting Nrf2: new opportunities for liver IR protection

The findings described so far provide a rationale for a new therapeutic strategy for the management of IR-induced liver injury. Different strategies that could effectively activate Nrf2/HO-1 pathway proved to lead a better outcome during hepatic IR. Table 2 shows several compounds that, by activating Nrf2 signaling pathways, protect the liver from IR injury [48,116,121–128].

In hepatic warm and cold IR models in mice, the activation of Nrf2 signaling using the organosulfur compound oltipraz, activated the anti-inflammatory functions of ATF3 [116]. Geraniol (a natural essential oil) was also used in a model of liver IR in rats. The hepatoprotective effect of the oral administration of geraniol, prior to liver IR, was mediated through the activation of Nrf2/HO-1 signaling, which resulted in the improvement of the antioxidant defense system with the reduction of oxidative stress [122]. Intraperitoneal pretreatment with the triterpenoid CDDO-imidazole activates Nrf2 and improves IR liver injury in mice by attenuating necrosis and apoptosis and reducing ROS levels [121]. These protective effects were attributed to the Nrf2/HO-1 signaling pathway.

Table 2. Compounds that activate Nrf2 signaling with potential protective effects on IR liver injury.

Compound	Model	Nrf2 signaling	Mediators Effects and Outcome		References
Oltipraz	Mice	Activation of Nrf2/HO-1 pathway	Activate ATF3 anti-inflammatory function	Regulate TLR4 innate immune response	[116]
CDDO-imidazolide	Mice	Activation of Nrf2/HO-1 pathway	Enhanced autophagy	Reduced mtDNA release Reduced ROS Reduced inflammatory response	[121]
Telluric acid	Rats	Activation of Nrf2	TLR4 and p13K/AKT pathway	Reduction of Oxidative stress Suppressed TNF-α, increased eNOS,	[134]
Geraniol	Rats	Activation of Nrf2/HO-1 pathway	Increased antioxidant capacity and GSH levels	Anti-inflammatory effects: Suppressed TNF-α, iNOS, COX-2. Antiapoptotic effects: reduced Bax, caspase 3-9	[122]
Bardoxolone-Methyl	Rats	NRF2 activator	Change in hepatocyte membrane transporters(HMT) regulation	Mitigate cholestasis associated with hepatic IRI	[123]
Sevoflurane	Rats and Hepatocytes	Activation of Nrf2/HO-1 pathway	Promoted Nrf2 nucleation Antioxidant and anti-inflammatory	Reduced LDH, MDA, TNF-α and IL-6, lowered apoptosis rate and inhibited ROS production	[126]
Fisetin	Mice and Hepatocytes	Activation of Nrf2/HO-1 pathway	Reduced oxidative stress and apoptosis	Reduced ROS, MDA, Bax, cleaved-caspase-3, cleaved-PARP, and enhanced Bcl-2.	[128]
Bracteanolide A	Rats	Upregulated expression of Nrf2, NQO1and HO-1.	Antioxidant and anti-inflammatory	Decreased the levels of ALT, AST,IL-1β and TNF-α	[124]

Accelerated autophagy and clearance of damaged mitochondria, reduced mtDNA release, and ROS overproduction, decreased inflammatory responses and subsequent secondary liver injury were the effects reported. Bardoxolone-Methyl, a synthetic triterpenoid derivative, mitigates tissue damages in mouse and rat models of hepatic IRI [46,123]. Bardoxolone-Methyl binds to Keap1, inhibits the Keap1-Nrf2 interaction, leading to the activation of NRF2 as a transcription factor. Sevoflurane, a volatile anesthetic, used as a pre- and post-conditioning agent, exhibits protective effects against liver IR injury [125] through a mechanism related to the Nrf2/HO-1 pathway regulation [126].

The potential of phytochemicals against IR liver injury has also been explored. The flavonoid fisetin alleviates IR-induced liver damage [127]. Inhibition of the Nrf2/HO-1 pathway reverses the effects of fisetin on cell viability, apoptosis, and oxidative stress [128]. Pretreatment of bracteanolide A, the major active compound of *Tradescantia albiflora*, attenuates oxidative stress, and reduced the levels of the inflammatory cytokines through activating Nrf2, and its downstream effectors, NQO1 and HO-1 [124].

Among the therapeutic measures that have been developed with the aim of protecting the liver from I/R injury, we have highlighted the usefulness of hypothermic preconditioning [4]. Treatment with mild hypothermia against cerebral IR promoted the nuclear localization of Nrf2, and increased the levels of NQO and HO-1 reducing oxidative stress and mitochondrial dysfunction [129]. In the hippocampus, hypothermia significantly elevated nuclear Nrf2 localization and HO-1 expression, reduced lipid peroxidation and increased SOD activity after cardiac arrest [130]. The effect of hypothermia treatment inducing a decrease in cytosolic Nrf2 was also confirmed in liver IR [131]. At the same time, the levels of TNF-α, IL-1β, IL-6, and IL-12 decreased and the levels of IL-10 increased [131]. In cell lines, the response to cellular hypothermia is associated with higher levels of Nrf2 that affect the antioxidant system. In contrast, NF-κB, a transcription factor involved in the control of immune and inflammatory responses, was not induced by hypothermia [132].

In view of the evidences that Nrf2 activation provides cytoprotection against liver IR, it is reasonable to ask whether Nrf2-inducing compounds could be used therapeutically for liver preservation. Some Nrf2-inducers are ongoing clinical trials in 2021 for the prevention or the treatment of different diseases (https://clinicaltrials.gov). Such is the case of synthetic triterpenoids, like bardoxolone methyl (CDDO-Me) in patients with chronic kidney disease (NCT04702997).

The imidazol derivative (CDDO-Im) has been proved to be protective during liver IR in mice [121]. An additional inducer of Nrf2 with ongoing clinical trials is dimethyl fumarate (DMF). DMF has several pharmacological benefits, including immunomodulation and fibrosis prevention, which are dependent on Nrf2 pathways [133]. DMF has been used since 1994 for the use in psoriasis and from 2013 has been approved by several countries as a first-line oral treatment for people with relapsing-remitting multiple sclerosis (RRMS).

The organosulfur compound oltipraz has been shown to be effective for the prevention of liver diseases in liver IR in mice [116] and in clinical trials of liver fibrosis and cirrhosis. Oltipraz is currently used in an ongoing trial for the reduction of liver fat in nonalcoholic fatty liver disease (NCT04142749). As such, it is highly likely that potential compounds that induce Nrf2 pathways could be candidates for avoiding IR injury in organ resection and liver transplantation.

Concluding comments and future perspectives

Research results of the last decade have established the importance of Nrf2 in regulation of redox balance, inflammation and cytotoxicity, and its involvement in many diseases, such as cancer, neurodegeneration, aging, and IR injury, all of them associated with oxidative stress. In addition to antioxidant and anti-inflammatory responses, Nrf2 is involved in diverse cellular processes, including metabolism. A review of the literature shows that the functions currently described go beyond those originally intended.

During liver IR, ROS cause lipid, protein and DNA oxidation, mitochondrial dysfunction, and subsequent liver inflammation, apoptosis, and necrotic cell death. Different strategies have been adopted to limit ROS damage in IR, including the administration of pharmacological agents, along with the use of preconditioning models. Advances in the study of the regulation of antioxidant and anti-inflammatory systems by Nrf2 could provide new and interesting potential therapeutic targets as in the case of organ transplantation, when there is an inherent damage due to the ischemia and reperfusion protocol. Therefore, therapeutic measures that activate Nrf2 signaling pathways have attracted the interest of new research.

Today's unhealthy lifestyles along with other factors such as aging are responsible for the accumulation of fat in the liver. This leads to varying degrees of hepatic steatosis. Steatotic livers show greater vulnerability against IR, increasing primary failure, and compromising the result of the graft after transplantation. Due to the lack of available organs, non-optimal livers will be required for transplantation regardless. Induction of Nrf2 against cold ischemic injury in fatty livers promises to be a good strategy to reduce the extent of subsequent oxidative damage against reperfusion (Fig. 3).

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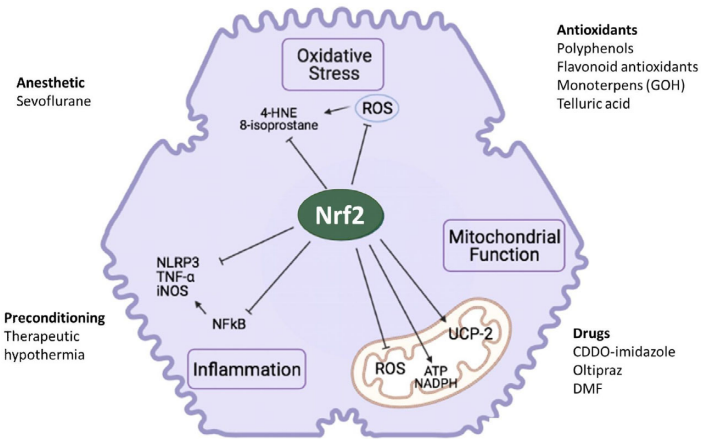

Fig. 3. Nrf2 activates different cellular mechanisms in response to IR damage. Nrf2-inducing compounds protection against IR injury has been shown at different cellular levels. First, against oxidative stress and ROS by activating phase II detoxification genes. Second, decreasing the production of ROS, through the UCP, and preventing the fall of ATP in the mitochondria. Finally, decreasing the inflammatory response through the regulation of NF-kB, cytokines, iNOS and NLRP3. Targeting Nrf2 through different treatments are good strategies to prevent liver damage.

Conflict of interest

The authors declare no conflict of interest.

Author contributions

All the authors participate in the elaboration of the text. Conceptualization: TC; Writing-Original Draft Preparation: RGB and TC; Writing-Review and Editing: RGB; APR; S.S.N.; NA; JRC and TC, RGB; and TC designed figures.

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CAPÍTULO II. Role of PEG35, Mitochondrial ALDH2, and Glutathione in Cold Fatty Liver Graft Preservation: An IGL-2 Approach

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Role of PEG35, Mitochondrial ALDH2, and Glutathione in Cold Fatty Liver Graft Preservation: An IGL-2 Approach

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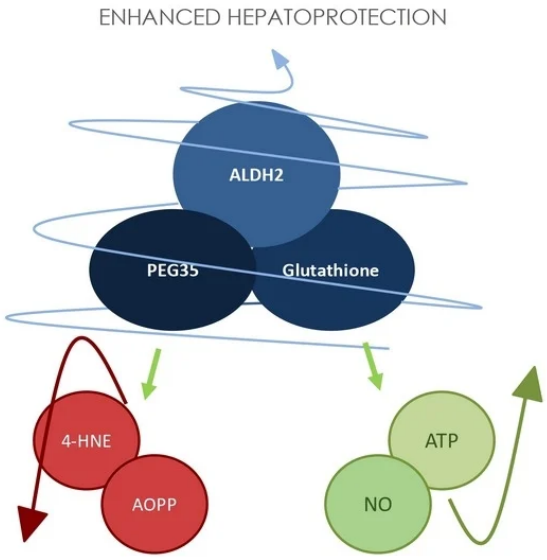
Graphical Abstract:



Resumen:

En los últimos años, el equipo del Dr. Joan Roselló (Mecanismos de daño y recuperación de la Isquemia, IibB, CSIC) en colaboración con la Dra. Teresa Carbonell (Grupo de Fisiología Adaptativa: Ejercicio e Hipoxia, Facultad de Biología, UB), han realizado diversos estudios para evaluar el efecto protector del medio de preservación IGL-1 en comparación con los medios utilizados de forma clásica (UW y HTK) en la preservación

estática fría del injerto hepático. Estos estudios demostraron que la solución de preservación IGL-1 previenen del daño al tejido, representado con niveles inferiores de estrés oxidativo, una menor activación del proteasoma, protección contra la autofagia y menores niveles de apoptosis [128–130].

En base a estos estudios, se evaluó el papel del polietilenglicol 35 (PEG35) y el GSH, presentes en la solución IGL-1 (3 mmol/L GSH + 1 g/L PEG35), en la protección mitocondrial y la mejora del estado redox en la preservación fría de hígado esteaótico. Para ello, se desarrollan dos soluciones de preservación modificadas de IGL-1: IGL-0 (sin GSH ni PEG35) e IGL-2 (9 mmol/L GSH + 5 g/L PEG35). Se aislaron hígados de ratas Zücker obesas y fueron distribuidos de forma aleatoria en cuatro grupos: el control (Sham) y preservados en IGL-0, IGL-1 e IGL-2 en frío (24 h a 4 ºC). Los resultados demuestran que la preservación en la solución IGL-2 promueve la expresión de la enzima protectora mitocondrial ALDH2, cuya función es prever la formación de aductos aldheidos (4-HNE) y la oxidación de proteínas (AOPP). Además, se observa una mayor concentración de óxido nítrico a y de la Óxido Nítrico Sintasa endotelial (eNOS), favoreciendo la vasodilatación, lo cual mejora la microcirculación de los hígados grasos durante la preservación y, en consecuencia, en la revascularización. En definitiva, la solución IGL-2 es la mejor opción para la preservación estática en frio de hígados esteatósicos. Será necesaria una investigación en profundidad para conocer los mecanismos moleculares en que actúa el PEG35 en la mejora de la calidad del injerto, de manera que se pueda aplicar clínicamente en la preservación previa al trasplante hepático.

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Role of PEG35, Mitochondrial ALDH2, and Glutathione in Cold Fatty Liver Graft Preservation: An IGL-2 Approach

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Abstract: The total damage inflicted on the liver before transplantation is associated with several surgical manipulations, such as organ recovery, washout of the graft, cold conservation in organ preservation solutions (UW, Celsior, HTK, IGL-1), and rinsing of the organ before implantation. Polyethylene glycol 35 (PEG35) is the oncotic agent present in the IGL-1 solution, which is an alternative to UW and Celsior solutions in liver clinical transplantation. In a model of cold preservation in rats (4 °C; 24 h), we evaluated the effects induced by PEG35 on detoxifying enzymes and nitric oxide, comparing IGL-1 to IGL-0 (which is the same as IGL-1 without PEG). The benefits were also assessed in a new IGL-2 solution characterized by increased concentrations of PEG35 (from 1 g/L to 5 g/L) and glutathione (from 3 mmol/L to 9 mmol/L) compared to IGL-1. We demonstrated that PEG35 promoted the mitochondrial enzyme ALDH2, and in combination with glutathione, prevented the formation of toxic aldehyde adducts (measured as 4-hydroxynonenal) and oxidized proteins (AOPP). In addition, PEG35 promoted the vasodilator factor nitric oxide, which may improve the microcirculatory disturbances in steatotic grafts during preservation and revascularization. All of these results lead to a reduction in damage inflicted on the fatty liver graft during the cold storage preservation. In this communication, we report on the benefits of IGL-2 in hypothermic static preservation, which has already been proved to confer benefits in hypothermic oxygenated dynamic preservation. Hence, the data reported here reinforce the fact that IGL-2 is a suitable alternative to be used as a unique solution/perfusate when hypothermic static and preservation strategies are used, either separately or combined, easing the logistics and avoiding the mixture of different solutions/perfusates, especially when fatty liver grafts are used. Further research regarding new therapeutic and pharmacological insights is needed to explore the underlying mitochondrial mechanisms exerted by PEG35 in static and dynamic graft preservation strategies for clinical liver transplantation purposes.

Keywords: fatty liver; PEG35; IGL-1 solution; ALDH2; 4-HNE; nitric oxide

1. Introduction

Unhealthy lifestyles associated with alcohol consumption and inappropriate diet, along with other factors such as aging, are responsible for the accumulation of fat in the liver, which leads to varying degrees of undesirable hepatic steatosis [1]. Considering the urgent lack of organs for transplantation, physicians have been obliged to take advantage

of fatty livers [1,2] to increase the donor pool and thus shorten waiting lists for clinical transplantation [3]. However, it is clear that steatotic livers show higher vulnerability against cold ischemia and reperfusion injury [4], and their use increases primary failure and compromises graft outcome after transplant [4,5].

Ischemic injury in liver transplantation is associated with mechanical organ manipulation by the physician prior to transplantation. The process includes organ recovery, washing of the graft, cold storage in an organ preservation solution, and finally, rinsing of the graft. Due to the combined action of ischemia and cold preservation, the graft may undergo damage. As a result, the cumulative injury in both mandatory steps before transplantation must be minimized in order to achieve the recovery of the graft's function after liver transplantation, especially in the case of steatotic grafts [1–4].

The most frequently used preservation solutions for liver transplant are UW, HTK, Celsior and, more recently, IGL-1 [6,7]. Certain limitations regarding the use of HTK [8,9] have been pointed out by the United Network for Organ Sharing (UNOS) and the European Liver Transplant Registry (ELTR). IGL-1 emerged as a good alternative to UW solution, which is the gold standard [9]. The only differences in their composition are the oncotic agent (HES for UW and PEG35 for IGL-1) and the reversal of Na+/K+ concentrations conferring IGL-1 the property of extracellular low potassium solution [10,11]. In addition, replacing the HES present in UW for PEG35 lessens red blood cell aggregation and favors organ rinsing, preservation, and perfusion [12]. All these changes in IGL-1 have shown to provide benefits in clinical liver transplantation in terms of reducing early allograft dysfunction [13] and improving graft survival according to the European Liver Transplant Registry (ELTR) [9].

Moreover, PEG35 presence in rinse solution for graft washing out promotes several cytoprotective factors, conferring hepatoprotection [14]. This includes the generation of nitric oxide (NO) whose vasodilation properties counterbalance the microcirculation disturbances in fatty liver grafts, favoring graft preservation and revascularization [15–18]. Keeping in mind the beneficial PEG35 properties, we recently proposed the use of IGL-2 solution (containing PEG35) as a suitable perfusate for dynamic hypothermic oxygenated strategies (HOPE) with promising results [19,20]. This might improve the only perfusate for machine perfusion currently available, Belzer-MPS and generics, containing HES [21], given that the use of a unique solution, such as IGL-2, for static preservation and machine perfusion (MP) would facilitate logistics and avoid the mixture of different solutions [19–21] when both techniques are combined. This is the criterion by which we evaluated the IGL-2 benefits in static hypothermic preservation in the present study.

Aldehyde dehydrogenase-2 (ALDH2), a liver mitochondrial enzyme that was initially implicated in the liver alcohol metabolism, has been associated with the pathophysiology of ischemia–reperfusion injury in several organs, including the liver [22–24]. Several authors reported that the use of Alda-1 (an activator of ALDH2) protects against liver ischemia–reperfusion injury (IRI) in the rat [25,26], but currently, no evidence exists on the direct PEG35 effect as a regulator of mitochondrial ALDH2 in cold old ischemic preservation strategies, although its cytoprotective action was indirectly evidenced when different organ preservation solutions were used for cold storage of fatty liver grafts [23,24]. ALDH2 could play a constitutive housekeeping role essential for the development and regulation of recycling and survival processes as those occurring in cold ischemia preservation [22,27].

With this in mind, we explored the relevance of PEG35 in IGL-2 solution (Table 1) on ALDH2. In this communication, we demonstrated for the first time how the direct effects of PEG35 on mitochondrial ALDH2 contribute towards maintaining mitochondrial functionality during ischemia preservation. Mitochondrial ALDH2 could act as a gatekeeper of ROS overproduction protecting the liver graft from the damaging effects of transient aldehydes produced [22–24] besides other additives in preservation solutions such as labile glutathione [28] that play an important role against oxidative stress.

Table 1. Composition of IGL-2 and IGL-1 solutions.

Preservation Solution	IGL-1	IGL-2
Electrolytes (mmol/L)		
K^+	25	25
Na^+	125	125
Mg^{2+}	5.5	
SO_4		5.5
Zn^{2+}		0.091
Buffers (mmol/L)		
Phosphate	25	25
Histidine		30
Impermeants (mmol/L)		
Mannitol	60	60
Lactobionic acid	80	
Colloids (g/L)		
Polyethylene glycol- 35	1	5
Antioxydants (mmol/L)		
Glutathione	3	9
Metabolic precursors (mmol/L)		
Adenosine	5	5
$NaNO_2$ (nmol/L)		50
pH	7.4	7.4
Osmolarity (mosmol/L)	320	320
Viscosity (cP)	1.2	1.4

The results reported here reveal the superior antioxidant capacity of IGL-2 (due to ALDH2 combined with increased glutathione content) against the ischemic insult during graft preservation, presenting an interesting tool to be considered for improving hypothermic fatty liver preservation by using static and dynamic approaches.

2. Results

The presence of PEG35 and glutathione (group IGL-1) is determinant for preventing liver cold ischemic injury (AST/ALT) and mitochondrial damage (GLDH) during fatty liver graft preservation and the prevention of energy breakdown during cold storage, at 4 °C during 24 h. As revealed in Figure 1, a higher ATP content is shown in liver preserved in IGL-1 solution (containing PEG35 as oncotic agent) than in liver preserved in IGL-0 (the same solution as IGL-1 but without PEG35).

Since PEG35 in IGL-1 preserved liver mitochondrial status during cold ischemia insult during experimental models [23], we evaluated its effect on mitochondrial ALDH2 and compared it to IGL-0 (which is the same as IGL-1 but without PEG35 in its composition). As shown in Figure 2, PEG35 presence in IGL-1 promoted significant increases in ALDH2, contrasting with the significantly lower levels of ALDH2 found in fatty liver grafts preserved in IGL-0 (without PEG35). (Figure 2).

Considering the relevance of PEG35 concentration according to the previously known results, we expanded our study to one additional group using IGL-2 solution [19,20]. We focused mainly on parameters relevant to the mitochondria (such as ALDH2) that regulate other cytoprotective responses. As demonstrated in Table 1, the IGL-2 solution is mainly characterized by higher concentrations of PEG35 and glutathione when compared to IGL-1 (Table 1).

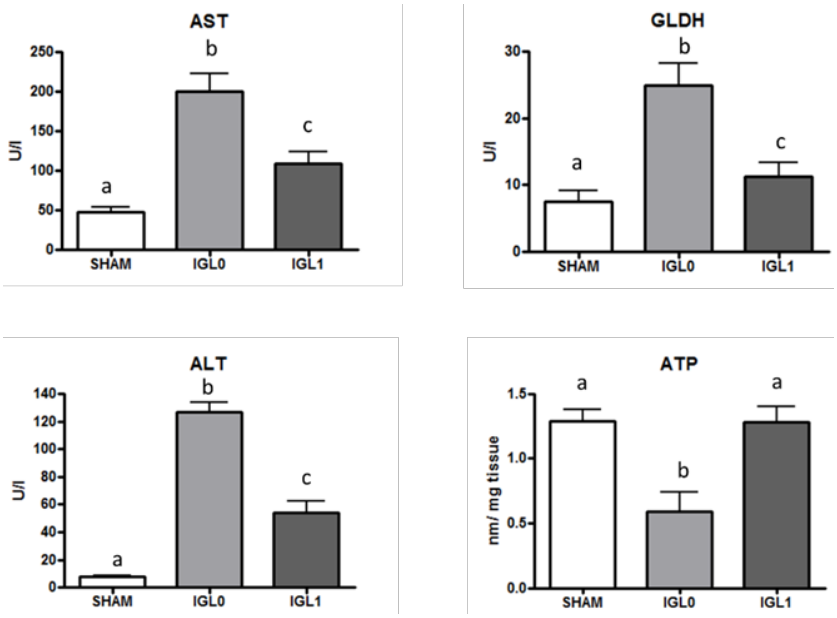

Figure 1. Transaminases (AST/ALT), mitochondrial damage (measured as GLDH), and ATP levels in steatotic livers preserved in IGL-0 (without PEG35) and IGL-1 solutions (PEG35: 1 g/L) vs. SHAM. Results are expressed as mean ± SEM (n = 6). Different lowercase letters indicate significant differences among treatments $p < 0.05$.

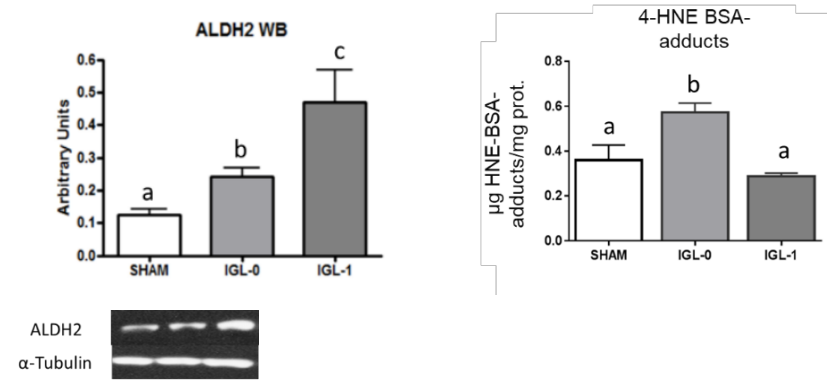

Figure 2. ALDH2 expression and 4-HNE protein-adducts (expressed as µg 4-HNE-BSA-adducts/mg protein) levels in steatotic livers preserved in IGL-0 (no PEG35) and IGL-1 solutions (PEG35: 1 g/L) vs. SHAM. Results are expressed as mean ± SEM (n = 6). Different lowercase letters indicate significant differences among treatments $p < 0.05$.

Firstly, we analyzed transaminases (AST/ALT) release and mitochondrial damage (GLDH), from which the total liver damage could be inferred. As shown in Figure 3, the PEG35 presence was a major factor in preventing transaminases and GLDH release with a dose-dependent PEG35 tendency. Although no significant differences between IGL-2 and IGL-1 were observed (except for AST), the presence of PEG35 seems to be a determinant factor in preventing the release of transaminases and GLDH in a dose-dependent manner.

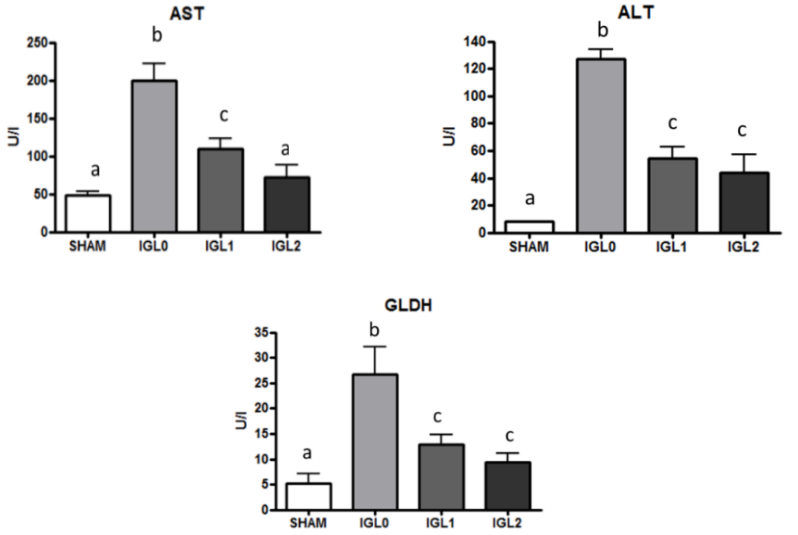

Figure 3. AST, ALT, and GLDH levels in steatotic liver samples for SHAM, IGL-0, IGL-1, and IGL-2 groups. Results are expressed as mean ± SEM (n = 6). Different lowercase letters indicate significant differences among treatments $p < 0.05$.

With this in mind, we evaluated the incidence of PEG35 on the energy breakdown prevention during cold storage by measuring ATP content in preserved livers. Data reported in Figure 4 revealed higher ATP levels in PEG-containing solutions and evidenced the PEG35-dependent energy breakdown prevention during cold preservation.

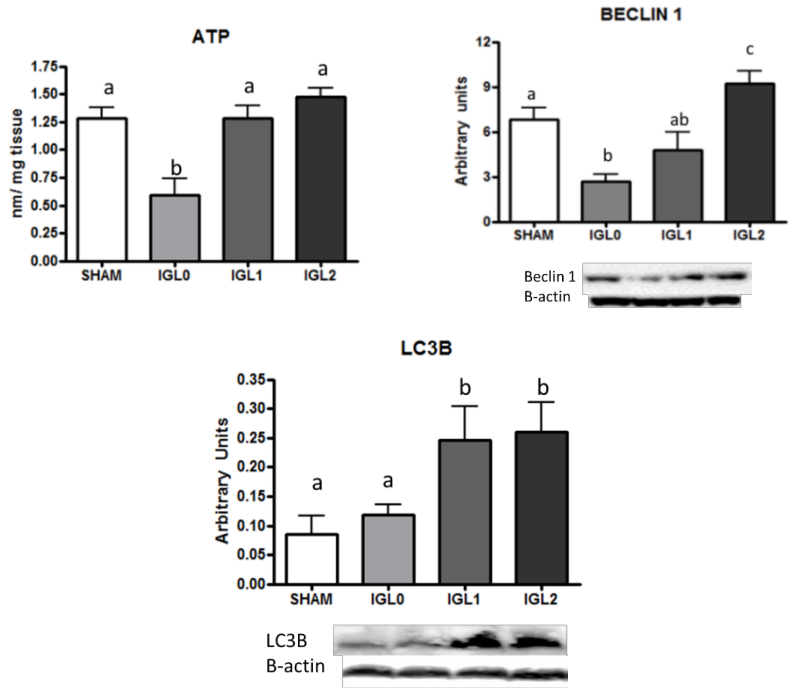

Figure 4. ATP, Beclin-1, and LC3B in steatotic liver samples for SHAM, IGL-0, IGL-1, and IGL-2 groups. Results are expressed as mean ± SEM (n = 6). Different lowercase letters indicate significant differences among treatments (one-way ANOVA, $p < 0.05$).

Additionally, and considering the cytoprotective autophagy as a recycling mechanism of nutrients and energy to cope with stress situations existing in cold ischemia conditions [27], we evaluated Beclin-1 and LC3B as autophagy marker [29]. Beclin-1 and LC3B levels correlated with the tissue ATP levels in preserved livers in IGL-0, IGL-1, and IGL-2. As shown in Figure 4, there was a significant upregulation of cytoprotective autophagy in the IGL-2 group compared with the others, which correlated with ATP levels in PEG35 groups, showing a positive tendance in ATP prevention for IGL solutions.

We correlated mitochondrial aldehyde dehydrogenase 2 (ALDH2) with the toxic aldehydes (4-HNE) and oxidized protein (AOPP) levels. Figure 5 shows an increase in the expression of the ALDH2 enzyme, which is concomitant with a decrease in the 4-HNE protein adducts formation, notably decreasing the levels of oxidized proteins going from IGL-0 to IGL-1 and IGL-2. When comparing IGL-0 and IGL-1, which only differ in the presence or absence of PEG, it can be seen that ALDH2 is augmented, the 4-HNE protein adducted, and AOPP decreased solely by the effect of PEG.

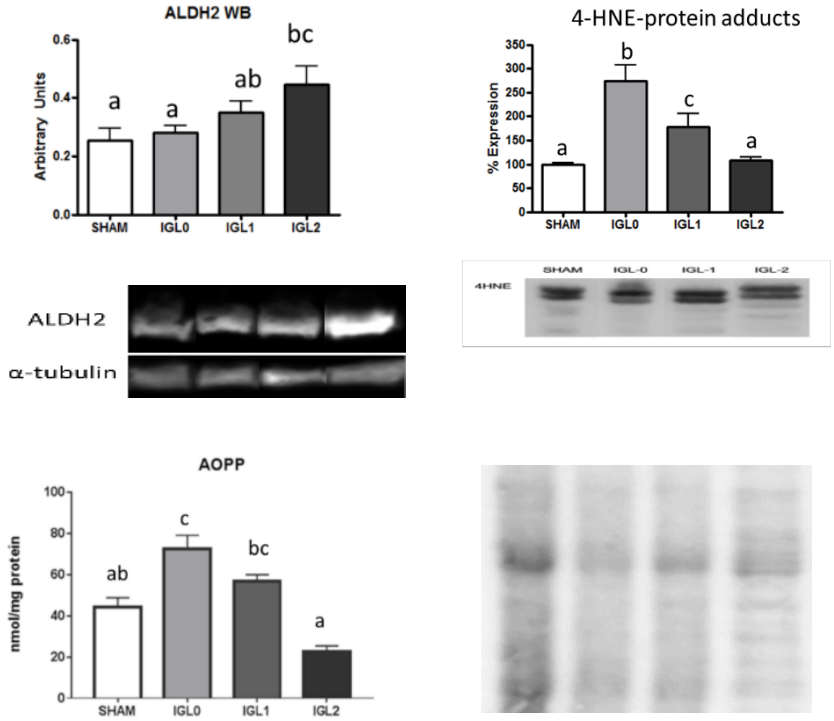

Figure 5. ALDH2 expression and levels of AOPP and 4HNE protein adducts in steatotic liver samples for SHAM, IGL-0, IGL-1, and IGL-2 groups. Results are expressed as mean ± SEM (n = 6). Different lowercase letters indicate significant differences among treatments (one-way ANOVA, $p < 0.05$).

Therefore, to observe the impact of glutathione on the reduction of oxidative stress (and not only by PEG35 itself, which happens when comparing IGL-0 and IGL-1), reduced glutathione was measured. There was a significant difference of reduced glutathione in IGL-2 solution, which was to be expected due to its initial increased dosage; however, high variance in the IGL-1 group might suggest that part of its glutathione (if compared to IGL-0) might be spared (therefore, not oxidized) due to other antioxidant capacities derived from PEG35 (Figure 6).

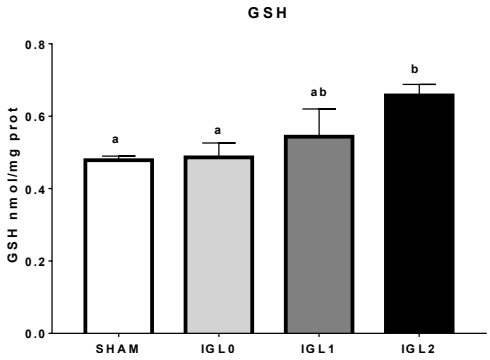

Figure 6. Reduced glutathione (GSH) in IGL-2 solution compared to IGL-1 and IGL-0 (no PEG35) in steatotic liver samples. Different lowercase letters indicate significant differences among treatments (one-way ANOVA, $p < 0.05$).

Finally, we evaluated the direct effect of PEG35 on NO production and oncotic pressure to avoid oedema. As shown in Figure 7, we evidenced respective augments in NOx products and endothelial NO synthase expression levels. Significant (endothelial NO synthase) eNOS activity and NOx levels were observed for PEG35 groups (IGL-1 and IGL-2). This fact was correlated with a significant upregulation of mitochondrial ALDH2 expression in these groups. A positive trend towards increased eNOS expression and NOx levels in IGL-2 vs. IGL-1 was observed, but statistical differences were not found.

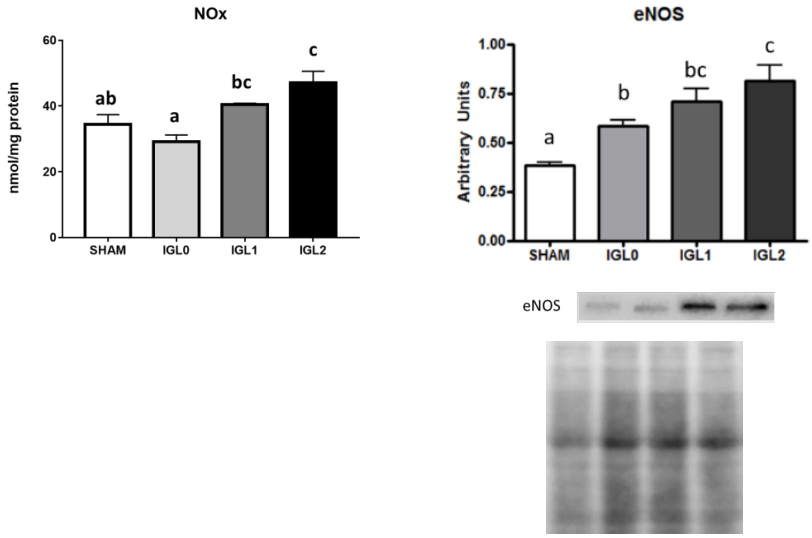

Figure 7. NOx and eNOS in steatotic liver samples for SHAM, IGL-0, IGL-1, and IGL-2 groups. Results are expressed as mean ± SEM ($n = 6$). Different lowercase letters indicate significant differences among treatments (one-way ANOVA, $p < 0.05$).

3. Discussion

It is well known that fatty livers are more susceptible to cold ischemia during preservation than normal livers. Therefore, the aim of our research was to increase the performance of their preservation by developing a new solution (IGL-2).

PEG35 is the oncotic agent present in IGL-1 solution [11], and its protective role in IRI [30], rinse solution [14], and cold storage [23,24] was previously described. It is an established fact that any preservation solution must contain antioxidants to counteract ox-

idative stress during IR. Considering this, the IGL-2 benefits reported here for hypothermic static preservation are consistent with its suitability to be used in HOPE over the currently used perfusates (Belzer-MPS or its generics) [19,20]. Both reasons constitute a solid basis for simplifying the logistics and avoiding mixing different solutions when both static and HOPE need to be combined. This is especially interesting for rescuing fatty liver graft liver transplantation purposes [21].

PEG35 solutions (IGL-2 and IGL-1) are related to reduced AST/ALT and especially to GLDH, showing better mitochondrial protection, as previously described [14,19,20]. PEG35 improved mitochondrial machinery and ALDH2 functionality, reducing graft cold ischemic injury [23,24]. Consequently, this calls for an exploration of the underlying protective mechanisms by which PEG35 confers mitochondrial protection on fatty liver grafts preserved in IGL-1 and IGL-2 solutions whose mechanisms substantially differ from the ones induced in HOPE [31], where the transient oxygenation during hypothermic preservation is responsible for maintaining mitochondrial activity function at basal levels. This confirms the relevance of organ preservation strategies on the mitochondrial status and their subsequent benefits in liver transplantation, as recently published by Horvath et al. [32].

Recently, it has been reported that aldehyde dehydrogenase 2 ALDH2 activation is linked to protective mechanisms in several organs, such as the heart, brain, kidney, and intestine [22,33]. The cardioprotective and neuroprotective role of ALDH2 in myocardial ischemia–reperfusion has been demonstrated [34], with recent evidence showing that ALDH2 inhibition alters endothelial functions along with a deterioration of bioenergetic functions [35]. ALDH2 arises as an important gatekeeper of ROS overproduction, making the cell more tolerant to it [36]. In fact, the main function of mitochondrial ALDH2 is to protect mitochondria and cells from the damaging effect of aldehydes (by oxidizing the substrates into their corresponding non-toxic carboxylic acids), which are involved in the oxidative stress associated with IRI [22]. Zhang et al. [25] demonstrated that Alda-1, an ALDH2 activator, protects the liver against warm IRI preventing oxidative stress. In this sense, our work revealed that the oncotic agent PEG35 could be considered as an enhancer of mitochondrial ALDH2 upregulation, whose underlying protective mechanisms against cold ischemic insult have not been assessed in depth [22].

The prevention of liver injury exerted by solutions containing PEG35 contrasts with the injury observed in livers preserved in IGL-0 (without PEG35) with depleted energetic levels after 24 h of cold storage. Remarkably, the comparison of IGL-1 to IGL-0, which only differ in the presence or absence of PEG, suggests that PEG35 by itself leads to an ALDH2 upregulation. This is consistent with the IGL-2 solution, where the augmented PEG35 content further prevented oxidative stress through ALHD2 upregulation and promoted cytoprotective autophagy.

Nevertheless, the total antioxidant effects of the IGL-2 solution are mediated by the contribution of ALDH2, besides the antioxidant action of glutathione present as an additive, which should be considered as well. Thus, the higher IGL-2 antioxidant capacity compared to that of IGL-1 (due to increased PEG35 and glutathione concentrations) is also reflected by preventing AOPP and 4-HNE protein adduct formation in PEG35 solutions (IGL-1 vs. IGL-0) and, even more importantly, with the presence of increased PEG35 and glutathione content (IGL-2 vs. IGL-1). Indeed, IGl-1 shows more ALDH2 than IGL-0, and more IGL-2 than IGL-1, suggesting that ALDH2 increases with PEG35 in a concentration-dependent manner.

In addition, it must be considered that the increased expression of eNOS synthase increased the hepatoprotection mechanisms during cold preservation [15]. The beneficial effects of PEG35 on ALDH2 mitochondrial machinery are also increased by the concomitant presence of glutathione to prevent the action of toxic aldehyde adducts (4-HNE) and lipoperoxide generation [22] associated with hypothermic storage. Alternatively, PEG35, through the increased e-NOS activation and subsequent NO generation, prevents the microcirculatory disturbances that occur in fatty liver graft revascularization [37]. Furthermore, some of this NO could act as a scavenger of ROS, thus reducing the number of oxidizing

particles. IGL-2 is a suitable alternative solution for increasing cold graft preservation strategies when static cold preservation and HOPE need to be combined.

In conclusion, we demonstrated the relevance of the oncotic agent PEG35 in modulating the redox state through mitochondrial ALDH2, thus reinforcing the protection mechanisms of fatty liver graft in cold preservation in combination with glutathione. This could improve the preservation of fatty liver grafts and may help to design new static and dynamic preservation strategies using PEG-containing solutions/perfusates.

Further, in-depth research should be conducted to clarify the role of mitochondrial ALDH2 and its direct relationship with polyethylene glycols features as efficient tools for preventing IRI.

4. Materials and Methods

4.1. Animals

Homozygous (obese (ob)) Zücker male rats aged 16–18 weeks were purchased from Charles River (Charles River, Lyon, France). They were housed in a temperature-controlled environment (25 °C) with a 12 h light/dark cycle and provided water and standard chow ad libitum. The rats presented a rate of steatosis between 60% and 70%. All procedures were carried out according to the EU rules for animal experiments (EC guideline 86/609/CEE) and were approved by the University of Barcelona's Ethics Committees for Animal Experimentation (#483/16). The animals underwent general anesthesia with isoflurane inhalation.

4.2. Experimental Groups

Zücker Rats aged (16–18) weeks were divided into three groups. The abdomen was cut with a midline incision, and following bile duct cannulation, the portal vein and the splenic and gastroduodenal veins were ligated. After organ recovery, the livers were flushed with IGL-0, IGL-1, or IGL-2 (Table 1) and stored in each solution for 24 h at 4 °C. Animals were randomly distributed in different groups (n = 6), as follows:

Group 1 (SHAM): Obese Zücker rats underwent transverse laparotomy, and silk ligatures of right suprarenal and diaphragmatic veins and hepatic artery were performed before retrieving the liver.
Group 2 (IGL-0 solution): After organ recovery, fatty livers were flushed with 40 mL of IGL-0 preservation solution and were then stored in IGL-0 at 4 °C for 24 h.
Group 3 (IGL-1 solution): After organ recovery, fatty livers were flushed with 40 mL of IGL-1 preservation solution and were then stored in IGL-1 at 4 °C for 24 h.
Group 4 (IGL-2 solution): After organ recovery, fatty livers were flushed with 40 mL of IGL-1 preservation solution and were then stored in IGL-2 at 4 °C for 24 h.

After 24 h of cold preservation or right after surgery (in the case of Sham), liver samples were rinsed with Ringer's lactate (20 mL), and samples were taken from the flush. They were then stored at −80 °C for subsequent biochemical determinations.

4.3. Biochemical Analyses

Transaminase Assay

Liver injury was assessed by alanine aminotransferase (ALT) and aspartate aminotransferase (AST) commercial kits, purchased from RAL (Barcelona, Spain) following the manufacturer's instructions. Briefly, 100 mol of effluent washout was added to 1 mL of substrate provided by the commercial kit. Transaminase activity was measured at 340 nm using a UV spectrometer.

4.4. Glutamate Dehydrogenase (GLDH) Activity

Mitochondrial damage was measured by GLDH activity, following the manufacturer's instructions of the commercial kit purchased from RANDOX (Crumlin, United Kingdom).

4.5. Energy Metabolism (ATP Breakdown)

The determination of ATP in liver samples homogenized in a perchloric acid solution was performed using the ATP assay kit for fluorimetry (Sigma Aldrich ATP colorimetric/fluorometric assay kit, Madrid, Spain). The ATP concentration was determined by the phosphorylation of glycerol, which is a detectable product for the fluorimeter (excitation/emission 535 nm/587 nm) at 37 °C and proportional to the amount of ATP in the sample. Energy breakdown during cold storage was measured through the changes in ATP levels.

4.6. 4-Hydroxynonenal Protein Adducts Assay

4-Hydroxynonenal (4-HNE) protein adducts were measured in liver homogenate using the OxiSelect™ HNE Adduct Competitive ELISA Kit (Cell Biolabs, Inc. San Diego, CA, USA). Liver was homogenized in 10% (*w*/*v*) with a Teflon bar in a RIPA solution, (Tris 50 M pH 7.4, 1% Triton 100×, NaCl 150 mM, NaF 5 M, 0.1% sodium dodecyl sulphate, and 1% sodium deoxycholate) with antiprotease solution (aprotinin at 1.7 mg/mL, 2 µg/mL pepstatin, 2 µg/mL leupeptin and 1 mM phenylmethylsulfonyl fluoride, and sodium orthovanadate at 1 mM). The suspension was centrifuged at 2000 g for 5 min and the pellet discarded. Liver homogenates were added to an HNE conjugate preabsorbed ELISA plate. After a brief incubation, an anti-HNE polyclonal antibody was added, followed by an HRP conjugated secondary antibody. The quantity of HNE adduct in protein samples was determined by comparing its absorbance with that of a known HNE-BSA standard curve.

4.7. Advanced Oxidation Protein Products (AOPP)

Advanced oxidation protein products (AOPP) are biomarkers of oxidative damage to proteins, detecting dityrosine-containing and cross-linking protein products. The formation of AOPP in the liver homogenates was spectrophotometrically measured at 340 nm. Results were obtained through a standard calibration curve using 100 µL of chloramine-T solution (0–100 µmol/L). AOPP concentration was expressed in nmol/mg protein. Advanced oxidation protein products (AOPPs) in the liver were assayed by a modification of Witko-Sarsat's method [38].

4.8. Glutathione Analysis

Reduced glutathione (GSH) was measured in the liver extracts using the procedure previously described [28]. Liver samples were homogenized in cold buffer containing 5 mm phosphate–EDTA buffer (pH 8.0) and 25% HPO3. The homogenates were ultra-centrifuged at 100,000× *g* and 4 °C for 30 min, and the resulting supernatant, with the fluorescent probe o-phthalaldehyde, was used to determine GSH concentration. Fluorescence was determined at a wavelength emission of 420 nm and excitation at 350 nm. Results are expressed as GSH nmol/mg protein.

4.9. Nitrite/Nitrate Analysis

NO production in the liver was determined by tissue accumulation of nitrite and nitrate using a colorimetric assay kit (Cayman, Tallinn, Estonia) according to the manufacturer's instructions.

4.10. Western Blot Analysis

ALDH2, Beclin-1, and LC3B

Separated on 6–15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels, proteins were blotted into poly-vinylidene fluoride (PVDF) membranes (Bio-Rad, Madrid, Spain) and immunoblotted overnight at 4 °C using antibodies against ALDH2 (Abcam, Cambridge, UK. ref: ab133306), Beclin-1 (Sigma Aldrich, San Louis, Missouri, ref: SAB5700251), and LC3B (Abcam, Cambridge, UK. ref: ab48394). Detection was performed with anti-IgG-HRP (Santa Cruz Biotechnology, Inc., Heidelberg, Germany). In all cases, the

chemiluminescence signals were quantified ChemiDoc (Bio-Rad, Madrid, Spain). Both β-actin (Abcam ref: ab8226) and α-tubulin (Abcam ref: ab7291) were used as loading controls.

4.11. 4-HNE Protein Adducts and eNOS

Liver samples were homogenized in RIPA (50 M Tris (pH 7.4), 1% Triton X-100, 150 mM NaCl, 5 M NaF, 0.1% sodium dodecyl sulfate, and 1% sodium deoxycholate) and centrifuged for 20 min at 10,000 g. The supernatant was denatured with the addition of Bromophenol Blue (1/2) and heating at 95 °C for 5 min. A total of 50 mg of protein per sample was loaded onto the 10% agarose gel, and wet blotting was carried out on a PVDF membrane (Bio-rad, Irvine, CA, USA). The membranes were blocked for 1 h in Odyssey® Blocking Buffer (LI-COR Biosciences GmbH, Germany) diluted in Tris Base Buffer (TBS, Tris-buffered saline) (pH = 7.4) with 0.05% Tween (TTBS). The membranes were incubated overnight with anti-4-hydroxynonenal (4-HNE) protein adducts and anti-eNOS (BD Biosciences-Europe) antibodies according to the manufacturers' recommendations.

Detection and analysis were carried out by incubation with secondary fluorescence (800 W) with the Odyssey® Fc system (LI-COR Biosciences GmbH). To quantify the expression, Image Studio 5.2.5 software (LI-COR Biosciences) was used, correcting for the total protein analyzed with the REVERTTM solution (Li-COR Biosciences) according to the manufacturer's protocol and expressing the results as a percentage with respect to the sham group.

4.12. Statistics

Data are expressed as mean ± standard error and were compared statistically by variance analysis, followed by the Student–Newman–Keuls test using GraphPad Prism version 8.1.0 for Windows (GraphPad Prism software, San Diego, CA, USA, 2018) and one-way ANOVA. A level of $p < 0.05$ was considered significant. Significant differences between groups are represented with different letters in the graphs. A group labeled with a letter has significant statistical differences compared to a group labeled with a consecutive letter with a $p < 0.05$.

Author Contributions: R.G.B., R.T.d.S., and A.P.-R. carried out surgical procedures and the experimental. E.F.-P. and J.R.-C. carried out the data analyses evaluation. E.F.-P., C.P., J.P., R.A., and J.R.-C. analyzed data and wrote the paper. T.C. and A.P.-R. designed the experiments, coordinated the study, and wrote the paper. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: All procedures were carried out according to the EU rules for animal experiments (EC guideline 86/609/CEE) and were approved by the University of Barcelona's Ethics Committees for Animal Experimentation (nº 483/16).

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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CAPÍTULO III. Peg35 and Glutathione Improve Mitochondrial Function and Reduce Oxidative Stress in Cold Fatty Liver Graft Preservation

antioxidants

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Article

PEG35 and Glutathione Improve Mitochondrial Function and Reduce Oxidative Stress in Cold Fatty Liver Graft Preservation

Raquel G. Bardallo [1], Idoia Company-Marin [1], Emma Folch-Puy [2,3], Joan Roselló-Catafau [2,3], Arnau Panisello-Rosello [2,3] and Teresa Carbonell [1,*]

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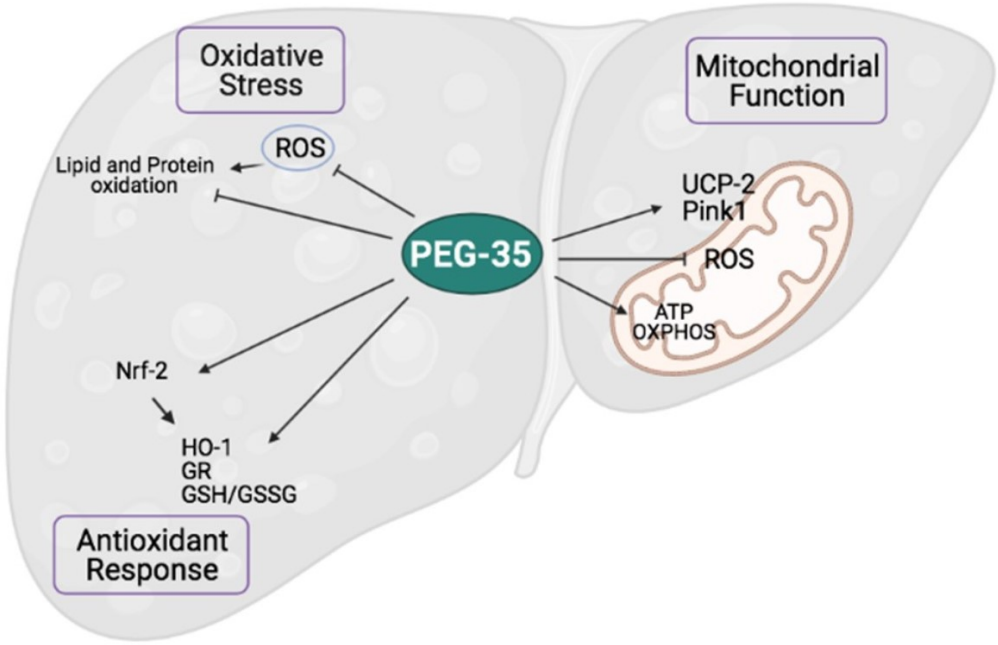
Graphical Abstract:



Resumen:

En el segundo capítulo, se observó la función protectora mitocondrial del PEG35 y del GSH de la solución IGL-2 en la preservación fría de hígado esteatósico. En el presente estudio, se propuso analizar los mecanismos celulares que han sido modificados por el PEG35 y el GSH, como el estado redox y energético. Para ello, se aislaron hígados de ratas Zücker obesas y fueron distribuidos de forma aleatoria en cuatro grupos: el control (Sham), preservado (24 h a 4 ◦C) en IGL-0 (sin PEG35 y 3 mmol/L GSH), IGL-1 (1 g/L PEG35

y 3 mmol/L GSH) e IGL-2 (5 g/L PEG35 y 9 mmol/L GSH). Se determinaron los metabolitos energéticos (ATP y succinato) y la expresión de complejos de fosforilación oxidativa mitocondrial (OXPHOS). Además, se analizaron la proteína de desacoplamiento del portador mitocondrial 2 (UCP2), la cinasa 1 inducida por PTEN (PINK1), el factor nuclear-eritroide 2 relacionado con el factor 2 (Nrf2), la hemoxigenasa-1 (HO-1) y el inflamosoma (NLRP3). Como biomarcadores del estrés oxidativo, se determinó la oxidación (AOPP) y la carbonilación (derivados de DNP) de proteínas, así como la peroxidación lipídica (MDA, aductos de malondialdehido) Además, se determinaron las capacidades antioxidantes del GSH, a través de su forma oxidada y reducida (GSH y GSSG) y actividad enzimática (Cu-Zn superóxido dismutasa (SOD), CAT, GSH S-T, GSH-Px y GSH-R). Nuestros resultados mostraron que durante la preservación en frío del injerto de hígado graso se consume el ATP disponible, se acumula succinato y se produce un incremento del estrés oxidativo. En cambio, la conservación con la solución de IGL-2 mantuvo los niveles de ATP, no se produce una acumulación de succinato y se observó una expresión aumentada de los complejos OXPHOS I y II, UCP2 y PINK-1, manteniendo así la integridad mitocondrial. IGL-2 también muestra protección contra el estrés oxidativo al aumentar la expresión HO-1 y los niveles de GSH, presumiblemente gracias a la activación de Nrf2 y la inhibición del inflamosoma NLRP3. Por lo tanto, la presencia de PEG35 y GSH en las soluciones de preservación pueden ser una opción muy considerable como agente antioxidante para la preservación de órganos en el trasplante clínico.

antioxidants

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PEG35 and Glutathione Improve Mitochondrial Function and Reduce Oxidative Stress in Cold Fatty Liver Graft Preservation

Raquel G. Bardallo [1], Idoia Company-Marin [1], Emma Folch-Puy [2,3], Joan Roselló-Catafau [2,3], Arnau Panisello-Rosello [2,3] and Teresa Carbonell [1,*]

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Abstract: The need to meet the demand for transplants entails the use of steatotic livers, more vulnerable to ischemia-reperfusion (IR) injury. Therefore, finding the optimal composition of static cold storage (SCS) preservation solutions is crucial. Given that ROS regulation is a therapeutic strategy for liver IR injury, we have added increasing concentrations of PEG35 and glutathione (GSH) to the preservation solutions (IGL-1 and IGL-2) and evaluated the possible protection against energy depletion and oxidative stress. Fatty livers from obese Zücker rats were isolated and randomly distributed in the control (Sham) preserved (24 h at 4 °C) in IGL-0 (without PEG35 and 3 mmol/L GSH), IGL-1 (1 g/L PEG35, and 3 mmol/L GSH), and IGL-2 (5 g/L PEG35 and 9 mmol/L GSH). Energy metabolites (ATP and succinate) and the expression of mitochondrial oxidative phosphorylation complexes (OXPHOS) were determined. Mitochondrial carrier uncoupling protein 2 (UCP2), PTEN-induced kinase 1 (PINK1), nuclear factor-erythroid 2 related factor 2 (Nrf2), heme oxygenase-1 (HO-1), and the inflammasome (NLRP3) expressions were analyzed. As biomarkers of oxidative stress, protein oxidation (AOPP) and carbonylation (DNP derivatives), and lipid peroxidation (malondialdehyde (MDA)–thiobarbituric acid (TBA) adducts) were measured. In addition, the reduced and oxidized glutathione (GSH and GSSG) and enzymatic (Cu–Zn superoxide dismutase (SOD), CAT, GSH S-T, GSH-Px, and GSH-R) antioxidant capacities were determined. Our results showed that the cold preservation of fatty liver graft depleted ATP, accumulated succinate and increased oxidative stress. In contrast, the preservation with IGL-2 solution maintained ATP production, decreased succinate levels and increased OXPHOS complexes I and II, UCP2, and PINK-1 expression, therefore maintaining mitochondrial integrity. IGL-2 also protected against oxidative stress by increasing Nrf2 and HO-1 expression and GSH levels. Therefore, the presence of PEG35 in storage solutions may be a valuable option as an antioxidant agent for organ preservation in clinical transplantation.

Keywords: antioxidants; glutathione; Nrf2; OXPHOS; polyethylene glycol; UCP2; IGL-2

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1. Introduction

The increasing incidences of obesity and unhealthy lifestyles in the world population are both responsible for the metabolic syndrome and the consequent accumulation of fat in the liver. Given the global shortage of livers for transplantation, studies on the conditions that allow the use of steatotic livers are needed to broaden donation criteria [1]. However, the use of steatotic liver grafts and therefore suboptimal increases the risk of later impaired function leading to cell necrosis, which is caused by the higher vulnerability to suffer an ischemia-reperfusion (IR) injury [2,3].

So far, the most widely used preservation method has been static cold storage (SCS). The purpose of the researchers is to find and define the optimal composition of commercial solutions used during cold ischemia, so that the functionality of the stored organ is maintained, ensuring the viability of the steatotic organ after reperfusion [4].

Among the most used solutions for liver transplantation is the Institut Georges Lopez-1 (IGL-1) solution [5,6]. IGL-1 contains polyethylene glycol (PEG) with a molecular weight of 35 kDa (PEG35), glutathione (GSH), and a higher concentration of Na^+ in relation to that of K^+. PEG is a variable-weight and neutral polymer with high solubility, due to its hydrophilicity and low toxicity, so it does not trigger an immunological response [7]. It protects against necrosis, prevents oedema formation and contributes to cell membrane stabilization and cytoskeleton integrity maintenance [8]. PEG helps to repair and maintain multiple cell structures, such as the plasma membrane in hypoxic conditions, as it happens in IR procedures [4]. Those facts entail several beneficial effects for IGL-1 compared to other solutions [9–11], both in healthy and steatotic livers. The benefits of the IGL-1 solution (1 g/L PEG35 and 3 mmol/L GSH) include the prevention of liver damage [12] and lipid peroxidation [13,14]. We recently used a new solution, IGL-2, (5 g/L PEG35 and 9 mmol/L GSH). The detailed compositions of IGL-1 and IGL-2 have been described in Bardallo et al. [15]. Our previous results showed that IGL-2 reduces fatty liver damage due to cold storage. Furthermore, we observed an increase in the mitochondrial expression of ALDH2, which could act as a guardian of the overproduction of reactive oxygen species (ROS), therefore protecting the steatotic liver graft from the damaging effects of cytotoxic aldehydes.

Since IR injury will significantly determine the functionality of the fatty liver graft after transplantation, it is necessary to explore the cellular mechanisms that lead to tissue damage. Among them, it is known that the mitochondrial response during ischemia stimulates ROS production and reduces adenosine triphosphate (ATP) levels, which have been identified as the main cause of reperfusion damage [16], by producing oxidation and tissue inflammation. Our objective is to address whether the beneficial mechanisms of the addition of PEG35 and GSH in storage solutions may be due to the maintenance of mitochondrial function and the modulation of the redox state in cold fatty liver preservation.

Energy metabolites, mitochondrial oxidative phosphorylation complexes, mitophagy, oxidative stress, and antioxidants were analyzed. We found that liver grafts preserved in IGL-2 maintained mitochondrial integrity and redox status; thus, this solution would be recommended for clinical liver transplantation purposes.

2. Materials and Methods

2.1. Animals and Liver Isolation and Perfusion

Male homozygous obese (Ob) Zücker rats from Charles River (France), aged 16–18 weeks, were used. Zücker rats constitute a well-characterized model of nutrition-induced obesity, closely simulating the most common cause of steatosis in the Western Hemisphere, according to Selzner et al. [17]. Homozygous Ob Zücker rats lack the brain leptin receptor and develop obesity at the age of 8 weeks due to markedly increased food intake and decreased energy expenditure. Steatosis in the Ob Zucker rats was previously determined by Serafin et al. [18], using specific lipid staining. In this study, Ob Zücker rats showed severe and macrovesicular and microvesicular fatty infiltration in hepatocytes (between 60% and 70% steatosis). The rats were housed under conditions of conventional animal facilities, with controlled temperature, humidity, and twelve hours dark/light cycles. Animals had free access to water and dry-food-standard diet, and they were distributed into different experimental groups as described below. This study was performed following the European Union Directive for animal experiments (2010/63/EU), and all the experiments were conducted in accordance with protocols approved on 14 July 2016 (No. 483116). Concurrently, they were validated by the Ethics Committees for Animal Experimentation of the University of Barcelona (Directive 483/16).

For this study, a Sham group and three experimental groups were performed, as described below. The abdomen was cut with a midline incision, and following bile duct cannulation, the portal vein and the splenic and gastroduodenal veins were ligated. After organ recovery, the livers were flushed with IGL-0 (without PEG35 and 3 mmol/L GSH), IGL-1 (1 g/L PEG35, and 3 mmol/L GSH), or with IGL-2 (5 g/L PEG35 and 9 mmol/L GSH) and stored in each solution for 24 h at 4 °C. The animals were randomly distributed as follows:

Sham: Ob Zücker rats underwent transverse laparotomy, and the silk ligatures of right suprarenal and diaphragmatic veins and hepatic artery were performed.

IGL-0: After effecting the organs collection, livers were flushed with 50 mL of an IGL-0 solution and straightaway stored in an IGL-0 preservation solution for a 24 h period at 4 °C.

IGL-1: After the organs collection, livers were flushed with 50 mL of an IGL-1 solution and immediately stored in an IGL-1 preservation solution for a 24 h period at 4 °C.

IGL-2: After the organs collection, livers were flushed with 50 mL of an IGL-2 solution and next stored in an IGL-2 preservation solution for a 24 h period at 4 °C.

After the cold storage period described, organs maintained in their corresponding preservation solutions were flushed with 20 mL of Ringer Lactate solution. Then, all samples were stored at −80 °C for subsequent biochemical analysis.

2.2. ATP and Succinate Determination

The measurements of ATP and succinate concentrations in liver grafts homogenized in a perchloric acid solution were performed using the ATP assay kit (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) and the succinate assay procedure (Megazyme, Wicklow, Ireland). ATP was based on a fluorometric assay, which determined ATP amount through the phosphorylation of glycerol, which resulted in the formation of a fluorometric product (excitation/emission: 535/587 nm) proportional to the amount of ATP in the sample. Results are expressed in nmol of ATP per mg of weight of fresh tissue. Succinate was utilized by Succinyl-CoA Synthetase to form an intermediate, which underwent a series of reactions. The amount of NAD^+ formed in the last reaction pathway was stoichiometric with the amount of succinic acid. It was NADH consumption, which was measured by the decrease in the absorbance at 340 nm. Results are expressed in nmol of succinate per mg of weight of fresh tissue.

2.3. Oxidant and Antioxidant Assays in Liver

2.3.1. Lipid and Protein Oxidation

Lipid peroxidation was measured by the Thiobarbituric Acid Reactive Substances assay, which mainly detected malondialdehyde (MDA), one of the end-products of lipid peroxidation. For this assay, livers were homogenated at 10% in RIPA (Tris-HCl 50 mM; NaCl 150 mM; NaF 5 mM; SDS at 0.1%; Triton x-100 at 1%; DOC at 1%; pH 7.4). Thiobarbituric acid (TBA) reacted with the MDA of the samples, and the formation of MDA–TBA adducts was fluorometrically measured at an excitation wavelength of 515 nm and an emission wavelength of 550 nm. The calibration curve was determined using tetraethoxypropane. Values are expressed as MDA–TBA adducts in nmol/mg protein.

The identification of oxidative damage caused to proteins was conducted via advanced oxidative protein products (AOPP) test. This protocol is founded on Witko-Sarsat assay [19], where the AOPP content is determined from a standard of chloramine-t. The formation of AOPP in the liver homogenates was spectrophotometrically measured at 340 nm. The AOPP concentration is expressed in μmol Chloramine-t/mg protein.

Carbonyl groups, as a hallmark of oxidative modification of proteins, were also measured. The carbonyl groups in the protein side chains were derivatized to 2,4-dinitrophenylhydrazone (DNP hydrazone) by reaction with 2,4-dinitrophenylhydrazine (DNPH). Derivatization with 2,4-dinitrophenylhydrazine (DNPH) was performed according to the procedure of Nakamura and Goto [20]. Briefly, a mixture was prepared by combining equal amounts (30 μg) of samples, precipitated by trichloroacetic acid, and then incubated in 10 mM

DNPH in 2 N HCl at room temperature for 1 h. The DNP-derivatized protein samples were separated by polyacrylamide gel electrophoresis followed by Western blotting, as explained below.

2.3.2. Antioxidant Enzymes Activity

The measurement of the antioxidant enzymes superoxide dismutase (SOD, EC 1.15.1.1), glutathione S-transferase (GSH S-T, EC 2.5.1.18), glutathione peroxidase (GSH-Px, EC 1.11.1), and glutathione reductase (GSH-R, EC 1.6.4.2) were performed with the Sigma-Aldrich Determination Kit (SOD, Cat. 19160, Sigma, St. Louis, MO, USA) and Cayman Kits (GSH S-T, 703302; GSH-Px, 703102; GSH-R, 703202, Cayman Chemical, Ann Arbor, MI, USA), following the manufacturer's instructions. The catalytical activity of catalase (EC 1.11.1.6) was determined according to Aebi method (1984) [21], by the spectrometric analysis of the H_2O_2 consumption at 240 nm. Results are expressed as enzymatic activity units (U) in relation to the total protein of the samples.

The total protein content in liver (µg/mL) was determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA) based on the Bradford dye-binding method.

2.3.3. GSH

Reduced glutathione (GSH) and oxidized glutathione (GSSG), and the redox ratio (GSH/GSSG) were determined following the protocol of Hissin and Hilf, modified by us [22,23]. The o-phthaldeyde (OPA) reagent was added to the samples and the standard calibration curve, so that it reacted with the GSH at pH = 8, and it was fluorometrically measured at an excitation wavelength of 350 nm and an emission wavelength of 420 nm. GSSG was detected through previous incubation with N-ethylmaleimide, which was added to GSH in order to avoid its interference. Results are expressed in nmol of GSH relative to g of fresh tissue or as the GSH/GSSG ratio.

2.4. Western Blotting Analysis and Quantification

The detection of DNP-protein carbonyl groups (Novus), uncoupling protein 2 (UCP2), PTEN-induced kinase 1 (PINK1), nuclear factor-erythroid 2 related factor 2 (Nrf2), heme oxygenase-1 (HO-1), NOD-, LRR-, and pyrin domain-containing protein 3 (NLRP3) (Santa Cruz Biotechnology, Heidelberg, Germany), and mitochondrial oxidative phosphorylation complexes (OXPHOS) (Abcam, Cambridge, UK) was carried through Western blotting. Liver samples were homogenated in an RIPA buffer (as described below), separated in 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and plotted into PVDF membranes. After overnight blocking with an Odyssey® Blocking Buffer (PBS 927-40000, LI-COR Biosciences, Lincoln, NE, USA), membranes were incubated with the primary antibody for 3 h at room temperature. Detection was performed with chemiluminescence (anti-IgG-HRP, Santa Cruz Biotechnology, Inc., Heidelberg, Germany) or fluorescence (IRDye, LI-COR Biosciences, Lincoln, NE, USA) signals exposed and quantified by LI-COR Odyssey system and the Image Studio programme (LI-COR Biosciences, Lincoln, NE, USA). Revert total protein stain, REVERT-TM (LI-COR LI-COR Biosciences, Lincoln, NE, USA), was used as a loading control [24]. (The revert development of each membrane and complete blots are shown in Figure S1 in Supplementary Materials).

2.5. Statistical Analysis

Data obtained are expressed as the means and SDs of each group for n = 4–6. The variance analysis was carried through the one-way ANOVA and Tukey's post-hoc test for multiple comparisons in terms of significant results. A p-value of <0.05 was considered statistically significant. The graphs and statistic analysis were performed with GraphPad prism 7 (GraphPad software, San Diego, CA, USA).

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3. Results

3.1. IGL-2 Prevents ATP Breakdown and Succinate Accumulation and Affects Mitochondrial OXPHOS Complex Expression

ATP depletion and succinate accumulation is a major feature in ischemic tissues. Our results showed that after 24 h of SCS at 4 °C, ATP levels (Figure 1a) decreased significantly in the samples preserved with IGL-0 (without PEG35 and 3 mmol/L GSH) when compared to in the control group (Sham) ($p < 0.001$) and when compared to in the samples preserved in IGL-1 (1 g/L PEG35, and 3 mmol/L GSH) ($p < 0.01$) and IGL-2 (5 g/L PEG35 and 9 mmol/L GSH) ($p < 0.001$). Cold ischemia induced the accumulation of succinate, as observed in the IGL-0 and IGL-1 groups (Figure 1b). However, this accumulation of succinate was prevented in IGL-2-preserved livers.

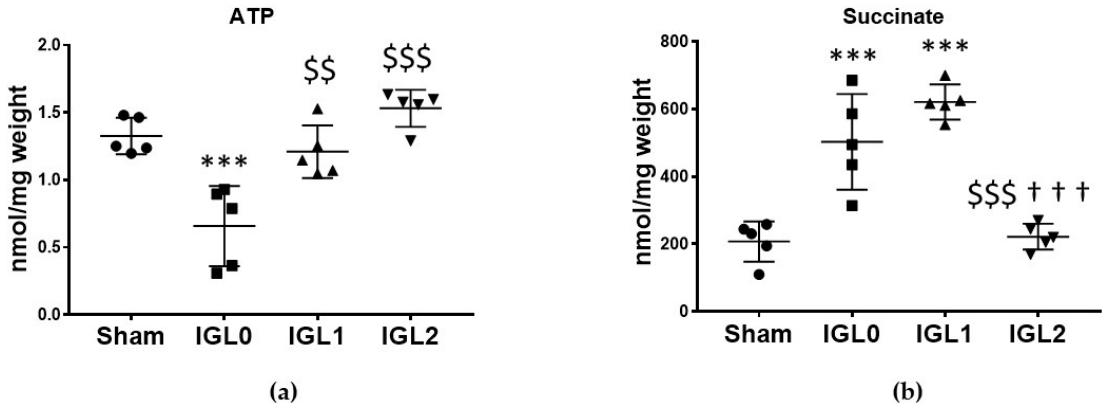

Figure 1. Energy metabolites as ATP (**a**) and succinate (**b**) in steatotic livers preserved (4 °C, 24 h) in the IGL-0, IGL-1, and IGL-2 solutions, vs. Sham. Bars represent mean values ± SDs of each group ($n = 5$). Differences are shown comparing groups (* vs. Sham, $^{\$}$ vs. IGL-0, and † vs. IGL-1) according to the one-way ANOVA test and the Tukey post-hoc test (one symbol indicates $p < 0.05$; two symbols indicate $p < 0.01$; three symbols indicate $p < 0.001$).

Mitochondrial OXPHOS complex expression is shown in Figure 2. The oxidation of NADH and $FADH_2$ and the subsequent phosphorylation of ADP to form ATP took place in the inner mitochondrial membrane through electron transport chain protein complexes (I–IV) and ATP synthase (complex V). Complex I NADH–coenzyme Q oxidoreductase expression was significantly increased in the IGL-2 group (Figure 2a). Complex II succinate–coenzyme Q oxidoreductase expression was significantly increased in the IGL-1 and IGL-2 groups (Figure 2b). Complex IV cytochrome C oxidase had an increasing tendency in the IGL-2 group without statistic difference (Figure 2d). There were no changes in the levels of hepatic mitochondrial complexes V ATP synthase (Figure 2e) and III coenzyme Q–cytochrome C oxidoreductase (Figure 2c), among groups.

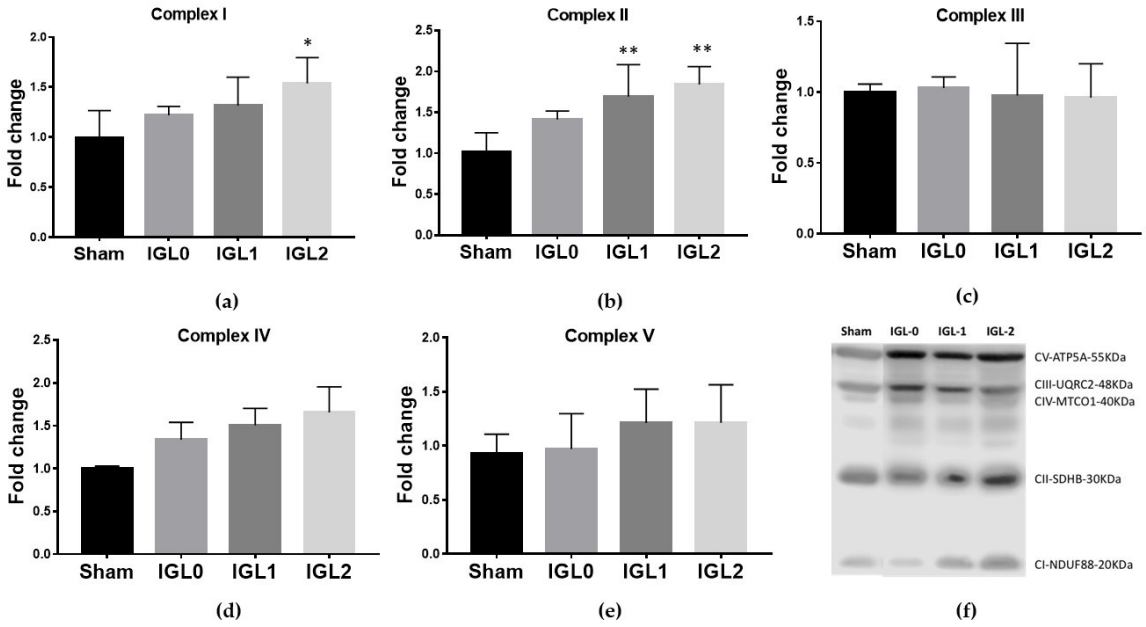

Figure 2. Oxidative phosphorylation complexes (OXPHOS) expression levels in complex I NADH–coenzyme Q oxidoreductase (**a**), complex II succinate–coenzyme Q oxidoreductase (**b**), complex III coenzyme Q-cytochrome C oxidoreductase (**c**), complex IV cytochrome C oxidase (**d**), complex V ATP synthase (**e**), and representative blots of OXPHOS in steatotic livers preserved (4 °C, 24 h) (**f**) in IGL-0, IGL-1 and IGL-2 solutions, vs. Sham. The bars represent the mean values ± SDs of each group (n = 4–6). Differences are shown comparing groups (* vs. Sham), according to the one-way ANOVA test and the Tukey post-hoc test (one symbol indicates $p < 0.05$; two symbols indicate $p < 0.01$; three symbols indicate $p < 0.001$).

3.2. UCP2 Expression and Mitophagy Increases in IGL-2 Preservation

The mitochondrial protein UCP2 acts as an anion carrier, and it is involved in the regulation of various processes such as cellular homeostasis, oxidative stress, and cell survival. Recently, a link has emerged between UCP2 cytoplasmic accumulation and mitophagy stimulation, particularly with respect to cardiomyopathies, including IR injury [25]. In this sense, we determined whether the PEG35 and GSH addition to preservation solutions could exert a protective effect through UCP2 upregulation. The results showed a significant increase in the expression of UCP2 in those livers preserved with IGL-2 (Figure 3a). We also evaluated the expression levels of PINK1, the main regulator of mitochondrial biogenesis [26], and observed a significant increase in the PINK1 expression (Figure 3b) in those livers preserved in IGL-2.

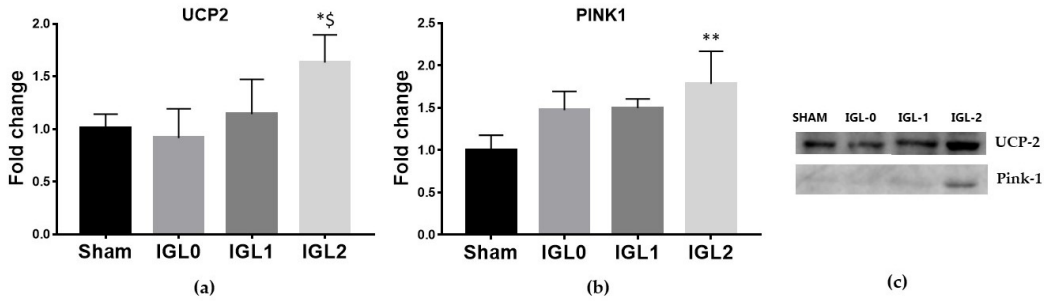

Figure 3. Expression levels of uncoupling protein 2 (**a**) and mitophagy marker protein PTEN-induced kinase 1 (PINK1) (**b**) in steatotic livers preserved (4 °C, 24 h) in the IGL-0, IGL-1, and IGL-2 solutions, vs. Sham. (**c**) Representative blots. The bars represent the mean values ± SDs of each group (n = 4–6). Differences are shown comparing groups (* vs. Sham, $ vs. IGL-0) according to the one-way ANOVA test and the Tukey post-hoc test (one symbol indicates $p < 0.05$; two symbols indicate $p < 0.01$; three symbols indicate $p < 0.001$).

3.3. Nrf2 Transcription Factor Increases in IGL-2 Solution Preservation

Nrf2 acts as a transcription factor by inducing the expression of cytoprotective gene products and plays a key role in activating cellular defense mechanisms. In order to explore whether the protective effects of PEG35 and GSH are associated with the Nrf2 response, we analyzed its expression, finding a dramatically increase of the transcription factor as well as its downstream enzyme HO-1 in IGL-2-preserved livers (Figure 4a,b). Inverse relationships between Nrf2 pathways and NLRP3 inflammatory pathways have also been reported at different levels [27,28]. In this regard, the inflammasome NLRP3 expression was found to be significantly increased in IGL-0 and IGL-1 preserving livers, while no differences were found when using IGL-2 (Figure 4c).

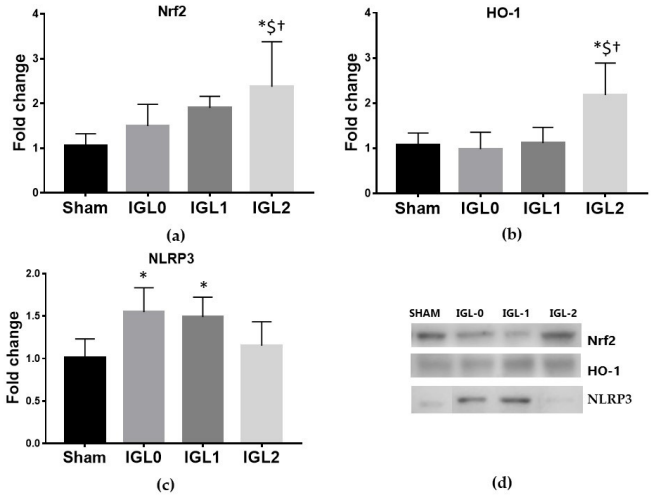

Figure 4. Nrf2-mediated response to oxidative stress in steatotic livers preserved (4 °C, 24 h) in the IGL-0, IGL-1, and IGL-2 solutions vs. Sham: (**a**) Nrf2 transcription factor; (**b**) HO-1; (**c**) NLRP3; and (**d**) representative blots. The bars represent the mean values ± SD of each group (n = 4–6). Nrf2 and HO-1 expression followed the same trend, being higher in the IGL-2 group compared to in the other groups. Differences are shown comparing groups (* vs. Sham, $ vs. IGL-0, and † vs. IGL-1) according to the one-way ANOVA test and the Tukey post-hoc test (one symbol indicates $p < 0.05$; two symbols indicate $p < 0.01$; three symbols indicate $p < 0.001$).

3.4. IGL-2 Preservation Solution Better Protects Steatotic Livers from Oxidative Stress

The cold preservation of steatotic livers increased oxidative stress damage, as measured by lipid peroxidation (MDA–TBA adducts) and protein oxidation (AOPP and carbonylated proteins). With the known antioxidant capacity of GSH, we investigated whether these oxidative stress markers were altered in the livers preserved with the different solutions. A significant rise on MDA–TBA adducts (Figure 5a) was observed in IGL-0 when compared to in Sham, IGL-1, and IGL-2. AOPP has been identified as a biomarker of oxidative damage to proteins, detecting dityrosine and cross-linking protein products [29]. AOPP levels (Figure 5b) significantly increased in the IGL-0 group compared to those in the Sham, IGL-1, and IGL-2 groups, while significant lower levels were observed in the IGL-2 group. Carbonylated proteins, the most general and widely used marker of severe protein oxidation, increased in the IGL-0 and IGL-1 groups when compared to in the Sham group (Figure 5c). Altogether, these results showed less oxidation of lipids and proteins in SCS livers when the increasing concentrations of PEG35 and GSH were added to the preservative.

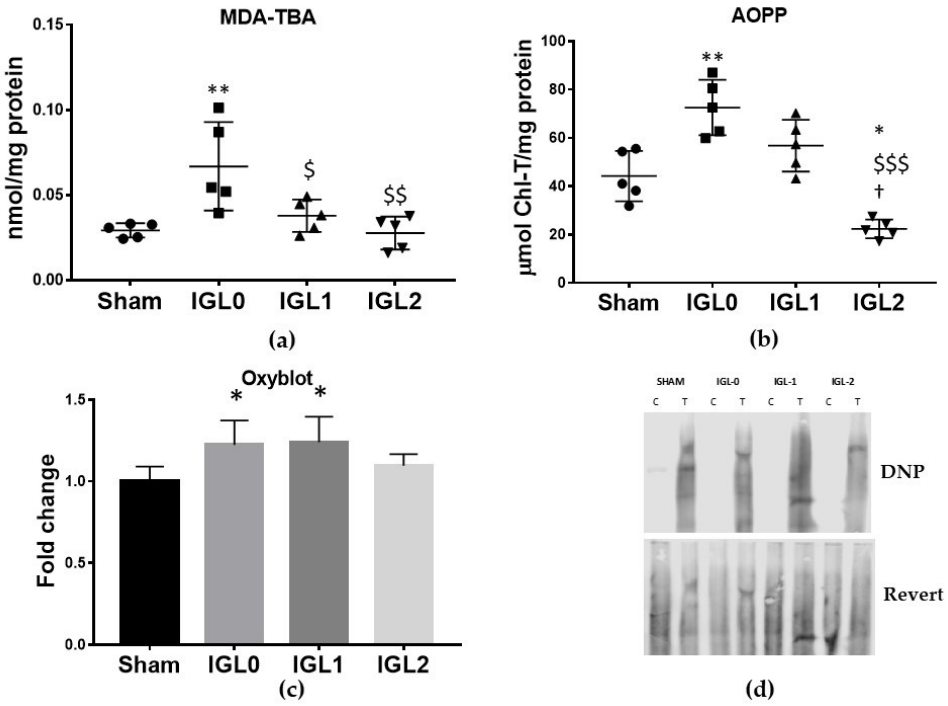

Figure 5. Hepatic oxidative stress damage in steatotic livers preserved (4 °C, 24 h) in the IGL-0, IGL-1, and IGL-2 solutions vs. Sham: (**a**) MDA-TBA adducts expressed in nmol/mg of total protein; (**b**) advanced oxidative protein products (AOPP) expressed in µmol chloramine-t/mg of total protein; (**c**) protein carbonyl formation (Oxyblot) analyzed by immunoblotting and expressed as a relative unit; (**d**) representative blots for protein oxidation. The bars represent the mean values ± SDs of each group (n = 4–6). Differences are shown comparing groups (* vs. Sham, $^{\$}$ vs. IGL-0, and † vs. IGL-1) according to the one-way ANOVA test and the Tukey post-hoc test (one symbol indicates $p < 0.05$; two symbols indicate $p < 0.01$; three symbols indicate $p < 0.001$).

3.5. Antioxidant Enzymatic Activity

Regarding antioxidant enzymatic activity, the group preserved in IGL-0 showed a higher activity in the antioxidant enzymes—Cu–Zn SOD (Figure 6a), GSH S-T (Figure 6c) and GSH-Px (Figure 6d), when compared to the Sham group. This correlated with the

higher oxidative stress previously exposed in the IGL-0 group. The liver preservation in the IGL-1 solution increased the activities of Cu–Zn SOD and catalase, when compared to Sham livers, and decreased the activity of GSH S-T compared to that in the IGL-0 solution. When comparing the IGL-2 solution (with higher amounts of PEG35 and GSH) to IGL-0, the results showed significantly lower antioxidant activities for Cu–Zn SOD and GSH S-T. No significant differences were found between IGL-2 and Sham and in any antioxidant enzymatic activity, which correlated with a lower amount of oxidative stress biomarkers in livers preserved in the IGL-2 group.

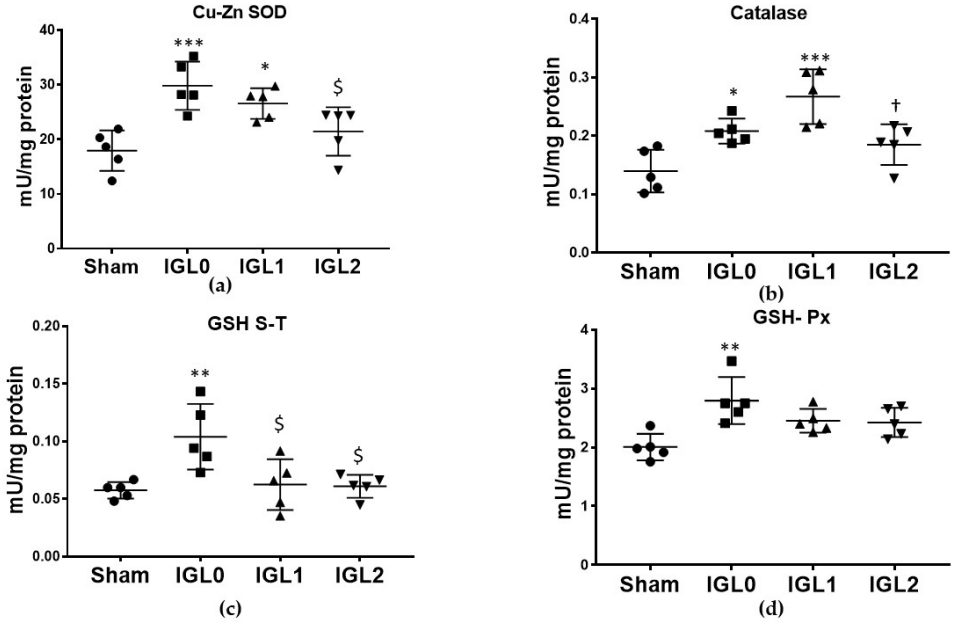

Figure 6. Enzymatic antioxidant capacity in steatotic livers preserved (4 °C, 24 h) in the IGL-0, IGL-1, and IGL-2 solutions vs. Sham: (**a**) superoxide dismutase (Cu–Zn SOD) expressed in mU/mg of total protein; (**b**) catalase expressed in mU/mg of total protein; (**c**) glutathione S-transferase (GSH S-T) expressed in mU/mg of total protein; (**d**) glutathione peroxidase (GSH-Px) expressed in mU/mg of total protein. The bars represent the mean values ± SDs of each group (n = 5). Differences are shown comparing groups (* vs. Sham, $ vs. IGL-0, and † vs. IGL-1) according to the one-way ANOVA test and the Tukey post-hoc test (one symbol indicates $p < 0.05$; two symbols indicate $p < 0.01$; three symbols indicate $p < 0.001$).

In contrast to other enzymes, the GSH-R, the enzyme responsible for reducing GSSG to GSH, showed a different pattern of activity, being significantly higher, in livers preserved in IGL-2 compared to in all the other groups (Figure 7c). This result is of great interest, since the reason why livers preserved in IGL-2 showed a significantly higher amount of GSH (Figure 7a) and a significantly higher GSH/GSSG ratio (Figure 7b) were not only attributable to higher content in the medium, but also to the increased activity of the GSH-R.

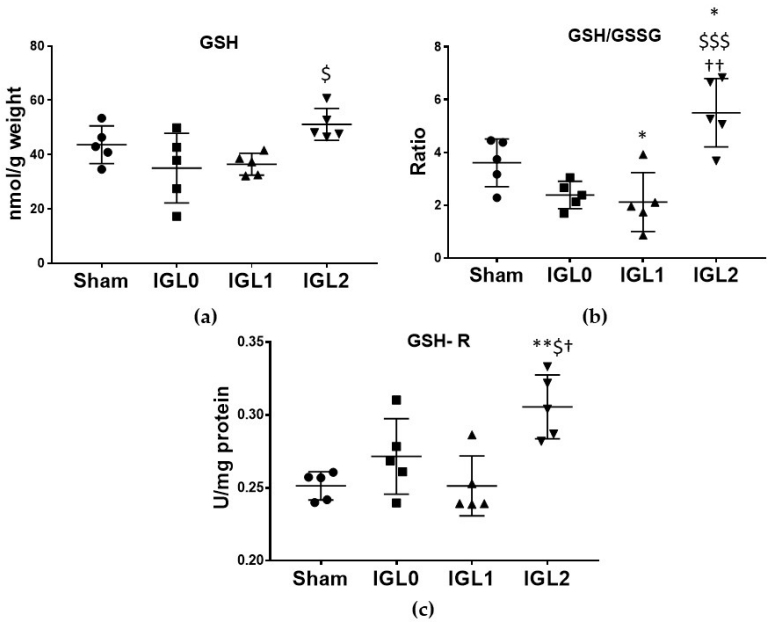

Figure 7. Glutathione status in steatotic livers preserved (4 °C, 24 h) in the IGL-0, IGL-1, and IGL-2 solutions vs. Sham: (**a**) reduced glutathione (GSH) expressed in nmol GSH/g fresh weight; (**b**) reduced glutathione/oxidized glutathione ratio (GSH/GSSG); (**c**) glutathione reductase (GSH-R) expressed in mU/mg of protein. The bars represent the mean values ± SDs of each group. Differences are shown comparing groups (* vs. Sham, $ vs. IGL-0, and † vs. IGL-1) according to the one-way ANOVA test and the Tukey post-hoc test (one symbol indicates $p < 0.05$; two symbols indicate $p < 0.01$; three symbols indicate $p < 0.001$).

4. Discussion

SCS remains the standard for liver preservation, although the effects due to anaerobic metabolism during the ischemic phase result in succinate accumulation and ATP depletion. These effects have been identified as the leading cause of reperfusion injury, causing oxidative stress and tissue inflammation [16]. This is of particular relevance in the case of fatty livers, as steatosis affects the activity of oxidative phosphorylation during cold liver preservation [30].

Our work showed the protective role that PEG35 and GSH play in the prevention of energy degradation and in the modulation of the redox state in cold steatotic livers preservation when using homozygous Ob Zücker rats.

During the cold ischemia phase, ATP comes from glycolysis, a significantly less efficient route of ATP production, while some anaerobic metabolites accumulate. The selective accumulation of the citric acid succinate is a feature of the ischemia response. With the particularity that succinate can control reperfusion injury through the formation of mitochondrial ROS by reverse electron transport at mitochondrial complex I. The pharmacological manipulation of the pathways that generate succinate accumulation has been shown to improve IR injury in murine models of heart attack and stroke [31]. In this regard, it should be noted that we observed lower succinate levels in livers preserved in IGL-2. In addition, PEG35 (IGL-1 and IGL-2) maintained ATP levels in the graft. This together may suggest a protective role of these components in the preservation medium.

Mitochondrial oxidative phosphorylation activity is decreased by cold ischemia in fatty livers, contributing to the reduced capability of steatotic grafts to restore the ATP after transplantation [30]. Until now, the OXPHOS mitochondrial expression of cold-preserved fatty liver has not been addressed. We observed enhanced expression of complex I and complex II in livers preserved with PEG35 and GSH. Recently, increased expression of

complexes I, II, III, and V has been reported in a murine model of cardiac IR in young mice. In contrast, the expression is lower in older individuals [32]. Therefore, the increased expression of OXPHOS components would be aimed at maintaining mitochondrial integrity under IR stress.

The protection of mitochondria is crucial to prevent IR injury [16]. In an early investigation, we found that SCS preservation with PEG35 improves mitochondrial biomarkers and the mitochondrial enzyme ALDH2 functionality [10,15]. In the present work, we found that the preservation with IGL-2 induces a significant increase in the expression of mitochondrial protein UCP2, an anion carrier involved in the regulation of various processes, such as maintaining mitochondrial membrane potential and limiting the generation of reactive oxygen species [33,34]. In fact, UCP2 silencing reduces the levels of GSH and the GSH/GSSG ratio in HepG2 cells [35]. UCP2 can also function as an oxidative stress sensor, not directly involved in antioxidant defense, but through other adaptation mechanisms [36]. Moreover, UCP2 activates IR-induced mitophagy [25], and the inhibition of mitophagy abolishes UCP2 cardioprotective effects in IR [37]. Accordingly, we found a significant increase in PINK1 expression linked to enhanced UCP2 expression in IGL-2-preserved fatty livers. This PINK1 pathway is one of the best characterized among mitophagy signaling pathways [26], and such enhanced expression is consistent with our previous results, in which we also found an increase in the mitophagy markers Beclin1 and LC3B in the fatty livers preserved with PEG35 [15].

The protective role of PEG35 is also evidenced in the Nrf2-mediated response. The activation of the Nrf2 transcription factor could prevent IR injury, both through the activation of antioxidant and anti-inflammatory pathways [38–40]. In our study, the preservation of steatotic livers in the IGL-2 solution increased the Nrf2 expression and that of the antioxidant system, HO-1. Nrf2 is also associated with the constitutive expression of GSH-dependent enzymes [41]. Regarding the anti-inflammatory response, the expression of the NLRP3 inflammasome is reduced. It has also been reported that Nrf2 can control mitophagy by directly binding to the promoter regions of PINK1, which would provide an advantage for cell survival [42]. Decreased NLRP3 expression, along with increased PINK1, indicates that Nrf2 could regulate the cytoprotective mechanism of IGL-2 preservation.

Nrf2 has recently been proposed as a predictive marker for organ recovery and donor expansion in human allografts [43]. Since we found that the IGL-2 solution significantly increased Nrf2 expression, we can expect the better viabilities of these steatotic grafts against IR damage after transplantation.

We have recently demonstrated how the direct effects of IGL-2 on mitochondrial ALDH2 protect the steatotic liver graft from the damaging effects of cytotoxic aldehydes generated by ROS overproduction [15]. In the present work, we found a reduction in lipid peroxidation and oxidized proteins correlated with increased concentration of PEG35 and GSH in the preservation solution. However, only the use of IGL-2 solution completely reversed the increase found with IGL-0. Carbonylated proteins decreased when fatty grafts were preserved with the combination of PEG35 and a high dose of GSH. The importance of several endogenous antioxidants has been demonstrated during ischemic heart injury in early studies [44,45]. Cardiac ischemia induces a significant increase of cytoplasmic GSH-Px activity, while superoxide dismutase activity is unmodified. The greater the oxidative damage, the greater the antioxidant enzymatic activity. In our results, the enzymatic activity was higher in the groups with higher levels of oxidation of lipids and proteins, except for the activity of GSH-R, which was the enzyme that recycled GSH from its oxidized form, increasing its activity and the GSH level in the IGL-2 group. Since ROS regulation is a therapeutic strategy for liver IR injury, decreased lipid and protein damage and antioxidant properties appear to be one of the most relevant mechanisms mediating the beneficial effects of PEG35 used in cold storage solutions.

5. Conclusions

The steatotic liver is especially vulnerable to IR lesions that significantly determine graft function after transplantation. An in-depth understanding of the molecular mechanisms that lead to tissue and cell damage is therefore essential. Cold fatty liver preservation with PEG35 and GSH (IGL-2 solution) maintained ATP production, decreased succinate accumulation and increased the expression of the OXPHOS complexes I and II, UCP2, PINK-1, Nrf2, and HO-1. IGL-2 protected against lipid and protein oxidation, increased the GSH/GSSG ratio and decreased the inflammasome NLRP3 expression. Taken together, its effects included protecting mitochondrial integrity, maintaining ATP levels, and reducing oxidative stress and inflammation. Further research on whether these conditions would protect the graft during the reperfusion phase would be necessary in view of its potential use in clinical transplantation.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/antiox11010158/s1, Figure S1: Membranes and loading control (Figure 2f). Figure S2: Membranes and loading control (Figure 3c). Figure S3: Membranes and loading control (Figure 4d). Figure S4: Membranes and loading control (Figure 5d).

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Conflicts of Interest: The authors declare no conflict of interest.

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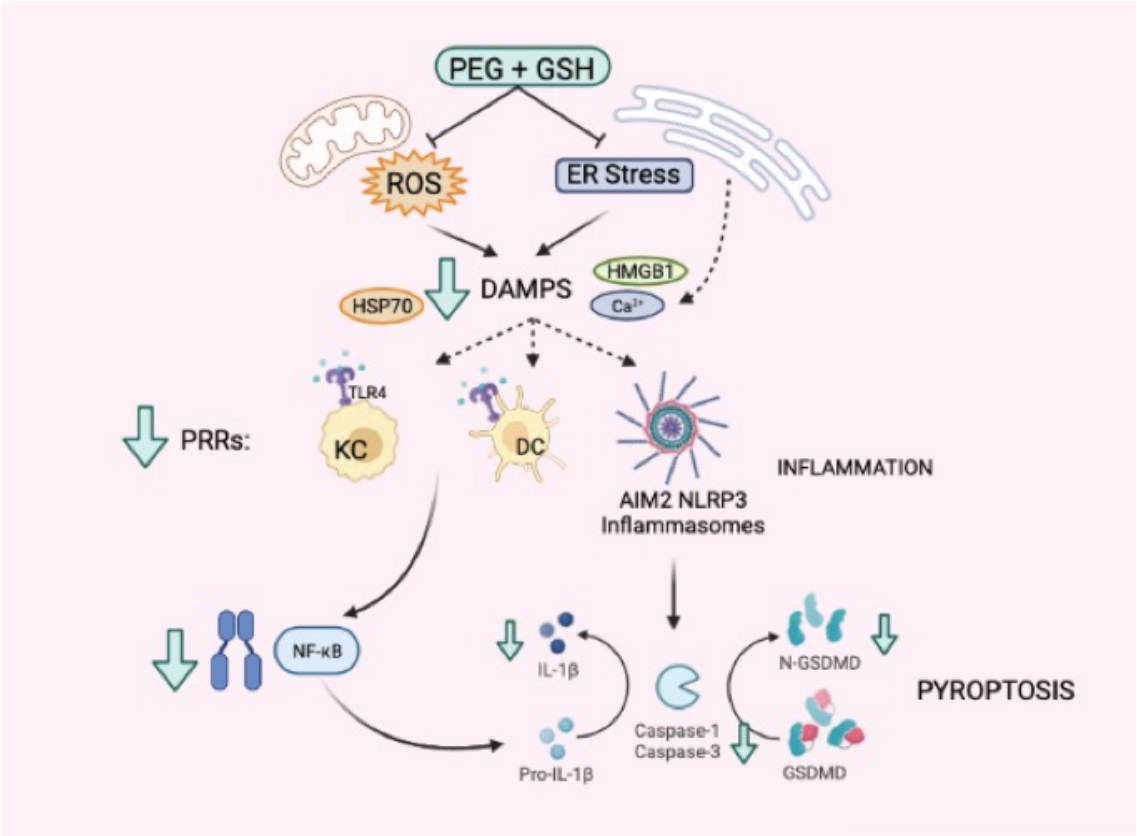
CAPÍTULO IV. DAMPS induce inflammatory injury in cold-static fatty liver graft preservation. The key role of redox and ER stress.

Graphical abstract:



Resumen:

Ante la falta de donantes para el trasplante clínico, se han establecido criterios de donantes extendidos (ECD, por sus siglas en inglés) que incluyen entre otros el uso de hígados con un 30 % de grasa infiltrada. El uso de estos hígados subóptimos supone un importante reto a la investigación básica y clínica. Los hígados grasos se caracterizan por un incremento del estrés oxidativo de base así como una mayor inflamación crónica, con la consecuente vulnerabilidad al daño por isquemia-reperfusión. El aumento del estrés oxidativo asociado con el estrés del retículo endoplásmico (ER) y la excreción de patrones moleculares asociados al daño (DAMPS) durante la isquemia darán lugar a la inflamación y la muerte de las células hepáticas. El objetivo del presente estudio es evaluar los mecanismos que desencadenan tanto el daño celular como la protección celular durante el almacenamiento en frío estático (SCS) antes del trasplante.

Se evaluaron tres soluciones de conservación, dos de ellas disponibles comercialmente: la solución de histidina-triptófano-cetoglutarato (HTK) de la Universidad de Wisconsin (UW) y una solución de conservación Instituto Georges López (IGL-2) modificada, con

mayor concentración del agente oncótico (5 g/L PEG35) y del antioxidante (9 mmol/L GSH). Se preservó el hígado graso de ratas Zücker macho obesas (11 semanas de edad) (24 h, 4 °C) en las soluciones IGL-2, UW y HTLK y se han analizaron las vías de señalización redox, estrés del ER, DAMPS, inflamación, apoptosis y piroptosis.

Nuestros resultados muestran que la SCS aumenta el estrés del retículo y el estrés oxidativo, la inflamación, la apoptosis y la piroptosis en injertos de hígado graso UW y HTK conservados en frío. En cambio, la preservación en la solución IGL-2, con presencia de PEG35 y GSH, previene la oxidación y la inflamación. Proponemos profundizar en la investigación de estas vías de muerte , la señalización del estrés oxidativo y las estrategias para abordar la inflamación mediada por DAMPS durante la SCS en soluciones de preservación con el fin de mejorar la viabilidad de los hígados esteaóticos y aumentar la disponibilidad de hígados disponibles para el trasplante.

DAMPS induce inflammatory injury in cold-static fatty liver graft preservation. The key role of redox and ER stress.

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Abstract

Oxidative stress is a key feature of liver steatosis, due to the production of reactive oxygen species induced by fatty acids in the liver. Increased oxidative stress associated with endoplasmic reticulum (ER) stress and the promotion of damage-associated molecular patterns (DAMPS) will eventually lead to inflammation and liver cell death. Extended criteria donor livers (ECD) include the use of fatty livers, which represents an increasing challenge in transplantation. Our goal is to study the mechanisms that trigger both cell damage and cell protection during static cold storage (SCS) prior to transplantation.

We evaluated three preservation solutions, two of them commercially available: the University of Wisconsin (UW) and histidine-tryptophan-ketoglutarate (HTK) solution and a modified preservation solution from the Georges Lopez Institute (IGL-2) with an augmented oncotic agent (5 g/L PEG35) and glutathione (9 mmol/L GSH). Fatty liver from obese male Zücker rats (11 weeks old) were preserved (24 h, 4°C) in the IGL-2, UW and HTLK solution groups. Redox signaling pathways, ER stress, DAMPS, inflammation, apoptosis, and pyroptosis have been analyzed.

Our results show that SCS increases ER and oxidative stress, inflammation, apoptosis, and pyroptosis in cold-preserved UW and HTK fatty liver grafts. IGL-2 solution prevents oxidation and inflammation. A deeper understanding of ER and oxidative stress signaling and strategies to address DAMPS-mediated inflammation during SCS are essential to rescuing fatty livers for clinical use.

Keywords: Antioxidants, Cold static preservation, Damage-associated molecular patterns, ER, Glutathione, liver ischemia-reperfusion injury, Polyethylene glycol, Redox signaling.

Introduction

A direct consequence of an ever-increasing number of candidates on the list for liver transplantation is the use of marginal organs such as fatty liver grafts. Hepatic steatosis is known to increase the risk of functional impairment after transplantation [1,2].

Excessive fatty acid production mediates endoplasmic reticulum stress (ER) inducing reactive oxygen species (ROS) accumulation in the liver [3–5]. ROS promotes lipid peroxidation, generates oxidative stress-mediated injury inhibiting mitochondrial respiration and ultimately produces cytokine release, which is associated with the leading to inflammation and hepatic cell-death [6,7]. ER stress is caused by the activation of the unfolded protein response (UPR) pathway. In unstressed cells, the most abundant ER chaperone, glucose-regulated protein 78 kDa (GRP78), binds to the luminal domains of three sensors, including the ER-like kinase PKR (PERK), in order to keep them in an inactive state. When misfolded proteins accumulate in the ER, GRP78 binds to misfolded or unfolded proteins to assist their correct folding and consequently dissociates from UPR sensors. These initiate UPR signaling cascades that encode for ER chaperones, folding enzymes, pro-apoptotic proteins and antioxidants, with the aim of restoring ER homeostatic balance [8].

Due to increased oxidative stress, ER stress and diverse pathological factors, the recognition of endogenous host-derived damage-associated molecular patterns (DAMPS) was activated [9–11]. In IR damage, the best characterized DAMPS are the high mobility group 1 box (HMGB1), Ca2+ from reticulum stress, and the histone/DNA complex, which activate TLR4 and the Nod-like receptor protein 3 (NLRP3) and AIM2 inflammasomes, but the role of HSP70 as DAMPS has also been described [12] . Activation of TLR4 promotes nuclear factor-kB activation, which activates pro-IL-1β transcription in the nucleus. In turn, detection of DAMPS by cells induces the assembly of NLRP3 and AIM2 inflammasomes. Inflammasome triggers an inflammatory response through Caspase 1 activation, which cleaves pro-IL-1β and also with pp38 MAPK increases the transcription of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α). Next, gasdermin D (GSDMD) can be cleaved by cleaved-caspase 1 into

GSDMN-terminal with pore-forming characteristics [13]. Recently, it has been proved that Caspase 3, a key protein of apoptosis, can also cleave GSDM and induce pyroptosis [14]. Activation of inflammasome and cleavage of GSDM are important processes in the canonical [12] pyroptosis activation. Pyroptosis is a newly discovered inflammatory programmed cell death, and it has been shown in both basic and clinical research that it may play a key role in the progression of fatty liver disease [15–17] disease and prevention of IR injury during transplantation [18]. Therefore, the increase in liver steatosis donors constitutes a growing challenge for the liver transplantation procedure. [19]

The current challenge is therefore to study the mechanisms involved at different levels to prevent ischemic injuries. This could be modulated by the composition of the preservation solution in SCS [20]. Among solutions used, the University of Wisconsin (UW) solution became the gold standard. This solution contains an oncotic support, the hydroxyethyl starch (HES) and other additives such as adenosine, as an ATP precursor, and glutathione (GSH) and allopurinol as antioxidants. However, it has been demonstrated that hydroxyethyl starch (HES) promotes red blood cell aggregation and confers high viscosity which difficult the proper flushing and preservation of the graft [21]. In addition UW also triggers other complications in the organ preserved due to its high concentration of K^+. For these reasons, other preservation solutions, with lower viscosity and K^+ , like Histidine-tryptophan-ketoglutarate (HTK) and Institute Georges Lopez solution (IGL-1) were developed. IGL-1 contains Polyethylene glycol (PEG) with a molecular weight of 35 KDa (PEG-35) instead of HES and includes a low concentration of K^+. This fact entails several beneficial effects as compared with other solutions, since apoptosis, ER stress and inflammation are reduced [22–25] As the benefits of using the IGL-1 solution include the prevention of oxidative damage, with the aim of having a more efficient solution against oxidative stress, a new solution, named IGL-2, enriched in PEG35 (5 g/L) and glutathione (9 mmol/L), has been proposed (Table 1) [26].

The aim of this work is to analyze the pathways that can lead to pyroptosis, ER production and oxidative stress, cytokine release and the establishment of an inflammatory response during cold storage of the fatty liver graft. A comprehensive

investigation of these pathways in different conservation media may provide us with targets that could represent a promising strategy against IRI.

Materials and Methods

Animals and Liver Isolation and Perfusion

All the experiments have been conducted in accordance with protocols approved on 14 July 2016 (NO. 483116) following the European Union Directive for animal experiments (2010/63/EU). Protocols have been validated by the Ethics Committees for Animal Experimentation of the University of Barcelona (Directive 483/16). Male homozygous obese (Ob) Zücker rats 16-18 weeks of age,with a rate of steatosis between 60% and 70%, were used for this study (Charles River, France). They were housed under conventional animal facilities conditions. Animals had free access to water and dry food standard diet. Rats were randomly distributed into different experimental groups as described below. Sham group and three experimental groups were used, as described below. The abdomen was cut with a midline incision, and following bile duct cannulation, the portal vein and the splenic and gastroduodenal veins were ligated. After organ recovery, the livers were flushed either with IGL-2, UW or HTK solutions and stored in each solution for 24 h at 4ºC. Animals were randomly distributed as follows:

- Sham: Obese Zücker rats underwent transverse laparotomy, and silk ligatures of right suprarenal and diaphragmatic veins and hepatic artery were performed.
- IGL-2: After organs collection, livers were flushed with 50 mL of IGL-2 solution and next stored in IGL-2 preservation solution for a 24-hour period at 4 °C
- UW: After effecting organs collection, livers were flushed with 50 mL of UW solution and straightaway stored in UW preservation solution for a 24-hour period at 4 °C.
- HTK: After organs collection, livers were flushed with 50 mL of HTK solution and immediately stored in HTK preservation solution for a 24-hour period at 4 °C.

After the cold storage period described, organs maintained in their corresponding preservation solutions were flushed with 20 mL of Ringer Lactate solution. Samples were then stored at -80 ºC for subsequent biochemical analysis.

Histomorphometry

For histopathology, tissue samples were collected and fixed in 10% buffered formalin, dehydrated by an ascending alcohol series ending in xylol and finally embedded in paraffin. The specimens were sectioned at a thickness of 2–3 µm and sections were stained with hematoxylin-eosin (H&E). Images of the H & E (800X) stained liver tissue were captured using the light microscope (BX61; Olympus) connected to a digital camera (DP70; Olympus). A 35-point grid on the H & E stained slides determined the steatosis lesions. To assess the volume density of steatosis, a ratio of points touching lipid vacuoles (Pp) compared to the total number of test points (PT) was determined following Layman [27].

Antioxidant Enzymes Activity

The measurement of the antioxidant enzymes glutathione peroxidase (GPx, EC 1.11.1) and glutathione reductase (GR, EC 1.6.4.2) were performed with Cayman Kits (GPx, 703102; GR, 703202, Cayman Chemical, Ann Arbor, MI, USA) following the manufacturer's instructions. Results are expressed as enzymatic activity units (mU) in relation to the total protein of the samples.

Glutathione

Reduced (GSH) and oxidized (GSSG) glutathione and the redox ratio (GSH/GSSG) were determined following the protocol of Hissin and Hilf, modified by us [28,29].The o-phthaldeyde (OPA) reagent is added to the samples and standard calibration curve so that it reacts with the GSH at pH=8, and it is fluorometrically measured at an excitation wavelength of 350 nm and emission wavelength of 420 nm. GSSG is detected through previous incubation with N-ethylmaleimide, which is joined to GSH in order to avoid its interference. Results are expressed in nmol of GSH relative to g of fresh tissue or as the ratio (GSH/GSSG).

Caspase activities

The activity of the caspase 1was determined using the fluorimetric kit for the detection of caspase 1 activity from Abcam laboratories (ab39412). This protocol is based on the

detection of cleavage of the substrate YVAD-AFC (AFC: 7-amino-4-trifluoromethyl coumarin). Results are expressed in RFU (relative fluorescence units).

Caspase 3 activity was determined following the Sigma Laboratories colorimetric kit (CASP-3-C). The protocol is based on the hydrolysis of the substrate acetyl-Asp-Glu-ValAsp p-nitroanilide by this enzyme, leading to the release of p-nitroanilide (pNA), whose absorbance is measured at 405 nm. Results are expressed as pmol pNA /min/ mg of protein.

Cytokines determination

The concentrations of TNF-α (ab100785), and IL-1β (ab100768) in liver homogenates were determined using specific ELISA kits (Abcam), according to the manufacturer's instructions. Results are expressed as pmol/ mg of protein.

Western Blot Analysis and Quantification

The detection of 4-hydroxynonenal (4HNE, Novus NB100-63093); glucose regulatory protein 78 (GRP78, Abcam ab21685); PKR-like ER kinase (PPERK Cell signaling 16F8); Phospho-p38 MAPK (PP38, Cell signaling 9211); high mobility group protein 1 (HMGB1, Abcam ab18256); heat shock protein 70 (HSP70, BD Biosciences BD610607); TLR4 (Santa Cruz Biotechnology , sc-293072) nuclear factor kB (NFkB, Santa Cruz Biotechnology sc33022), NOD-,LRR- and pyrin domain-containing protein 3 (NLRP3, Novus NBP2-67639), AIM2 inflammasome (Santa Cruz Biotechnology sc-293174); and gasdermin (GSDM, Santa Cruz Biotechnology sc-393581) was carried through Western Blot. Liver samples were homogenated in RIPA buffer (as described below), separated in 10% sodium dodecylsulfate- polyacrylamide gel electrophoresis (SDS-PAGE) and plotted into PVDF membranes. After over-night blocking with Odyssey® Blocking Buffer (PBS 927-40000, LI-COR Biosciences, USA), membranes were incubated with the primary antibody for 3 hours at room temperature. Detection was performed with chemiluminescence (anti-IgG-HRP, Santa Cruz Biotechnology, Inc., Heidelberg, Germany) or fluorescence (IRDye, LI-COR Biosciencies, USA) signals exposed and quantified by LI-COR Odyssey system and the Image Studio programme (LI-COR Biosciencies, USA). Revert total

protein stain, REVERT-TM (LI-COR Biosciencies, USA) was used as a loading control [30]. (Revert development of each membrane is shown in supplementary material, S1).

Statistical Analysis

Data obtained is expressed as the mean of each group ± standard deviation (SD), for n=4-6. The variance analysis is carried through one-way ANOVA and Tukey's multiple comparisons test post-hoc test for multiple comparisons, when significant results. A p-value < 0.05 is considered statistically significant. SPSS was used for statistic analysis and graphs were performed with GraphPad prism 7 (GraphPad software, San Diego, CA, USA)

Results

Histomorphometry

No significant differences were observed in the volume of steatotic lesions between the groups as determined by a 36-point grid on the H & E stained slides (Fig 1a and 1b).

ER stress, oxidative stress, and antioxidants

Cold preservation of steatotic livers is known to increases ER and oxidative stress damage. As a misfolded protein indicator, increased GRP78 expression levels were found in UW preserved livers (Fig 2a), concomitant with increased PPERK UPR sensor (Fig 2b), which may indicate the initiation of the UPR signaling cascades that encode for ER chaperones, folding enzymes, pro-apoptotic proteins and antioxidants. No significant changes were observed in livers preserved with the modified preservation solution IGL2 when compared to sham fatty livers.

Oxidative stress levels were measured by 4-HNE adducts. A significant rise on 4-HNE adducts (Fig 2c) was observed in UW when compared to Sham and IGL-2 preserved livers. Our results so far indicate that IGL-2 protects better than other solutions by reducing oxidative lipid-protein products, probably due to the maintenance of high levels of reduced glutathione. This result is of great interest, since the reason why livers preserved in IGL-2 show a significantly higher GSH / GSSG ratio (Fig 2d) is not only attributable to higher content in the medium, but also to the increased activity of the GSH reductase (Fig 2e). Regarding to other antioxidant enzymatic activities, the group

preserved in UW and HTK solutions showed a higher activity in the antioxidant enzyme GSH peroxidase (Fig 2f) when compared to sham and IGL-2 groups. No significant differences were found between the antioxidant enzymatic activity of the IGL-2 and Sham groups, which correlates with a lower amount of oxidative stress biomarkers and redox signaling in preserved livers in the IGL-2 group.

DAMPS and inflammasome

Once it has been evaluated a difference in ER and oxidative stress in the different preservation solutions, we explored the impact of sterile inflammation. HMGB1 expression was shown in Fig 3a. A significant increase in the UW group in HMGB1 expression was found when compared to IGL-2 preserved livers. HMGB1 has dual function as a nonhistone nucleoprotein and an extracellular inflammatory cytokine. Stressed cells are known to passively release extracellular HMGB1 or actively secrete it. As a chemokine or cytokine, it binds to pattern recognition (PRR) receptors to play the role of DAMPS. Extracellular Hsp70 can also act as DAMPS and stimulate immune and inflammatory responses that lead to sterile inflammation and the spread of existing inflammation. In this sense, a significant increase in the expression of Hsp70 is observed in the UW and HTK groups (Fig 3b). The Hsp70 family of proteins and their cochaperones constitute a complex network of folding machines that cells use in many ways. Acting as DAMPS activates AIM2 (Fig 3c) and NLRP3 (Fig 3d) inflammasomes in the UW and HTK groups. Activation of TLR4 (Fig 3e) promotes the inflammatory pathway via NFκB, which is increased in the UW group (Fig 3f).

Cytokines, apoptosis and pyroptosis activation

The expression levels of DAMPS, and the impact of inflammasomes results in increased expression of proinflammatory cytokines such as PP38 (Fig 4a) and levels of TNF-α (Fig 4b) and IL-1β (Fig 4c) in UW preserved livers. The activities of caspase 1 (Fig 4d), and caspase 3 (Fig 4e) are dramatically increased in UW and HTK preserved livers. Overall results show that the processes of sterile inflammation, although inherent to the storage of cold grafts of fatty liver, are largely avoided in the preservation with IGL2 solution. Livers preserved in IGL2 are more efficiently prevented against inflammasome, inflammatory cytokines and caspases activation.

Caspase 1 and caspase 3 are involved in different pathways of the pyroptosis process, inducing the synthesis and cleveage of GSMDN. A significant rise on GSMDN (Fig 4f) was observed in UW when compared to Sham and IGL-2 preserved livers. During pyroptosis, the cleaved GSMDN forms a pore in the cell membrane and allows the release of inflammatory cytokines.

Discussion

While SCS remains the standard for liver preservation, accumulation of metabolites during the ischemic phase will pose a hazard after reperfusion, especially in the case of fatty liver grafts. Given that, our current research focuses on preventing the extent of damage using the appropriate components of the preservation solution. Several studies have reported the protective role of different molecular weight PEGs in cold preservation in different organs, including brain, liver, kidney, and heart [31–35]. We have previously demonstrated the protective effects of PEG35 addition to preservation solution for liver grafts [22]. Benefits have been linked to protecting the glycocalyx integrity [36] and improving mitochondrial machinery leading to a reduction in cold ischemic injury [25].

The relevance of mitochondria in cold ischemia of fatty liver is well established [37,38]. We recently observed that in liver preserved in IGL-2 solution, the mitochondrial enzyme ALDH2 was upregulated compared to livers preserved in UW and HTK solutions. These results were associated with reduced levels of transaminases, apoptosis, and lipid peroxidation. The mechanisms involved could be ALDH2 detoxification of 4-HNE, an important and damaging byproduct of lipid peroxidation. In the present work, our results showed that SCS of fatty livers in IGL-2 solution prevents 4-HNE protein adducts formation. IGL-2 also increases GSH levels and GSH reductase activity and reduced GSH peroxidase activity. This could well be the result of a synergistic effect of increased PEG35 and glutathione levels [26,39]. Moreover, endogen antioxidant synthesis is also promoted, which will be a key element in improving graft viability [39,40].

The lipotoxic environment in fatty livers directly influences ER homeostasis and ER stress activation [3,5]. The resulting signaling pathways lead to the triggering of the UPR response in order to start a repair process. However, if homeostasis is not restored, the

ER initiates inflammation and death-signaling pathways, which contribute to the pathogenesis of IRI.

Due to the increase in oxidative stress and ER stress and Ca^{2+} release, the activation of endogenous host-derived recognition of DAMPS occurs. Recently, a well-established DAMPS in sterile inflammation is HMGB1, which has been suggested as suitable marker to evaluate the graft performace in liver transplantation. In clinical procedures liver graft is subjected to cumulated damage associated with complex processes that include the organ recovery, liver washing-out and finally cold storage in preservation solution before clinical transplantation. HMGB1 and the Hsp70 family of proteins, acting as DAMPS, activate the inflammatory pathway through NFκB. Higher expression of the GRP78 chaperone and the PERK transmembrane receptor was found in UW-preserved fatty livers. In addition, the expressions of the DAMPS markers were found to be increased in livers cold-preserved in UW and HTK solutions, but not in the IGL-2 group. DAMPS will activate PRR. In the liver IR, TLR4 associated to Dentitric Cells (DC) or Kupffer Cells (KC), and cytosolic inflammasomes are the main PRR activated [41]. TLR-4 activation promotes NFkB release by KC, which promotes pro-IL-1B transcription. When liver are preserved in UW solution, TLR4 and NFkB expression are increased, as well as IL-1β activity, comparing with Sham and IGL-2 preserved groups.

Activation of inflammatory mechanisms, particularly the NLRP3 and AIM2 inflammasome, are emerging as a crucial outcome of oxidative [11] and ER stress [42]. The inflammasome triggers an inflammatory response through IL-1β, which increases the transcription of proinflammatory cytokines such as tumor necrosis factor-α (TNF-α). The increase in the activity of caspase 1 and caspase 3, has been described to be responsible for the GSDMN with characteristics of pore formation by canonical and other pathways [16], inducing pyroptosis [17]. Pyroptosis, unlike apoptosis, is a caspase-dependent unprogrammed cell death whose involvement in IRI has recently been reported [43]. GSDM role identification in pyroptosis execution was essential for understanding the lytic form of cell death. Caspase stimulation due NLRP3 and AIM2 activation, promotes GSDM scission in a N-terminal (GSDM-N) and C-terminal (GSDM-C) regions. When does it happen, the N-terminal fragment allows for its oligomerization, so is able to translocate to the plasma membrane and pore formation, increasing

cytotoxicity [14]. GSDM-N has been also involved in IL-1 β activation, increasing the inflammatory cascade [43].

A significant inhibition of NLRP3 and AIM2 inflammasomes and related markers as IL-1β, caspase activities and GSDM cleavage, were observed in fatty livers preserved in the IGL-2 solution when compared to fatty liver grafts preserved in UW and HTK solutions. IGL-2 solution, which contains PEG-35, could protect membrane pore formation due to PEG-35 adhesion to cell membrane glycerophospholipids, thereby increasing membrane integrity and preventing edema [34]. Therefore, IGL-2 protects against pyroptosis during liver SCS preservation.

In conclusion, our results show that sterile inflammatory processes, although inherent to cold storage of fatty liver grafts, are largely avoided in the IGL2 preservation that contains PEG35 and glutathione. This solution was more efficiently in preserving against oxidative and ER stress, inflammasome activation, inflammatory cytokines, and pyroptosis-inducing phenomena.

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Figure Legends

Figure 1. Histomorphometry in steatotic livers preserved (4 °C, 24-hours) in IGL-2, UW and HTK solutions, vs Sham. (a) Representative images of liver tissue stained with H & E 800X (Bar=30 μm) (b) Volume density of steatosis in H & E stained liver tissue. Bars represent mean values of each group ± SD (n=4).

Figure 2. ER stress, oxidative stress, and antioxidants in steatotic livers preserved (4 °C, 24-hours) in IGL-2, UW and HTK solutions, vs Sham. (a) Relative protein levels and representative western blot of GRP78. (b) Relative protein levels and representative

western blot of PPERK UPR sensor. (c) Relative protein levels and representative western blot of 4-HNE adducts. (d) GSH / GSSG ratio (e) Activity of GSH reductase. (f) Activity of GSH peroxidase. Groups represent mean values ± SD (n=4-6). Differences are shown comparing groups (* vs Sham and # vs IGL-2) according to one-way ANOVA test and Tuckey post-hoc (one symbol p<0.05; two symbols p<0.01; three symbols p<0.001).

Figure 3. DAMPS and inflammasome in steatotic livers preserved (4 °C, 24-hours) in IGL-2, UW and HTK solutions, vs Sham. (a) Relative protein levels and representative western blot of HMGB1. (b) Relative protein levels and representative western blot of Hsp70. (c) Relative protein levels and representative western blot of AIM2 inflammasome. (d) Relative protein levels and representative western blot of NLRP3 inflammasome (e) Relative protein levels and representative western blot of TLR4. (f) Relative protein levels and representative western blot of NFκB. Groups represent mean values ± SD (n=4-6). Differences are shown comparing groups (* vs Sham,# vs IGL-2 and $ vs UW) according to one-way ANOVA test and Tuckey post-hoc (one symbol p<0.05; two symbols p<0.01; three symbols p<0.001).

Figure 4. Cytokines, apoptosis and pyroptosis activation in steatotic livers preserved (4 °C, 24-hours) in IGL-2, UW and HTK solutions, vs Sham. (a) Relative protein levels and representative western blot of PP38. (b) Levels of TNF-α . (c) Levels of IL-1β (d) Activity of Caspase 1. (e) Activity of Caspase 3 (f) Relative protein levels and representative western blot of cleaved GSDM. Groups represent mean values ± SD (n=4-6). Differences are shown comparing groups (* vs Sham,# vs IGL-2 and $ vs UW) according to one-way ANOVA test and Tuckey post-hoc (one symbol p<0.05; two symbols p<0.01; three symbols p<0.001).

Graphical Abstract: Protective role of PEG35 and GSH in inflammation and pyroptosis during liver SCS. The decrease in oxidative stress and stress in the reticulum favor a decrease in DAMPS and, as a consequence, in PRR. Activation of inflammasomes and activation of NFκB, as well as caspases, are prevented. Finally, cleavage of the GSMD and the formation of membrane pores are prevented, avoiding pyroptosis.

Figure 1.

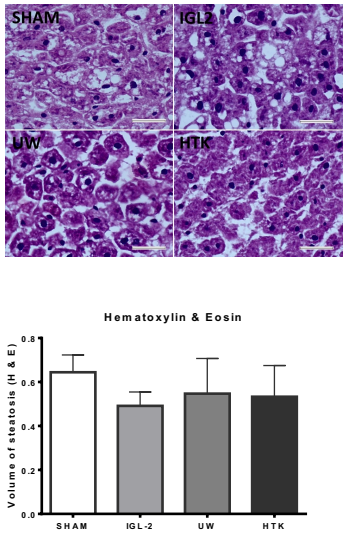


Figure 2.

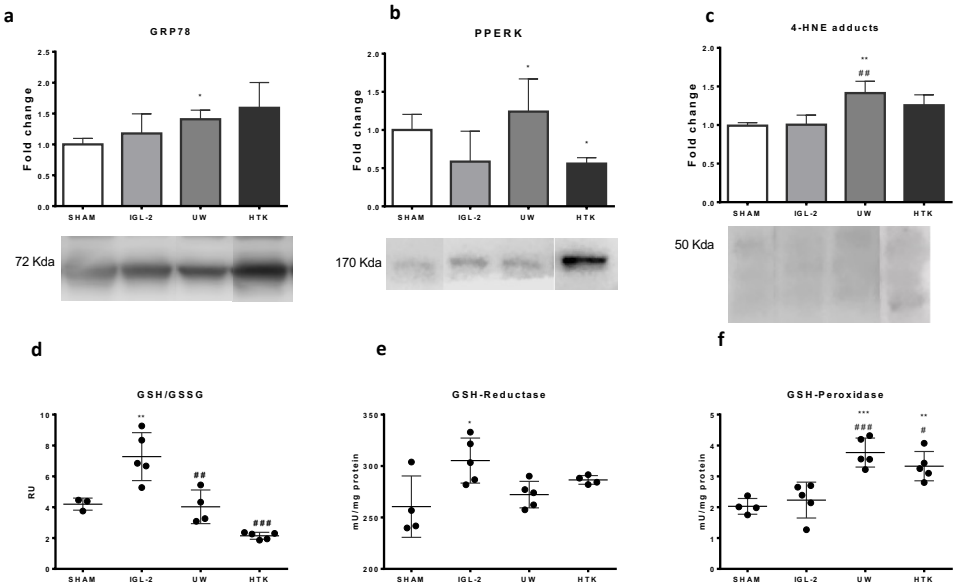


Figure 3.

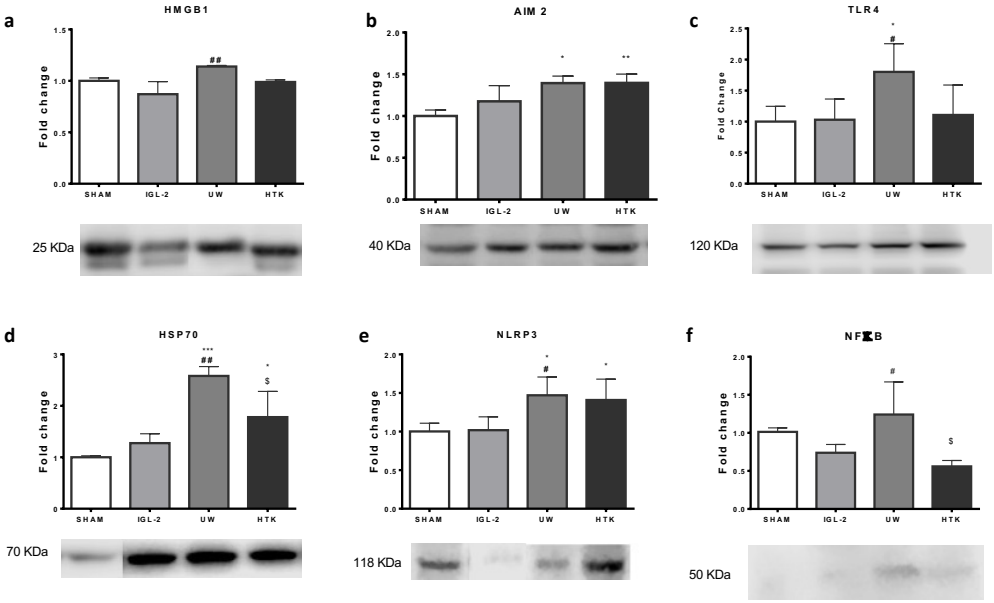
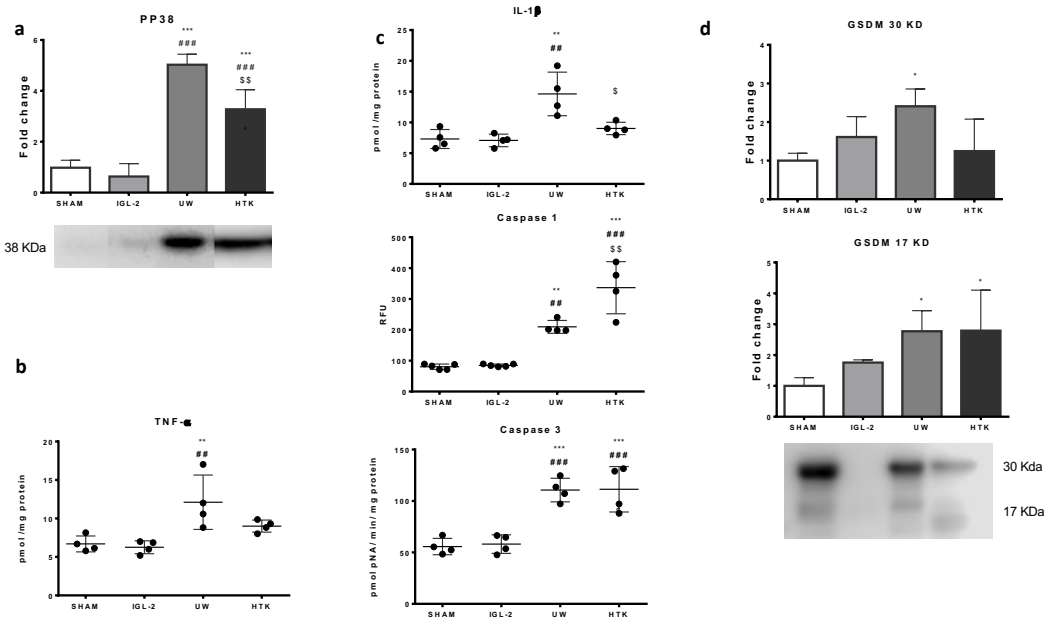
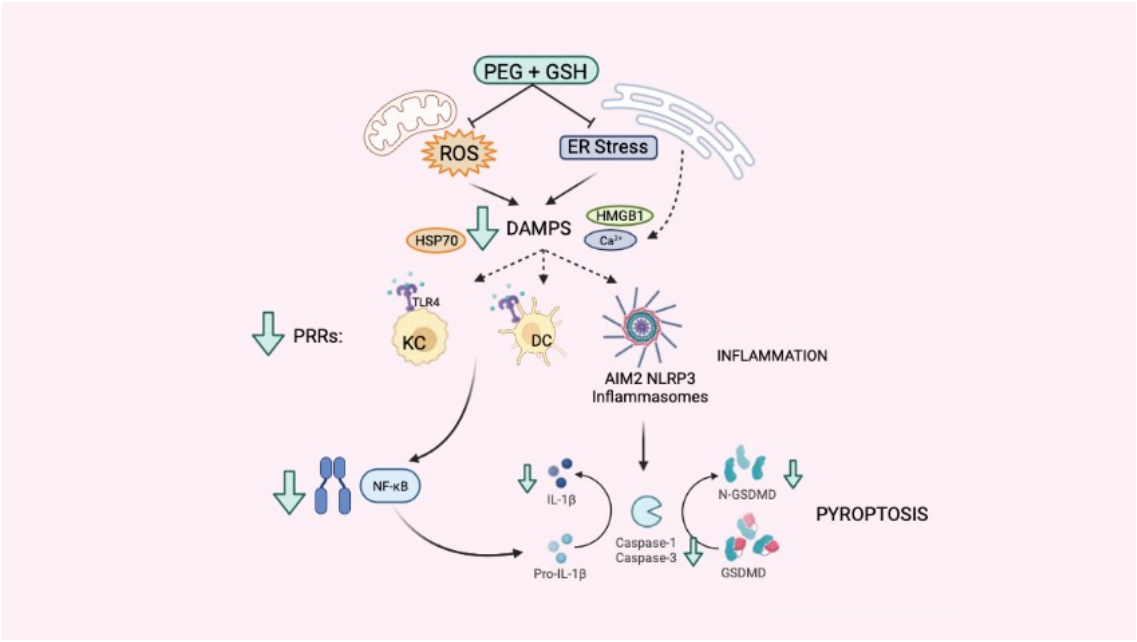


Figure 4.



Graphical Abstract.



CAPÍTULO V. IGL-2 as a Unique Solution for Cold Static Preservation Machine in Liver and Mitochondrial Protection

ELSEVIER

IGL-2 as a Unique Solution for Cold Static Preservation and Machine Perfusion in Liver and Mitochondrial Protection

Rui Teixeira Da Silva[a], Raquel G. Bardallo[b], Emma Folch-Puy[a], Teresa Carbonell[b], Carlos M. Palmeira[c], Constantino Fondevila[d], René Adam[e], Joan Roselló-Catafau[a], and Arnau Panisello-Roselló[a*]

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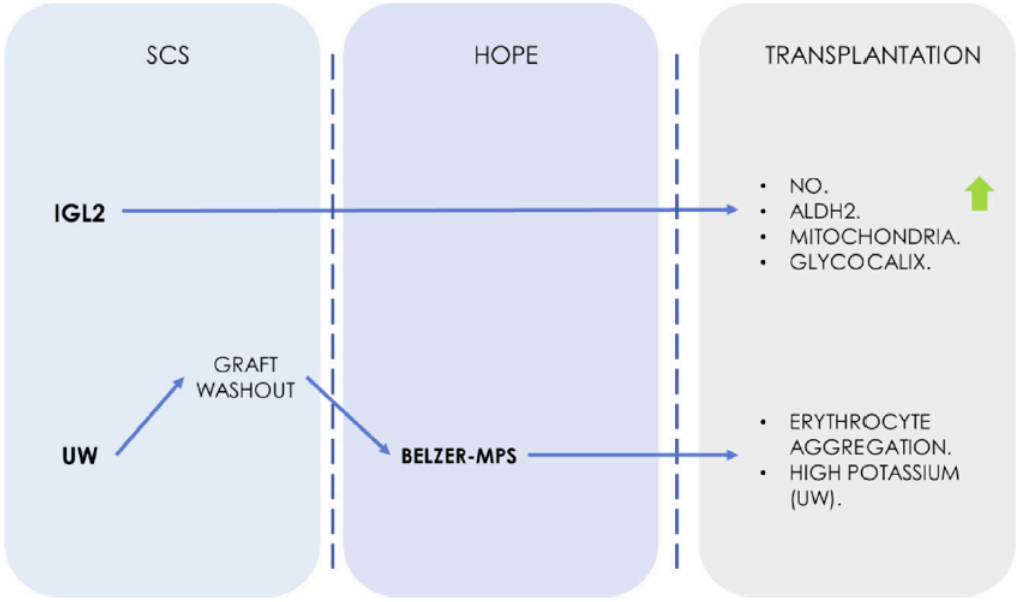
Graphical abstract:



Resumen:

En la clínica, la preservación estática en frio es el método más común de preservación para el trasplante de hígado gracias a su disponibilidad global y su sencilla aplicación. Además, en los últimos años se ha combinado esta estrategia con las máquinas de perfusión, que mejoran la viabilidad en la conservación del injerto hepático. Los aspectos de referencia para evaluar la viabilidad del órgano después de la reperfusión son la integridad mitocondrial y el mantenimiento energético de las células. Sin embargo, las complejas interacciones entre la protección mitocondrial del hígado y su relación con el uso de soluciones de perfusión han sido poco investigadas. En esta

revisión se propone el uso de la solución IGL-2 en la preservación estática del injerto hepático, así como alternativa a Belzer MPS en la perfusión oxigenada hipotérmica (HOPE), ya sea para su uso individual o combinado. La solución IGL-2, gracias a la presencia del agente oncótico PEG35, ha probado ser preservadora de la integridad mitocondrial, y se ha demostrado su efectividad en mecanismos protectores presentados en los anteriores capítulos, como es el incremento de la expresión de la enzima mitocondrial ALDH2. Además, su formulación con presencia de una incrementada concentración de GSH respecto a la solución Belzer-MPS, le confiere una alta capacidad antioxidante traducida en niveles inferiores de estrés oxidativo. Por tanto, su uso exclusivo permitiría evitar la combinación o modificación de las diferentes soluciones de preservación, tal y como se procede actualmente. Los retos de futuro en la conservación de injertos pasarán, por una parte, la mejora del estado mitocondrial y su estado energético durante la isquemia y, por otra parte, el desarrollo de estrategias para reducir los tiempos de isquemia a bajas temperaturas, lo que debería traducirse en un mejor resultado del trasplante.

IGL-2 as a Unique Solution for Cold Static Preservation and Machine Perfusion in Liver and Mitochondrial Protection

Rui Teixeira Da Silva[a], Raquel G. Bardallo[b], Emma Folch-Puy[a], Teresa Carbonell[b], Carlos M. Palmeira[c], Constantino Fondevila[d], René Adam[e], Joan Roselló-Catafau[a], and Arnau Panisello-Roselló[a*]

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ABSTRACT

Hypothermic static cold storage and machine perfusion strategies remain the clinical standard of care for liver graft preservation. Recently, the protection of the mitochondrial function and the energetic levels derived from it has emerged as one of the key points for organ preservation. However, the complex interactions between liver mitochondrial protection and its relation with the use of solutions/perfusates has been poorly investigated. The use of an alternative IGL-2 solution to Belzer MPS one for hypothermic oxygenated perfusion (HOPE), as well as in static cold storage, introduce a new kind of perfusate to be used for liver grafts subjected to HOPE strategies, either alone or in combination with hypothermic static preservation strategies. IGL-2 not only protected mitochondrial integrity, but also avoided the mixture of different solutions/perfusates reducing. Thus, the operational logistics and times prior to transplantation, a critical factor when suboptimal organs such as donation after circulatory death or steatotic ones, are used for transplantation. The future challenges in graft preservation will go through (1) the improvement of the mitochondrial status and its energetic status during the ischemia and (2) the development of strategies to reduce ischemic times at low temperatures, which should translate in a better transplantation outcome.

A.P.R. participated in the design, draft, revision, and approval of the work. The author agrees to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. R.T.d.S., R.G.B., and E.F.P. participated in the draft, revision, and approval of the work. The author agrees to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. T.C. and C.P. participated in the draft, revision, and approval of the work. The author agrees to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. C.F. participated in the draft, revision, and approval of the work. The author agrees to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. J.R.C. participated in the supervision, direction, revision, and approval of the work. The author agrees to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. R.A. participated in the supervision, direction, revision, and approval of the work. The author agrees to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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INTRODUCTION

TRADITIONALLY, liver graft preservation strategies have been based in static cold storage (SCS) [1,2], but more recently, a variety of dynamic perfusion techniques using machine perfusion (MP) strategies in normothermic and hypothermic conditions using the oxygenated perfusion techniques (hypothermic oxygenated perfusion [HOPE]) [2−9] resulted in a promising tool to rescue marginal livers, such as the ones presenting with steatosis for transplantation purposes [4,10,11].

Since the first investigations on hepatic perfusion carried out by Guarrera et al [3] in 2010, the interest has grown in the field of MP, both for normothermic hepatic perfusion [7,8] and for HOPE (with less operative complexity than normothermic perfusion) [3]. Recent advances have made it a very promising strategy to increase the donor pool in the face of the pressing shortage of organs for transplantation [4,10,11].

It is well known that the HOPE benefits are tied to the oxygenation of the perfusate, which is responsible of maintaining the integrity and function of mitochondrial machinery [12], and this applies either by using HOPE itself or in combination with SCS using a commercial preservation [13]. Recent investigations have shown that the emerging interest of mitochondrial protection during hypothermic graft preservation and its energetic status is growing [14]. In this sense, the induced HOPE protection mechanisms, defined recently by Schlegel et al [12], are associated with the sustaining of the mitochondrial state that contributes to: (1) the prevention of energy breakdown with the subsequent sustaining of intracellular ATP levels; (2) the prevention of damage-associated molecular pattern formation; and (3) the induction of underlying mechanisms related to mitochondrial repair and endothelial protection. However, the relevance of the interactions between mitochondrial graft protection and preservation solutions/effluents would need to be considered further.

In this context, the relevance of mitochondrial consequences of organ preservation techniques in organ transplantation should be specially considered in future organ hypothermic preservation strategies, especially when new solute/effluents could be a useful tool to increase mitochondrial protection for the liver graft [14].

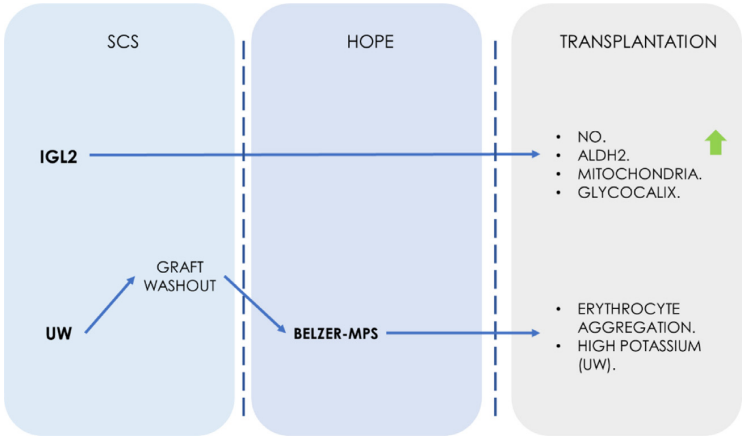
The perfusion solutions normally used for HOPE are a modification of Belzer's solution (Table 1) used for the static preservation of the graft [2,3], which on one hand contains hydroxyethyl starch (HES) as an oncotic agent, and on the other hand, shows a higher decreased K+ concentration than its analogue University of Wisconsin, among other components (Table 1). It is well reported that HES presence may lead to hyperaggregability of the red blood cells during static hypothermic preservation [15]. HES could also interfere with further HOPE strategies using Belzer MPS, where the presence of HES is responsible for increasing the viscosity of the perfusate during hypothermic perfusion vs IGL-2 (Table 1). This is especially relevant for steatotic liver grafts in which the fluid disturbances due to dynamic of fluids in HOPE [4] may destroy the luminal sugar thin layer covering liver endothelia, also known as glycocalyx [16,17]. However, the lower concentration of K+ in Belzer perfusate seems to be not relevant to affect to vascular resistance in hypothermic conditions given that it is well known that perfusates with physiologically low content prevented the vascular resistance increases when livers are subjected to cold perfusion [3,18].

Table 1. Composition of IGL-2 and Belzer MPS solutions

	Belzer-MPS	IGL-2
Electrolytes (mmol/L)		
K+	25	25
Na+	120	125
Mg2+	5	5
SO4 2-	5	5
Ca+		0.5
Zn2+		0.091
Buffers (mmol/L)		
Phosphate	25	25
HEPES		10
Histidine		30
Impermeants (mmol/L)		
Mannitol	30	60
Lactobionic acid		100
Dextrose	10	
Ribose	5	
Gluconate	85	
Colloids (g/L)		
Hydroxyethyl starch		50
Polyethylene glycol−35	5	
Antioxidants (mmol/L)		
Glutathione	3	9
Metabolic precursors (mmol/L)		
Adenosine		5
Adenine	5	
NaNO2 (nmol/L)		50
pH	7.4	7.4
Osmolarity (mosmol/L)	320	360
Viscosity (cP)	2.6	1.7

HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Recently, we have proposed the use of IGL-2 solution as a good alternative to Belzer MPS for HOPE strategies alone or combined with cold static preservation [19,20] (Table 1). The substitution of HES by polyethylene glycol 35 (PEG35); as well as the presence of glutathione (among other components) constitute the main difference between IGL-2 and Belzer MPS; glutathione content in IGL-2 (9 mM) is responsible for a higher antioxidant capacity compared with Belzer MPS (3 mM glutathione), which is translated as an enhanced protection against radical oxygen species formation and their potential damage against mitochondria in hypothermic static preservation followed by HOPE strategies [19−20], where the transient oxygenation sustains the liver mitochondrial machinery at basal levels. We compared Sprague Dawley rats' liver grafts subjected 1h HOPE after 7 hours SCS in both solutions (Belzer MPS and IGL-2)[20]. No significant differences in transaminases (alanine transaminase/aspartate transaminase) were found. However, significant lower levels of glutamate dehydrogenase (as a mitochondrial damage marker), were found in the IGL-2 rats' group vs Belzer MP, which were concomitant with



Scheme 1. IGL 2 Mechanisms of liver graft protection suggested for HOPE and hypothermic SCS preservation. The use of IGL-2 facilitates the logistics of using different solutions/perfusates besides favouring mitochondrial function, NO generation (vasodilation agent) and diminishing the disturbances associated with low viscosity that affect to endothelial glycocalyx.

higher levels of the mitochondrial enzyme aldehyde dehydrogenase 2 (ALDH2). These results are in agreement with the results of Schlegel et al [12], which confirm that the quality and protection of the mitochondria will greatly determine the capability of the graft to recover from ischemia-reperfusion injury insult, although further post-transplant studies are needed.

In addition, it is well known that in glutathione-based solutions, such as Belzer MPS and IGL-2, the presumed vs actual oxidation of glutathione over time is a key point that needs to be carefully overseen [21]. This is especially relevant when the hypothermic storage conditions of the preservation solution are not maintained properly and according to manufacturer instructions. To avoid the oxidation of the glutathione, additional factors such as high-quality package or the maintenance of the cold chain during transport are of utmost importance. However, these points are only valid if the initial quantity of glutathione is the optimal one, which is a differential point between original solutions and white brands. [22].

The use of IGL-2 in hypothermic preservation strategies also prevents the generation of aldehydes such as 4-hydroxynonenal through the activation of ALDH2 and its related protective mechanisms [13,19,20], contributing to HOPE benefits when PEG35 is used (Scheme 1). Scheme 1 summarizes the potential protective mechanisms of PEG35 solutions/perfusate in hypothermic static preservation [13] and HOPE [19,20] for liver transplantation purposes.

Scheme 1. IGL-2 mechanisms of liver graft protection suggested for HOPE and hypothermic SCS preservation. The use of IGL-2 facilitates the logistics of using different solutions/perfusates besides favoring mitochondrial function, nitric oxide generation (vasodilation agent), and diminishing the disturbances associated with low viscosity that affect to endothelial glycocalyx.

In accordance with the relevant investigations of Schlegel et al [12] and Horváth et al [14], we reported for the first time the benefits of using a novel IGL-2 solution for a combined use of SCS and HOPE strategies to rescue marginal livers, facilitating the logistics and avoiding the mixture of preservation solutions/perfusates for transplantation purposes. With this in mind, the use of a unique solution, such as IGL-2, for static and HOPE preservation strategies, could also be a useful tool in combination with "ex vivo" liver splitting and HOPE strategies, as recently reported by Mabrut et al [23].

In conclusion, the actual strategies used in liver graft hypothermic preservation suggest that the use of and unique preservation solution for the protection of mitochondrial functions should be considered as a priority in the actual studies of liver preservation solutions [24]. Future investigations on the mitochondrial protection induced by polyethylene glycols need to be explored in depth.

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CAPÍTULO VI. Liver Graft Hypothermic Static and Oxygenated Perfusion (HOPE) Strategies: A Mitochondrial Crossroads

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Review

Liver Graft Hypothermic Static and Oxygenated Perfusion (HOPE) Strategies: A Mitochondrial Crossroads

Raquel G. Bardallo [1,†], Rui T. da Silva [2,3,†], Teresa Carbonell [1], Carlos Palmeira [2], Emma Folch-Puy [3], Joan Roselló-Catafau [3], René Adam [4] and Arnau Panisello-Rosello [3,4,*]

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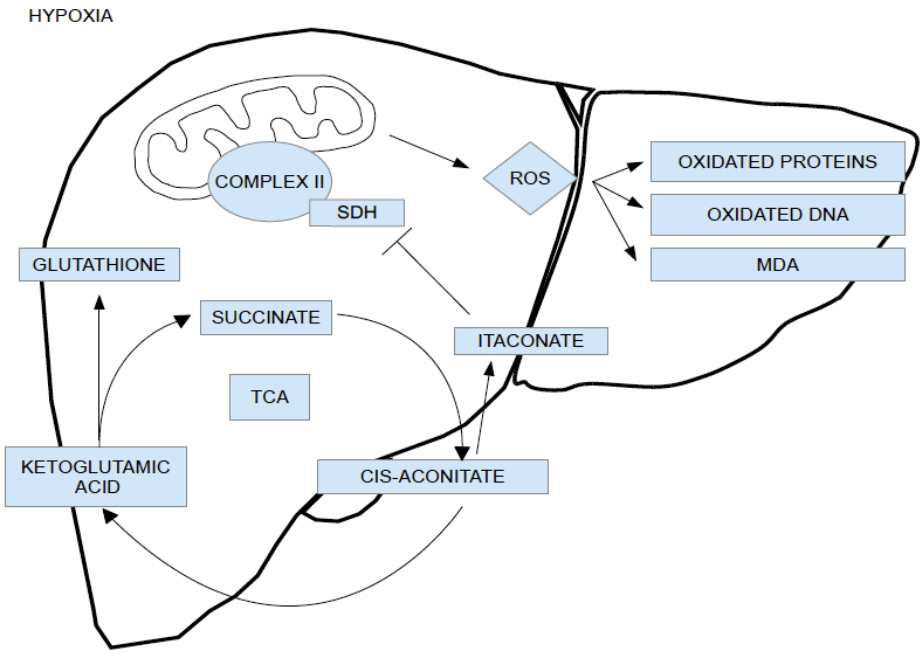
Graphical abstract:



Resumen:

En los capítulos anteriores se ha descrito como las soluciones de preservación en la conservación estática en frío del hígado graso pueden ser esenciales para la mejora de la viabilidad de los injertos hepáticos. Varios autores han descrito que los injertos

hepáticos marginales, como los hígados esteatósicos y aquellos de donantes con muerte cardiaca, son altamente vulnerables al daño por isquemia/reperfusión, producida desde la obtención del órgano hasta su revascularización. Además de la preservación estática en frío, descrita en los capítulos anteriores, recientemente se han desarrollado diversos métodos de preservación para preservar los injertos hepáticos basados en estrategias de preservación estática hipotérmica y perfusión oxigenada hipotérmica (HOPE), ya sea combinadas o solas. Sin embargo, sus efectos sobre las funciones mitocondriales y su relevancia en el uso clínico no han sido íntegramente descritos.

Durante el almacenaje en frio del órgano para el trasplante, el daño por isquemia se basa en la privación de oxígeno al injerto, lo cual provoca alteraciones en la integridad y función mitocondrial y ruptura del metabolismo energético. En esta revisión, se trata la relevancia de la función mitocondrial en la conservación estática en frío, así como la acumulación de succinato en las etapas finales de la isquemia fría y su modulación por parte de las diferentes soluciones de preservación, como son IGL-2, HTK y UW. En base a los resultados obtenidos en los capítulos anteriores, en que la preservación estática en frío en el medio de preservación IGL-2 ha probado ser clave para mejorar la funcionalidad mitocondrial y el mantenimiento energético, se propone extender la aplicación del IGL-2 durante la preservación HOPE en lugar de la solución Belzer MP, comúnmente utilizada. La oxigenación transitoria en HOPE sostiene la maquinaria mitocondrial a niveles basales e impide, en parte, la acumulación de metabolitos energéticos como el succinato, a diferencia de los que se producen en condiciones de conservación estáticas en frío.

Por otro lado, se presentan y discuten varios posibles aditivos aplicables a las soluciones de preservación para combatir la privación de oxígeno y la descomposición del metabolismo energético del injerto durante la preservación estática hipotérmica, como transportadores de oxígeno, ozono, los inductores de AMPK y los inhibidores mitocondriales de UCP2, y si se combinan o no con HOPE.

Finalmente, en esta revisión se reafirma que la solución IGL-2 es potencialmente ventajosa en la protección de la maquinaria mitocondrial del injerto, de forma que se simplificaría la logística en el trasplante clínico y se podría aplicar su uso tanto en la preservación clásica estática como en su combinación con estrategias más innovadoras, como es el caso de HOPE. El objetivo final no es otro que optimizar la utilización de los hígados marginales para aumentar la reserva de órganos viables para el trasplante y, por lo tanto, acortar las listas de espera de pacientes que esperan un órgano.

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Review

Liver Graft Hypothermic Static and Oxygenated Perfusion (HOPE) Strategies: A Mitochondrial Crossroads

Raquel G. Bardallo [1,†], Rui T. da Silva [2,3,†], Teresa Carbonell [1], Carlos Palmeira [2], Emma Folch-Puy [3], Joan Roselló-Catafau [3], René Adam [4] and Arnau Panisello-Rosello [3,4,*]

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Abstract: Marginal liver grafts, such as steatotic livers and those from cardiac death donors, are highly vulnerable to ischemia–reperfusion injury that occurs in the complex route of the graft from "harvest to revascularization". Recently, several preservation methods have been developed to preserve liver grafts based on hypothermic static preservation and hypothermic oxygenated perfusion (HOPE) strategies, either combined or alone. However, their effects on mitochondrial functions and their relevance have not yet been fully investigated, especially if different preservation solutions/effluents are used. Ischemic liver graft damage is caused by oxygen deprivation conditions during cold storage that provoke alterations in mitochondrial integrity and function and energy metabolism breakdown. This review deals with the relevance of mitochondrial machinery in cold static preservation and how the mitochondrial respiration function through the accumulation of succinate at the end of cold ischemia is modulated by different preservation solutions such as IGL-2, HTK, and UW (gold-standard reference). IGL-2 increases mitochondrial integrity and function (ALDH2) when compared to UW and HTK. This mitochondrial protection by IGL-2 also extends to protective HOPE strategies when used as an effluent instead of Belzer MP. The transient oxygenation in HOPE sustains the mitochondrial machinery at basal levels and prevents, in part, the accumulation of energy metabolites such as succinate in contrast to those that occur in cold static preservation conditions. Additionally, several additives for combating oxygen deprivation and graft energy metabolism breakdown during hypothermic static preservation such as oxygen carriers, ozone, AMPK inducers, and mitochondrial UCP2 inhibitors, and whether they are or not to be combined with HOPE, are presented and discussed. Finally, we affirm that IGL-2 solution is suitable for protecting graft mitochondrial machinery and simplifying the complex logistics in clinical transplantation where traditional (static preservation) and innovative (HOPE) strategies may be combined. New mitochondrial markers are presented and discussed. The final goal is to take advantage of marginal livers to increase the pool of suitable organs and thereby shorten patient waiting lists at transplantation clinics.

Keywords: liver graft preservation; AMPK; succinate; ALDH2; glycocalyx

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1. Introduction

In liver transplantation, graft quality is the key factor that determines procedural success and long-term survival and outcome, but this depends not only on its intrinsic performance but also on the time and quality of preservation and transportation from donor to recipient implantation. At Christmas in 1971, Belzer decided to ship a recovered

kidney in San Francisco (USA) to the Leiden transport for transplantation. After 37 h of in-flight preservation, the kidney was successfully transplanted into a 42-year-old truck driver with polycystic kidney disease, as planned [1,2].

Since the first investigations initiated by Belzer et al. [1,3–7], much progress has been made in the preservation of liver grafts [8–10]. The actual challenge in liver transplantation is organ scarcity and the pressing need for liver transplantation, which have led physicians to use marginal livers, such as steatotic livers, to increase the organ pool for transplantation [11–14]; however, its high vulnerability against ischemia and reperfusion injury [14–16] due to microcirculation exacerbated by fat accumulation in hepatic sinusoids could compromise graft viability and further outcomes with post-transplantation problems [17–19].

Cold static preservation is a mandatory step characterized by oxygen deprivation to the graft that provokes ischemic damage, leading to mitochondrial integrity changes and energy metabolism breakdown alterations that affect fatty liver graft integrity during cold storage in experimental settings. Fatty livers exhibit reduced tolerance against ischemic events, with further reduced ATP levels and greater injury levels when compared to nonsteatotic livers [20,21]. However, the cold ischemic time accepted for clinical purposes is 12 h [22,23], considered suitable against the apparition of "primary nonfunction", graft failure, and patient death, along with reduced long-term graft survival [22].

The mechanism of mitochondrial metabolic changes upon cold ischemia is relatively well known [24], as shown in Figure 1. During prolonged cold graft storage, the oxygen deprivation conditions allow diminishing the metabolic demands, and consequently, the mitochondrial respiration complexes are seriously altered, leading to succinate accumulation at the end of ischemia [24]. Succinate dehydrogenase (SDH) activity drives a significant portion of ROS generation, and it has been demonstrated that SDH inhibition by malonate is protective against reperfusion injuries. Notably, itaconate inhibits SDH in a dose-dependent manner, leading to succinate accumulation [25,26], and its exogenous administration modifies the host response to ischemia–reperfusion that is sufficient to suppress reperfusion-related injuries [24–28].

The accumulation of succinate is partly responsible for controlling graft injury after oxygenation [24–28], and consequently, the ability of itaconate to inhibit succinate dehydrogenase in complex II may also play a determining role, reducing the initial succinate metabolism after earlier oxygenation before reperfusion conditions (Figure 1) [24–28]. Moreover, the longer the ischemic period lasts, such as during liver graft cold storage, the more compromised the mitochondrial antioxidant electron transport chain (ETC) system, depleting substrates such as glutathione, which render the cells more susceptible to oxidative stress at reperfusion after graft revascularization.

With this in mind, it seems necessary to envisage new directions in graft cold static preservation strategies [8,29], possibly in combination with newer techniques developed, such as hypothermic oxygenated perfusion (HOPE) using machine devices [30,31], for better protection from harvest to revascularization. New protective strategies to increase graft protection are urgently needed to take advantage of marginal grafts and increase the donor pool to reduce waiting lists for liver transplantation. This review covers different aspects of hypothermic liver graft preservation as follows: cold ischemic insult and liver graft cold storage are discussed in Section 2; mitochondrial protection and organ preservation solutions in static cold storage are discussed in Section 3; new additives for improving cold static preservation are discussed in Section 4; HOPE, mitochondrial protection, glycocalyx preservation, and PEG35 effluents (IGL-2) are discussed in Section 5; and (5) some considerations and concluding remarks are presented in Section 6.

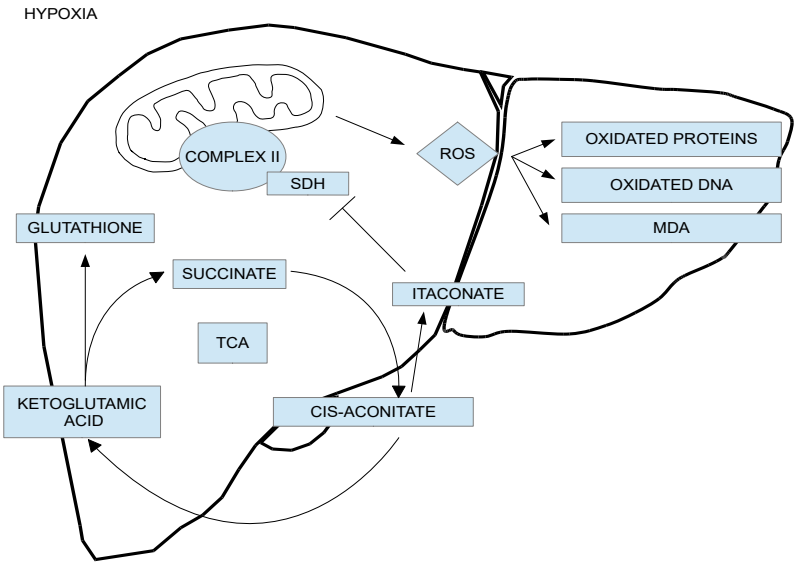

Figure 1. Intracellular mechanisms of ischemic injury. The lack of oxygen to the preserved graft during cold storage provokes a mitochondrial switch to anaerobic metabolism with the interruption of electron flow and mitochondria machinery, the accumulation of interacting energy metabolites succinate and itaconate, and rapid adenosine-triphosphate breakdown. These events that occur during graft cold preservation are modulated by organ preservation solutions. When oxygen is reintroduced under hypothermic oxygenated perfusion and/or with further normothermic conditions, mitochondria re-establish the electron flow with rapid and suitable consumption of the accumulation of succinate at the end of cold ischemia. Subsequent release of reactive oxygen species from complex I occurs. Mitochondria and energy breakdown appear, therefore, as the main targets not only to improve graft quality but also to identify a valid biomarker to predict the "graft status" after static cold storage in organ preservation solutions just before transplantation procedures.

2. Cold Ischemic Insult and Liver Graft Cold Storage

Crucial adverse consequences for liver graft during cold storage due to deprived oxygen conditions have led us to use organ preservation solutions such as UW, HTK, Celsior, and IGL-1 [8,29]. UW and IGL-1 solutions are characterized by an oncotic agent in their composition such as hydroxyethyl starch (HES) in UW and PEG35 in IGL-1 [8,29], in contrast to HTK and Celsior, which do not contain an oncotic agent in their formulations [8]. Moreover, IGL-2 showed higher concentrations of PEG35 and glutathione compared to IGL-1. In clinical transplantation, IGL-1, Celsior, and HTK were demonstrated to be suitable alternatives to UW (gold-standard reference), although some limitations for HTK were recently described in the European Liver Transplantation Register (ELTR) [32].

Since 2006, we have investigated the protective mechanisms exerted by polyethylene glycol 35 (PEG35) in IGL-1 solution, which was found suitable for fatty liver graft preservation [33]. In this line, the presence of PEG35 in IGL-1 [33] and rinse solution [34] was a determinant for protecting the graft against the deleterious effects accumulated from organ recovery and washout up to graft cold storage and the following reperfusion [33,34]. This confirms that PEG35 is a useful tool for preventing cold IRI associated with transplantation [35]. The benefits of PEG35 in rinse and IGL-1 solutions are mainly associated with the prevention of mitochondrial damage, activated protective cell signaling mechanisms such as AMPK (energy sensor), and nitric oxide generation through constitutive eNOS activation [33,34,36,37].

With this in mind, we recently proposed IGL-2 [38] in order to enhance fatty liver graft protection by preserving liver damage with mitochondrial integrity inherent in oxygen de-

privation conditions during static cold graft storage. As shown in Figure 2, IGL-2 protected liver injury more efficiently (measured as AST/ALT transaminases) and mitochondrial damage (measured as GLDH) than UW and HTK solutions.

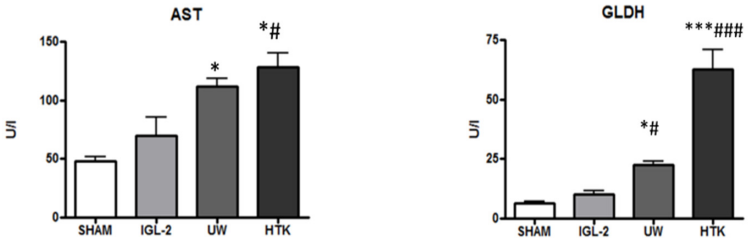

Figure 2. Liver injury and mitochondrial integrityof fatty liver graft preserved in IGL-2, UW, and HTK solutions (24 h; 4 °C). The bars represent the mean values ± SEM of each group (n = 4–6). Differences are shown comparing groups (* vs. Sham, # vs. IGL-2) according to the one-way ANOVA test and the Tukey post-hoc test (one symbol indicates $p < 0.05$; three symbols indicate $p < 0.001$).

3. Mitochondrial Protection and Preservation Solutions in Static Cold Storage

ATP energy metabolism breakdown is inherent to oxygen deprivation conditions during graft cold storage. As shown in Figure 3, noticeable prevention of ATP breakdown after 24 h cold storage occurred when IGL-2 was compared to UW and HTK.

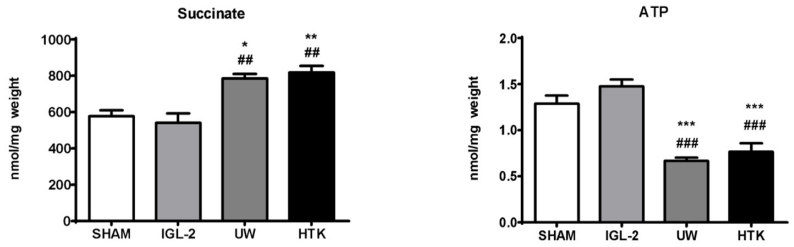

Figure 3. Succinate accumulation and ATP breakdown in fatty livers preserved in IGL-2, UW, and HTK solutions (24 h; 4 °C). The bars represent the mean values ± SEM of each group (n = 4–6). Differences are shown comparing groups (* vs. Sham, # vs. IGL-2) according to the one-way ANOVA test and the Tukey post-hoc test (one symbol indicates $p < 0.05$; two symbols indicate $p < 0.01$; three symbols indicate $p < 0.001$).

Moreover, ATP energy metabolism breakdown is associated with alterations in mitochondrial respiration complexes that occur as a consequence of oxygen deprivation, forcing the cell to adapt to anaerobic metabolism that leads to the accumulation of metabolites such as succinate (Figure 1), which will partly control graft damage when oxygen restoration occurs after reperfusion [24]. As shown in Figure 3, IGL-2 solution was capable of limiting succinate accumulation in a more efficient way compared to UW and HTK. The presence of PEG35 and glutathione in IGL-2 contributed to modulating succinate accumulation at the end of cold ischemia. Other metabolites such as itaconate, which accumulated at the same time, may partially contribute to inhibiting succinate dehydrogenase (SDH) during earlier stages of reperfusion [26–28]. Consequently, both succinate and itaconate levels accumulated at the end of the cold storage period will have an important role in determining the viability of the graft after revascularization.

It is well known that fatty livers show reduced tolerance to ischemic events with further reduced ATP levels and a greater injury level compared to nonsteatotic livers [21]. It has been discussed that mitochondrial uncoupling protein-2 (UCP-2), highly expressed in steatotic hepatocytes, may be responsible for the aforementioned higher fatty liver

sensitivity to ischemia [39]. UCP-2 acts as an inner-mitochondrial membrane proton carrier that uncouples ATP synthesis, facilitating proton leakage into the mitochondrial matrix and uncoupling mitochondrial respiration. Increased UCP-2 expression was associated with low ATP levels in hearts preserved in Celsior solution after static hypothermic preservation [40]. This would suggest that UCP-2 has a relevant role between mitochondrial machinery function and ATP preservation during cold graft storage [40]. The use of genipin, a UCP-2 inhibitor added to Celsior solution, would contribute to limiting ATP depletion and protecting fatty livers against cold ischemic insult during IRI [40]. UCP inhibitors, such as 2,4-dinitrophenol and others, have been proposed, but no clinical applications have been carried out [41,42].

Mitochondrial aldehyde dehydrogenase-2 (ALDH2) is best known for its critical detoxifying role in liver alcohol metabolism, but there is growing evidence that it also plays a role in IRI through the prevention of oxidative stress processes (oxygen free radicals and toxic 4-hydroxy-nonenal generation) [20,43]. PEG35 in rinse [34] and IGL-1/IGL-2 solutions [33,44] was a determinant for increasing mitochondrial protection and function, as well as preventing lipid peroxidation when compared to identical solutions with or without PEG35 [44]. The high antioxidant capacity of IGL-2 was corroborated by MDA and AOPP when compared to livers preserved in UW and HTK (Figure 4).

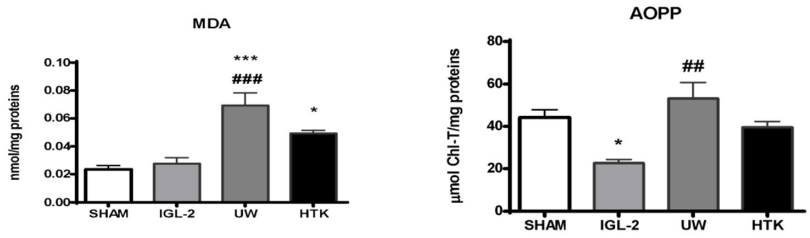

Figure 4. Lipid peroxidation (measured as MDA levels) and oxidized proteins (AOPP) in liver grafts in IGL-2, UW, and HTK solutions (24 h; 4 °C). The bars represent the mean values ± SEM of each group (n = 4–6). Differences are shown comparing groups (* vs. Sham, # vs. IGL-2) according to the one-way ANOVA test and the Tukey post-hoc test (one symbol indicates $p < 0.05$; two symbols indicate $p < 0.01$; three symbols indicate $p < 0.001$).

Although oxygen levels of the media embedding the organ (preservation solution) are very low, there is still an impairment of mitochondrial oxidative phosphorylation, leading to the generation of reactive oxygen species (ROS). In this sense, NO generation in IGL-1/IGL-2 solutions [33,38] could act, among many other functions, as an ROS scavenger in the lipoperoxidation process [44]. Interestingly, these involved cytoprotective mechanisms observed for PEG35 in IGL-1 and IGL-2 solutions were similar to those we proposed for liver ischemic preconditioning strategies in a rat model, where AMPK and NO are also well-known liver preconditioning cytoprotective factors [36,37].

With this in mind, we consider that preservation solutions can be considered preconditioning tools against IRI. This NO generation and its vasodilator properties are especially beneficial for steatotic liver grafts, in which fat accumulation in the sinusoidal space exacerbates microcirculation alterations [16,45].

4. New Additives for Improving Cold Static Preservation: Oxygen Carriers, Ozone, and AMPK Inducers

4.1. Oxygen Carriers: M-101

Static hypothermic preservation of liver grafts static hypothermic preservation implies a lack of oxygen, and its adverse consequences affect energy metabolism, blocking mitochondrial respiration, concluding with succinate accumulation. Under these circumstances, the use of a natural extracellular oxygen carrier added to the preservation solution could

help maintain the functionality of certain metabolic pathways, hence avoiding the nocive accumulation of succinate.

M-101 is a natural giant extracellular hemoglobin (Hb) extracted from the marine invertebrate *Arenicola marina* that was first used by Alix et al. as an additive to UW solution in pig liver transplantation [46]. The authors demonstrated that UW solution enriched with M-101 improved liver graft oxygenation when compared to simple UW solutions, but it did not reach the oxygenation level achieved with alternative dynamic hypothermic oxygenated machine perfusion (HOPE) strategies (with active flux of oxygen) [46]. This transient restoration of oxygenation achieved with M101 presumably contributes to maintaining liver mitochondrial machinery to function at basal levels, thus avoiding the accumulation of succinate during cold ischemia, according to Schlegel's mechanism [25]. Furthermore, the M-101 additive is also useful for fatty liver static preservation when combined with HOPE [47], but additional studies should investigate in depth its usefulness in maintaining graft quality when longer cold storage periods are applied, especially in vulnerable organs (DCD donors, pancreas, and fatty liver grafts).

4.2. Ozone

Ozone is a gas with antioxidant effects [48,49] that has been proposed as an additive in UW solution (gold-standard reference) to minimize cold ischemic damage during graft preservation in UW solution [50]. Ozone protection during cold storage modulates the XHD/XOD system, preserving adenosine storage and blocking the xanthine/xanthine oxidase pathway that promotes ROS generation [51]. Taking this into account, ozone persufflation strategies should be considered and further evaluated as a potential tool for hypothermic graft preservation strategies.

The deleterious oxidation consequences of oxygen generated by ozone conversion to oxygen during cold storage would be presumably even more negligible when glutathione and allopurinol are present in the composition of UW and IGL-2 solutions. Presumably, the persufflation of the liver graft before cold graft storage in ozonized UW solution could offer additional protection to preserve the liver graft.

4.3. Other Antioxidants

It is well known that glutathione is present in its reduced form and is a critical component often used in commercial UW and IGL-1 [8]. Reduced glutathione is a very labile component, and its auto-oxidation is accelerated by an increase in temperature and contact with oxygen [52]. With this in mind, we assume that long periods of storage at room temperature are not recommended for storing any preservation solutions containing glutathione that could affect the initial glutathione composition. [53]. This was one reason why we proposed increasing the content of glutathione in IGL-2 solution [44] vs. UW and IGL-1 [33]

In addition, well-known hydrosoluble antioxidants (including vitamin C, N-acetyl cysteine (NAC), alpha-tocopherol analog, and others) were used as additives to preservation solutions to reinforce their respective antioxidant features [54–57]. Further investigations are needed to explore new antioxidants for graft preservation purposes.

4.4. Adenosine Monophosphate Protein Kinase (AMPK) Inducers

Graft oxygenation is an alternative to solve the hypoxic conditions during cold storage; however, other studies focus on another of the main problems occurring during ischemia, such as the breakdown of energy metabolism and the dropping of ATP levels, which can be countered by the activation of the AMPK energy sensor [58–60]. In this line, AMPK inductors, such as 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) or metformin, have evidenced their benefits when added to UW solutions [61,62]. Other natural AMPK activators, such as 3,5,4′-trihydroxy-*trans*-stilbene (resveratrol) [63], could be good candidates as additives for preservation solutions in future approaches. This principle also applies to PEG35 solutions, in which there has been an increase in activated AMPK under

hypoxic conditions [64–66]. The mechanisms by which PEG35 activates AMPK in IGL-1 and rinse solutions remain to be investigated in depth.

5. HOPE, Mitochondrial and Glycocalyx Protection, and PEG35 Effluents (IGL-2)

The introduction of dynamic strategies by using machine devices for graft conservation, such as hypothermic oxygenated perfusion (HOPE), is a promising tool to not only increase graft quality for transplantation [67–72] but also to make useful marginal organs that were originally discarded for transplantation [31].

The use of transient and sustained oxygenation to the graft is the "key" that maintains the operational mitochondrial machinery at basal levels during HOPE, whose protective mechanisms were described by Schlegel and Cols [25]. Transient graft oxygenation during HOPE activates the mitochondrial machinery through mitochondrial complexes I and II, preventing preservation. Therefore, the importance of succinate metabolism by complex II/SDH during HOPE was shown to slowly re-establish a normal directed electron flow without significant ROS release, thereby slowing the breakdown of accumulated succinate (Figure 1) [24–27].

The mechanisms proposed by Schlegel et al. [25] also apply to static preservation, but in this case, the benefits of decreasing the accumulation of succinate can only be altered by preservation solutions, which can do so to a very limited extent. This is the case of IGL-2 solution, which better prevented succinate accumulation when compared to HTK and UW, the gold-standard reference (Figure 3), bringing the graft to more suitable conditions for the subsequent HOPE application. In clinical and experimental settings, the most used perfusate used in HOPE is modified UW solution, named Belzer MP, adapted for dynamic perfusion and maintaining HES as an oncotic agent in its formulation [25,67]. It is well known that HES can induce hyperaggregability of red blood cells [73], this being a limitation for graft washout and cold storage. This serious issue is not present with other oncotic agents such as PEG35 [74], which also confers more mitochondrial protection than HES.

Fluid dynamics constitutes one of the most differential features when HOPE and cold static preservation are compared. In any kind of dynamic perfusion, the perfusate applies pressure and shear stress that may involve the destruction of the superficial sugar thin layer covering the endothelium, known as "glycocalyx" [75,76]. The presence of PEG35 in perfusates (IGL-2 solution) contributes to favoring mechanotransduction processes inherent to fluid dynamics in HOPE, thereby also favoring better protection of the luminal glycocalyx [77]. Perfusates in a dynamic system, such as HOPE, can promote glycocalyx destruction, but this will greatly depend on factors such as viscosity, pressure, and other properties of the perfusate, which in turn depend on the components of the preservation solution, such as the oncotic agent, whose characteristics can be delimited by factors such as temperature [77].

Notably, PEG35 in IGL-2 lowers the viscosity in Belzer MPS currently used in HOPE by half [67]. The properties of PEG35 in effluents for HOPE and its effects in the organ justify a deep debate to change the paradigm on which is the better approach for dynamic preservation considering the aforementioned factors (high mitochondrial protection, diminished viscosity, no red blood cell hyperaggregability) that have been overlooked since the beginning of dynamic perfusion. Furthermore, PEG35 seems to be a more efficient agent for the protection of mitochondrial machinery and should be considered as a main strategy for hypothermic graft conservation in the complex route of the liver graft from "harvest to revascularization".

Recently, aldehyde dehydrogenase-2 has been described as a regulator of mitochondrial machinery during hypothermic machine perfusion to mitigate the deleterious effects of renal ischemia–reperfusion [78]. ALDH2 plays an important cytoprotective role in HMP, reducing the accumulation of 4-HNE and regulating the Akt/mTOR autophagy pathway in a similar way as in cold static preservation [79]. In addition, our previous observations in HOPE studies are consistent with better mitochondrial protection and increased ALDH2

in livers preserved for 7 h in IGL-2 after 1 h in HOPE and then 1 h of reperfusion with previous HOPE [67].

Taking this into account, mitochondrial GLDH and ALDH2 could be considered good indicators in HOPE to evaluate graft liver mitochondrial integrity and mitochondrial machinery function. This could also be presumably extended to cold static preservation enriched with oxygen inductors such as M-101, which could be combined with HOPE, as suggested by Alix et al. [46].

6. Some Considerations and Concluding Remarks

The challenge for the next decade is to develop technical strategies to rescue marginal organs. To do so, part of the effort should be aimed at mitochondrial protection and, therefore, at maintaining the mitochondrial machinery at basal levels to prevent energy breakdown and the subsequent generation of toxic metabolites, such as 4HNE. Certainly, HOPE is more efficient than static preservation because it provides the cell with an environment closer to physiological conditions, hence triggering less stress and coping mechanisms that inevitably lead to cell death. The continuous supply of oxygen, nutrients, and metabolic substrates for ATP generation and assuring the maintenance of mitochondrial machinery, which includes regulatory enzymes such as ALDH2, will in turn be proactive in the upregulation of other cytoprotective factors.

In HOPE, as mentioned above, one of the most common tools used is Belzer MP solution, containing an oncotic agent (HES) that provokes red blood cell aggregation [73], which can be fatal, and has some nonoptimal physical properties (high viscosity) that have been considered as a major factor of impact on shear stress occurring in the dynamic fluids system.

In this line, the use of PEG35 would be "killing two birds with one stone", avoiding red blood cell hyperaggregation and bringing the solution to more adequate parameters of viscosity in order not to damage the endothelial cells and their associated structures, such as glycocalyx, its application in the narrowed sinusoids of steatotic liver being especially interesting [16,45]. Furthermore, these benefits are not only applied at the physical level, as the use of IGL-2 confers higher mitochondrial protection and assures better conditions for the ALDH2 enzymatic function to prevent oxidative stress in hypothermic graft preservation [43,44].

The MP benefits of ALDH2 extend further, as demonstrated by Lin et al. [78], who evidenced that ALDH2 regulated autophagy via the Akt/mTOR pathway to mitigate renal ischemia–reperfusion injury and can activate the negative regulatory mammalian target of rapamycin (mTOR) pathway. We established similar protective autophagic mechanisms when IGL-2 and IGL-1 solutions were used [38,79]. Moreover, the fluid dynamics existing in HOPE may have adverse consequences due to the viscosity of the effluent generally used, Belzer MPS, leading to endothelial glycocalyx destruction and thus altering the induced transduction mechanisms involved in HOPE when compared to PEG35 solutions [77,80].

In this sense, the use of IGL-2/PEG35 solution would be a better alternative than Belzer MP/HES by two means: (1) protecting the glycocalyx due to reduced viscosity that produces less shear stress, which is especially relevant in narrowed sinusoids and peripheral microcirculation [77], and (2) as a consequence of a less damaged glycocalyx, mechanotransduction mechanisms are more preserved, promoting NO generation (with vasodilator properties), being, again, beneficial in narrowed sinusoids [45,77].

The scarcity of organs and the growing demand for liver transplantation have led to hospitals using marginal grafts for organ transplantation. For years, static preservation has been based on the principles of hypothermic and static storage; however, the limitation of oxygen supply is serious for graft survival, given that the conditions proposed for static preservation are far from physiological. The use of HOPE is very promising [67–72], as well as gas additives or hemoglobins such as M-101 that, combined with further HOPE, could be an alternative method to palliate the deficient oxygenation of the graft during static preservation conditions [46,47].

Further investigations should be carried out on the usefulness of oxygen carriers, such as M101, for transporting organs in countries with complex logistic operations that extend the static cold storage periods, since currently, the dynamic systems known to provide oxygen in a dynamic system (HMP/NMP) are not sufficiently developed/cheap enough to be applied at a real clinical level.

With all this in mind, we assume that new promising predictors may evaluate graft quality at the end of the cold storage/cold ischemia step such as glutamate dehydrogenase (mitochondrial damage), ALDH2 (mitochondrial function), succinate and itaconate accumulations, and flavin mononucleotide (FMN); in contrast to HOPE, succinate and itaconate metabolites have a predictive value at early oxygenation/reperfusion times, including FMN [24–27]. Moreover, we could logically assume that the use of a unique solution, such as IGL-2 [81], could be an efficient and interesting tool to protect graft mitochondrial machinery and avoid the cumulative damage induced by the mandatory complex processes before transplantation, including organ recovery and its earlier washout, subsequent graft cold storage, and additional washout to finally proceed with HOPE strategies [25,67–72]. Certainly, the suitable use of a unique solution such as IGL-2 would simplify the logistics of liver transplantation, including also the combination of split liver transplantation with HOPE [82,83].

Finally, it is clear that new markers are needed to assess graft quality. In a recent editorial, Schegel et al. [84] asked whether the current combination of biomarkers used in liver transplantation could be useful in predicting liver function or whether we are only measuring actual lesions. In this sense, the accumulation of energy metabolites such as succinate and itaconate metabolites at the end of ischemia, together with GLDH and mitochondrial ALDH2 and UCP2, including energy sensors such as activated AMPK, could be promising predictors for the evaluation of the graft at the end of cold storage prior to reoxygenation, a phase in which the role of those metabolites is unleashed and in which the harm of ROS is a major issue.

Summary

Mitochondrial preservation should be a prioritary direction for better conserving the graft when static and HOPE strategies are used in combination or alone. Promising markers such as GLHD, mitochondrial ALDH2, and accumulating succinate, itaconate, and flavin mononucleotide should be considered for future investigations in clinical transplantation. Glycocalyx and nitric oxide are altered in static preservation [80], but their use as predictors could be even more appropriate to HOPE strategies for evaluating the adverse effects of effluent dynamics and transduction mechanisms for future HOPE strategies, given the relevance of measuring glycocalyx alterations in clinical transplantation [85–88]. The benefit of a unique IGL-2 solution of static and dynamic HOPE preservation strategies is a topic that needs to be addressed for a simplified clinical procedure [81,83]. This will speed up current static and HOPE strategies in clinical liver transplantation, including liver split transplantation for HOPE application [82,83] and marginal livers. We assume that investigating in depth the underlying mitochondrial protection mechanisms [25,26] should be a priority direction for future approaches to liver hypothermic graft preservation strategies using PEG solutions. At this point, let us go back to 1989 when Belzer and Southard reported that the "mechanisms by which polyethylene glycols prevents cell swelling and thus maintains cell viability is not related to osmotic/oncotic properties but instead is apparently related to PEG/cell interactions that confer stability during hypothermia" [89].

Finally, due to their valuable insights, we now know that, in these interactions, the role of mitochondria, their integrity, and their function are determinants for hypothermic graft preservation, especially when marginal livers (steatotic and CDC) are used. In summary, Belzer and Southard [1,2] "left the door open for us" to return to the origins of preservation solutions and suggest new paradigms to increase liver cold graft preservation with PEG35 solutions for clinical transplantation purposes.

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Abbreviations

4H NE	4-Hydroxynonenal
ALDH2	Aldehyde dehydrogenase-2
ALT	Alanine aminotransferase
AMPK	AMP-activated protein kinase
AST	Aspartate aminotransferase
Belzer MPS	Belzer Machine Perfusion Solution
DCD	Donor after cardiac death
eNOS	Endothelial nitric oxide synthase
GCX	Glycocalyx
GLDH	Glutamate dehydrogenase
PEG35	Polyethylene glycol 35
HES	Hydroxyethyl starch
HOPE	Hypothermic oxygenated perfusion
HTK	Histidine-tryptophan-ketoglutarate
IGL-1/IGL-2	Institut Georges Lopez 1/Institut Georges Lopez 2
IRI	Ischemia–reperfusion injury
MP	Machine perfusion
NO	Nitric oxide
ROS	Radical oxygen species
SCS	Static cold storage
TX	Liver transplantation
UCP2	Uncoupling protein-2
UW	University of Wisconsin

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DISCUSIÓN

El daño por isquemia/reperfusión en el trasplante hepático

El trasplante hepático es un recurso de tratamiento quirúrgico para aquellos pacientes en los que la lesión hepática es irreversible. Lamentablemente, es un procedimiento cada vez más recurrente debido a la alta prevalencia de enfermedades relacionadas con el estilo de vida sedentario y malos hábitos de alimentación [9]. De hecho, esta inercia a la alza se ha visto truncada en los dos últimos años por el virus de la COVID-19; tanto por la mortalidad de los pacientes vulnerables como por la disponibilidad de órganos y de los centros hospitalarios, sin olvidar el miedo a la infección de aquellos pacientes en la lista de espera al ser llamados para ser trasplantados [131]. Superada esta etapa y una vez recuperada la actividad normal de los centros, el interés de los expertos no es otro que aumentar el *pool* de órganos disponibles para el trasplante, no solo para reducir las listas de espera sino también para ampliar los criterios selectivos de estas. Para ello, es de gran importancia la supervivencia del órgano después del trasplante, en la cual el fallo por el daño durante la isquemia es el principal hándicap después del rechazo por incompatibilidad.

El PEG35 como agente protector en las soluciones de preservación

Está descrito que el daño por IR provoca daño al tejido hepático y, tras la preservación del órgano para el trasplante, este daño o necrosis, apreciable a simple vista, será uno de los puntos a tener en cuenta. No obstante, se tendrán en cuenta también marcadores de daño hepático, entre los cuales, los más conocidos son los niveles de transaminasas (Alanina Transaminasa, ALT; Aspartato transaminasa, AST) y la Glutamato Deshidrogenasa (GLDH) como marcador de daño mitocondrial. Tras la isquemia, los niveles de ALT, AST y GLDH aumentan en hígados sanos, tras 24 h de preservación en frio (4 ºC). En el caso de los hígados grasos, en que partimos de niveles superiores basales, este aumento es aún más considerable (Tabla 2).

	ZUCKER LEAN	**ZUCKER OB**	**ZUCKER LEAN 24H 4ºC IGL-1**	**ZUCKER OB 24H 4ºC IGL-1**	**ZUCKER OB 24H 4ºC UW**	**ZUCKER OB 24H 4ºC HTK**
AST (U/L)	5,75 ±0,96	23,25±2,22	35±2,45	110,5±3,7	123±35	163±78
ALT (U/L)	5,75±0.96	13,5±2,02	23,75±2,2	71±4,24	76,65±27	131±40
GLDH (U/L)	20,5±3,11	35,25±5,97	46±6,78	109,25±5,91	117±6,3	135,1±53

Tabla 2. Daño hepático (AST y ALT) y daño mitocondrial (GLDH) en hígados sanos y esteatósicos (OB) control, preservados 24 h en IGL-1 e hígados esteatósicos preservados en UW y HTK. Los resultados se muestran en U/L como la media ± desviación estándar (n=4-6). Font: Zaouali et. al 2017; Panisello-Roselló et al. 2017; Panisello-Roselló et. al. 2018; Bardallo et. al. 2018 [107,114,128,132].

Resultados previos de nuestro grupo muestran como la solución de preservación IGL-1 previene de este daño si lo comparamos con hígados grasos preservados con las soluciones clásicas (UW y HTK), presumiblemente gracias a la presencia de PEG35

[12,56,128]. Los resultados mostrados en el Capítulo II corroboran este hecho, puesto que en caso de preservarlos sin PEG35 los niveles de transaminasas y GLDH aumentan considerablemente, dando lugar a un órgano menos asequible para el trasplante (Capítulo II, Figura 3). Además, se corroboró que la solución IGL-2 mejora el estado hepático respecto a las soluciones clásicas (Capítulo VI, Figura 2). Este primer filtro para la selección del órgano para ser trasplantado es de gran importancia, y nos hace preguntarnos los mecanismos en los cuales interviene el PEG35 para proteger el hígado durante su preservación.

Además de la necrosis, durante la reperfusión será de gran importancia la preservación de la microcirculación. En ese sentido, el papel del óxido nítrico (NO) es esencial en el precondicionamiento isquémico, de manera que incrementar su síntesis se considera una gran estrategia para evitar daño durante la reperfusión por su papel vasodilatador así como scavenger de ROS, principalmente el NO procedente de la óxido nítrico sintasa endotelial (eNOS) [133–135]. De hecho, se ha visto que el aumento del NO durante el precondicionamiento isquémico en hígado de rata previene del estrés oxidativo [101,136] y la aplicación de agentes donantes de NO previenen el daño por IR en pacientes trasplantados [137]. Por el contrario, la sobreproducción de NO derivada de iNOS, generalmente por la activación de las KC, es contraproducente debido a que durante la reperfusión, al reaccionar con oxígeno, puede dar lugar a superóxido, aumentando considerablemente el estrés oxidativo.

Resultados previos de nuestro grupo muestran que, durante la preservación hepática en frio, la solución IGL-1 tiene efectos positivos en la síntesis de NO procedente de eNOS, en comparación con las soluciones clásicas UW y HTK [112,138]. Según los resultados del Capítulo II, podemos corroborar que la expresión de eNOS y por tanto la producción de NO está activada por el PEG35, y, aunque no hay diferencias significativas, vemos una tendencia dosis-dependiente en la solución IGL-2 respecto IGL-1 (Capítulo II, Figura 7). Por tanto, podemos confirmar que el PEG35 incrementa la expresión de eNOS, dando lugar a una protección del tejido hepático en la preservación en frio del tejido.

El PEG35 protege la mitocondria durante la isquemia fría

La depleción del ATP durante la isquemia es una de las principales consecuencias de la preservación hipóxica hepática antes del trasplante, especialmente en hígados esteatósicos [139]. La irrupción del oxígeno durante la isquemia bloquea la cadena de transporte de electrones, fuente principal de ATP en el metabolismo aeróbico. La irrupción de la cadena de transporte de electrones así como la depleción del ATP y la acumulación de metabolitos como el succinato tendrán consecuencias graves durante la reperfusión, provocando acidosis y, en consecuencia, la necrosis del tejido. Se ha comprobado que algunas terapias que evitan este daño mejoran el daño por isquemia en modelos de isquemia en corazón [140,141]. Por tanto, cabe remarcar el papel del PEG35 en las soluciones de preservación IGL-1 e IGL-2. Los resultados del Capítulo II muestran como los niveles de ATP se mantienen en presencia de PEG35 en las dos soluciones, y reducen hasta niveles normales la acumulación de succinato en hígados preservados con IGL-2 (Capítulo II, Figura 1). Además, si lo comparamos con las soluciones clásicas UW y HTK, observamos que los niveles de ATP disminuyen en éstas, mientras que se mantienen en los hígados preservados en IGL-2 respecto a SHAM (Capítulo VI, Figura 3).

La disrupción de la cadena de transporte de electrones durante la preservación estática en frio comporta también la acumulación de succinato [45]. Durante la posterior reperfusión, la actividad de la succinato deshidrogenasa provocará la generación de ROS. Por ese motivo, una de las estrategias a seguir durante la reperfusión es la inhibición de la succinato deshidrogenasa. Como alternativa, la opción de reducir el succinato acumulado durante el proceso isquémico será ventajoso para evitar el daño del tejido tras la reactivación de la cadena de transporte de electrones. Dado el papel clave de este metabolito, se evaluaron los niveles de succinato en los diferentes medios de preservación. Los resultados obtenidos (Capítulo II, Figura 1; Capítulo VI, Figura 3) muestran que la solución de preservación IGL-2 es la única capaz de mantener los niveles de succinato basales, en comparación con SHAM, a diferencia de las soluciones clásicas UW y HTK, así como IGL-0 e IGL-1. La presencia de PEG35 y GSH en este medio podrían contribuir evitando la acumulación de succinato al final de la isquemia, mejorando la supervivencia del órgano tras la revascularización.

Vista la implicación que tiene la cadena de transporte mitocondrial, se procedió a estudiar sus complejos. La cadena de transporte de electrones, encargada de producir el ATP durante la fosforilación oxidativa (OXPHOS, Fosforilación Oxidativa), consta de cinco complejos. En condiciones normales, se produce un pequeño flujo de ROS durante la fosforilación oxidativa. En cambio, durante la isquemia, el desacoplamiento de la cadena de transporte de electrones dará lugar a un incremento de ROS. En nuestro estudio, observamos un incremento de la expresión del complejo I cuando preservamos con IGL-2 y un aumento del complejo II en hígados preservados con presencia de PEG35 y GSH, con las soluciones de preservación IGL-1 e IGL-2, respecto al SHAM e IGL-0 (Capítulo III, Figura 2). El complejo I oxida el NADH y transfiere los electrones a la ubiquinona, mientras transloca cuatro protones al espacio intermembrana. El complejo II o succinato deshidrogenasa, es el único complejo que forma parte también del ciclo de Krebs, y oxida el succinato a fumarato mientras reduce la ubiquinona. La expresión de los complejos de OXPHOS no había sido estudiada en preservación de hígados esteatósicos en frío hasta ahora. No obstante, en un modelo de isquemia cardíaca, se asocia el incremento de OXPHOS a una mejor integridad mitocondrial [142]. En otro estudio, se sugiere la hiperoxia para mantener dicha integridad mitocondrial, ya que mantiene activos e íntegros los complejos de OXPHOS, y por ende la producción de ATP [143]. Por tanto, nuestros resultados son alentadores y podemos confirmar que la preservación en frío en presencia de PEG35 y GSH ayuda a mantener la integridad mitocondrial.

Se ha estudiado la enzima mitocondrial ALDH2 como protectora del daño hepático, gracias a su actividad detoxificadora de aldehídos. De hecho, diversos autores han reportado que el uso de Alda-1, un activador de ALDH2, protege del daño por IR en hígado de rata [58,59]. En estudios previos, nuestro grupo de investigación demostró que la expresión del ALDH2 era significativamente mayor en hígados preservados con solución IGL-1 respecto a las soluciones clásicas UW Y HTK [144]. Para corroborar que este efecto es gracias a la adición de PEG35 en la solución de preservación, en el Capítulo II comparamos IGL-1 con las soluciones modificadas IGL-0 e IGL-2. Los resultados muestran un aumento significativo de la expresión en IGL-2 respecto IGL-0 y SHAM (Figura 5, Capítulo II). Además, se aprecia una tendencia dosis-dependiente, siendo mayor la expresión en IGL-1 que en IGL-0, aunque en este caso no es significativa. Por

tanto, el PEG35 podría ser activador de ALDH2. Por otro lado, correlacionamos la expresión de ALDH2 con los niveles de 4-HNE, como marcador de formación de aductos de proteínas, principalmente formados por aldehídos. En este caso, se da una correlación negativa y cuanto mayor es la expresión de ALDH2 menores son los niveles de 4-HNE (Figura 5, Capítulo II). El hecho de que ALDH2 reduzca los niveles de oxidación a través de 4-HNE ya se había demostrado anteriormente en isquemia, concretamente en ictus e infarto de miocardio [145,146]. Por ende, el PEG35 no es solo responsable de la activación de ALDH2, sino que además reduce los niveles de estrés oxidativo, tal y como se detallará a continuación.

Recientemente, los expertos proponen la preservación en máquinas de perfusión oxigenada hipotérmica, que han demostrado preservar la integridad mitocondrial así como los niveles de ATP [147], tal y como se plantea en el Capítulo V. Concretamente, y basado en los resultados previos, se propone la solución IGL-2 para optimizar el proceso y beneficiarnos del papel protector del PEG35. Además, se expone el uso de nuevos aditivos en la preservación estática en frio, como es el caso del transportador de oxígeno M-101, de manera que se mantengan las funciones metabólicas básicas para evitar tanto la depleción del ATP como la acumulación de succinato por bloqueo de la cadena de transporte de electrones.

Otro aspecto a tener en cuenta durante el daño por isquemia-reperfusión es la regulación de la mitofagia. El mantenimiento de la homeóstasis mitocondrial a través del balance de biogénesis y eliminación de las mitocondrias dañadas es esencial para la mantener la integridad celular. La inducción de la mitofagia se produce en diversos procesos fisiopatológicos, principalmente en respuesta al estrés oxidativo y la inflamación, para evitar la sobreproducción de ROS de las mitocondrias dañadas [148], por lo que será esencial en el daño por isquemia-reperfusión [149]. Se analizó la expresión de LC3 (Capítulo II, Figura 4), Beclina2, UCP-2 y PINK1 (Capítulo III, Figura 3), conocidos por su papel en la autofagia protectora [149,150]. La expresión de Beclina1 y LC3 se ve aumentada en presencia de PEG y GSH en hígados esteatósicos preservados en frío, mientras que UCP-2 y PINK1 solo se ven incrementados cuando preservamos con IGL-2. Estos resultados corroboran la protección mitocondrial en presencia de PEG y GSH, y además se correlaciona con la preservación de los niveles de ATP así como una disminución del daño oxidativo, que será analizado más adelante.

Además de estar implicado en la regulación de la mitofagia [151], UCP-2 es una proteína intermembrana cuya función es reducir el potencial electroquímico mitocondrial desacoplando el Complejo II de la cadena de transporte de electrones. En ese sentido, estudios recientes sugieren que la función de UCP-2 ayuda a controlar la generación de ROS por parte de la mitocondria, así como la síntesis de ATP [152], lo cual corrobora nuestros resultados.

La prevención del estrés oxidativo en presencia de PEG35

Como consecuencia de las ROS generadas en la mitocondria, se verán expuestos a la oxidación estructuras celulares, como son en DNA, los lípidos y las proteínas. La regulación de las ROS en el desarrollo de enfermedades ha sido desde hace años foco de la investigación [153–155]. En el caso de la IR hepática, la mediación de las ROS ha demostrado ser una importante diana terapéutica [156,157]. Algunas estrategias a

seguir son la aplicación de agentes antioxidantes como las vitaminas [158], el resveratrol [159], la melatonina [160], así como el precondicionamiento isquémico [101].

En los últimos años, Nrf2 se ha postulado como una de las dianas principales en la prevención del estrés oxidativo. Su función protectora se basa en la activación de una batería de genes asociados con el estrés oxidativo, la inflamación y la detoxificación celular, por lo que promover su expresión es una gran estrategia contra el estrés [26]. En las enfermedades en que el estrés oxidativo es el protagonista, como es la enfermedad del Alzheimer, el Parkinson, el infarto de miocardio, el daño por isquemia reperfusión y más recientemente el COVID19, Nrf2 ha cobrado protagonismo como diana terapéutica [93–96]. En el Capítulo I, se reportan diferentes tratamientos utilizados contra el daño por IR hepática que tiene la modulación de la expresión de Nrf2 como objetivo (Capítulo I, Tabla 2).

En nuestro modelo experimental, el PEG35 y el GSH presentes en la solución IGL-2 resultaron ser activadores de la expresión de Nrf-2, así como de la HO-1, unos de los genes diana (Capítulo III, Figura 4). Estos resultados se correlacionan con los citados previamente en cuanto a la expresión de UCP-2 y PINK1. Aunque no hay estudios concluyentes que asocien la expresión de Nrf2 y UCP-2, el grupo de la Dra. Cadenas en el centro de Biología Molecular Severo Ochoa (CSIC/UAM) sí demostró una relación directa de UCP-3 en un modelo de IR en cardiomiocitos de ratón [161]. En cuanto a PINK1, Murata y colaboradores demostraron que Nrf2 se une a regiones promotoras de PINK1, correspondiente a secuencias ARE, promoviendo así la autofagia protectora [162].

Para corroborar el efecto de la activación de Nrf2 en la preservación en frío de los hígados esteatósicos, se evaluó el daño oxidativo. Los marcadores de daño oxidativo más utilizados son los productos de oxidación de las ROS, como lípidos (MDA y 4-HNE) y proteínas (AOPP y proteínas carboniladas). Los resultados previos de nuestro grupo de investigación corroboraron que la solución de preservación IGL-1 presenta niveles inferiores de oxidación, analizados como MDA, AOPP y 4-HNE, respecto a las soluciones clásicas UW y HTK [128,144]. De nuevo, para comprobar el efecto del PEG35, se analizó el estrés oxidativo en dicha solución y sus modificaciones. Los resultados demuestran que la adición de PEG35 y GSH en IGL-2 disminuyen los niveles de 4-HNE, AOPP, MDA y proteínas carboniladas respecto a la solución original de IGL-1, mientras que la solución IGL-0, que carece de PEG35 y GSH, presenta mayores niveles de oxidación (Capítulo II, Figura 5; Capítulo III, Figura 5). Además, se aprecia un efecto dosis-dependiente, evidenciando que la concentración de PEG35 es crucial para disminuir el estrés oxidativo. Posteriormente confirmamos que la preservación en IGL-2 produce menos estrés oxidativo, oxidativo respecto a las soluciones clásicas, presentando niveles de MDA y AOPP inferiores (Capítulo VI, Figura 4).

Por otro lado, se analizó la actividad de las enzimas antioxidantes (Capítulo III, Figura 6). En este caso, los hígados preservados en IGL-0 presentaron mayor actividad de SOD, catalasa, GSH transferasa y GSH peroxidasa respecto a SHAM. En aquellos preservados con IGL-1, se vio incrementada la actividad de SOD y catalasa respecto a SHAM, mientras que disminuye la actividad de la GSH transferasa respecto a los preservados con IGL-0. Por el contrario, IGL-2 no presenta diferencias significativas en la actividad enzimática respecto a SHAM. Podríamos asociar el incremento de esta actividad antioxidante en el grupo IGL-0 a niveles superiores de estrés oxidativo, que provocó un aumento en la

respuesta antioxidante. Este aumento de la actividad de las enzimas antioxidantes no se ha visto reflejado cuando preservamos con IGL-2, posiblemente debido a que, por una parte, previene la formación de ROS a nivel mitocondrial y, por otra parte, por su contenido en GSH, un potente antioxidante, que las detoxifica más eficientemente. De hecho, nuestros resultados concuerdan con los del grupo de De Freitas Carvalho et. At. [163]. En su estudio, en que suplementan con *açaí* un modelo de ratones obesos, observaron una menor actividad de GPx, SOD y CAT, mientras que aumentaban los niveles de GSH y mejoraba el estrés oxidativo.

No obstante, la solución IGL-2, que contiene GSH aumentado, no solo previene el estrés oxidativo por la presencia de GSH en su fórmula, sino que además promueve la formación de éste, posiblemente a través de la activación de Nrf2. Cuando analizamos el sistema del GSH, observamos que, en contraste con las otras enzimas, IGL-2 aumenta la expresión de la GSH reductasa (Capítulo III, Figura 7c), enzima responsable de reducir GSSG a GSH. Por tanto, los niveles de GSH así como el ratio GSH/GSSG que encontramos incrementados en los hígados preservados en IGL-2 (Capítulo III, Figura 7ab) no solo derivan del aumento de GSH en la solución sino que además lo podemos asociar a un aumento de la actividad de la GSH Reductasa. Estos resultados son de gran importancia, puesto que se ha demostrado que la presencia de antioxidantes endógenos durante la isquemia protegen del daño por IR y son vitales para la viabilidad del órgano [164].

El estrés del Retículo Endoplasmático en la preservación hepática mejora en presencia de PEG35 y GSH.

La conservación en frío de los hígados esteatósicos aumenta el estrés del RE y el daño por estrés oxidativo, lo cual se ve exacerbado en los hígados grasos. En condiciones fisiológicas, existe un equilibrio entre las proteínas mal plegadas o desplegadas regulado por el RE. Durante la isquemia, el aumento del estrés en el RE provoca un incremento de las proteínas mal plegadas. En consecuencia, se liberará Ca^{+2} al citoplasma, provocando una serie de respuestas al estrés, entre ellas la UPR. Como indicador de proteína mal plegada, se encontró un aumento de los niveles de expresión de GRP78 y PPERK en hígados conservados en UW (Capítulo IV Figura 1b), concomitante con un aumento del sensor PPERK UPR, lo que puede indicar el inicio de las cascadas de señalización UPR que codifican para chaperonas del RE, enzimas plegables, proteínas pro-apoptóticas y antioxidantes. En cuanto al daño de las proteínas, resultados previos del grupo de investigación demostraron que la solución IGL-1 presenta niveles inferiores de activación del proteasoma y por tanto de la UPR respeto a hígados preservados en la solución HTK [128], lo cual se correlaciona con los resultados mostrados. La preservación en el medio IGL-2, que contiene PEG35 en mayor cantidad a IGL-1, previene el daño a proteínas así como el estrés del RE, respecto a la preservación en las soluciones clásicas UW y HTK.

El PEG35 y el GSH previenen del daño inflamatorio y la piroptosis

Una vez evaluado el papel del estrés oxidativo y del retículo en la preservación estática en frio en las diferentes soluciones de preservación, se exploraron los signos de la inflamación estéril. La inflamación estéril se produce en respuesta a mediadores pro-inflamatorios. Durante la exposición a la isquemia, el daño a los tejidos produce la liberación de DAMPS, entre los cuales destacan HMGB1 y Hsp70. Los DAMPS activan sus

respectivos receptores, los PRR, dando lugar a la activación de la vía inflamatoria a través de NF-κB.

La expresión de HMGB1 (Capítulo IV Figura 3a), muestra un aumento significativo en el grupo UW en comparación con los hígados conservados con IGL-2. A nivel hepático, HMGB1 tiene una doble función: como nucleoproteína no histona y como citocina inflamatoria extracelular. Las células estresadas liberan pasivamente HMGB1 extracelular, y también lo secretan activamente. Como quimiocina o citocina, se une a los receptores de reconocimiento de patrones (PRR) para desempeñar el papel de DAMPS. La proteína extracelular Hsp70 también puede actuar como DAMPS y estimular las respuestas inmunitarias e inflamatorias que provocan una inflamación estéril y la propagación de la inflamación existente, y ha sido especialmente estudiada en IR hepática. En este sentido, se observa un aumento significativo en la expresión de Hsp70 en los grupos UW y HTK (Capítulo IV, Figura 3d). Tanto HMGB1 como Hsp70, actuando como DAMPS activan la vía inflamatoria a través de NF-κB, que aumenta en el grupo UW (Capítulo IV, Figura 3f), a través de la activación de los receptores PRR [165,166]. En el caso del daño por isquemia, destacan los receptores tipo Toll, como TLR4, así como los inflamosomas citoplasmáticos NLRP3 y AIM2 [167]. La expresión de estos receptores se vio aumentada en la solución de preservación UW respecto a IGL-2 (Capítulo IV, Figura 4A, B y C). Además de promover la expresión de NFκB, la activación de los inflamasomas dará lugar a la escisión de la Caspasa 1. Estos dos factores pro-inflamatorios actuaran en paralelo: NFκB promueve la síntesis de la pro-IL-1β mientras que la Caspasa 1 la activará (IL-1β). La interleucina IL-1β, una vez activa, junto con la Caspasa 3, también promovida en el proceso inflamatorio, darán lugar a la escisión de la Gasdermina y al aumento de la expresión del factor inflamatorio TNF-α [168]. En consecuencia, se formarán poros en la membrana, liberando el contenido celular y aumentando la citotoxicidad, proceso conocido en su conjunto como piroptosis [169,170].

Los niveles de expresión de DAMPS y el impacto de inflamasomas como NLRP3 (Capítulo IV, Figura 3e) y otras citocinas proinflamatorias como pp38, TNF-α, caspasa 3, IL-1β y la actividad de Caspasa 1 (Capítulo IV, Figura 4) muestran que los procesos de inflamación estéril, aunque inherentes al almacenamiento de injertos fríos de hígado graso, se evitan en gran medida en la conservación con IGL-2. Esta solución resultó ser más eficiente durante la preservación contra la activación del inflamasoma y las citoquinas inflamatorias. Además, se observó un aumento significativo de GSDM en los hígados preservados en UW respecto a IGL-2 (Capítulo IV, Figura 4d), lo cual indica mayor formación de poros como evento final de la piroptosis.

La prevención de la inflamación estéril es una de las estrategias para prever el daño por IR, especialmente en hígados grasos en que la inflamación es crónica. Algunos grupos se han centrado en la inactivación de los DAMPS y los PRR, evitando la cascada inflamatoria causada por TLR4 y HMGB. Por ejemplo, la inhibición con eritoran, un agonista del receptor TLR4 utilizado como tratamiento de la sepsis, disminuyó el daño hepático así como las interleucinas inflamatorias en un modelo de isquemia caliente en ratón [171]. Por otro lado, la inhibición del inflamasoma NLRP3 a través de la molécula CY-09, un inhibidor directo, previene la inflamación crónica y la progresión de la esteatosis en un modelo de ratón obeso [172]. Aunque no se ha testado en isquemia, NLRP3 ha demostrado ser un factor clave en la progresión del daño por IR [173].

La presencia de PEG35 en la solución IGL-2 podría ser responsable de la protección frente al proceso inflamatorio, ya que el PEG35 se adhiere a los glicerofosfolípidos de la membrana, incrementando su integridad y previendo el edema. Este mecanismo de acción podría ser positivo contra la inflamación estéril en primer lugar en la liberación de DAMPS inicial, provocada por el edema, y en última instancia en la protección contra la formación de poros de en la membrana celular como consecuencia de la escisión de la gasdermina, lo cual encaja con los resultados expuestos en el capítulo IV. Por tanto, se propone la utilización de la solución IGL-2 como terapia antiinflamatoria en la preservación estática en frio de hígados grasos.

Perspectivas futuras: la utilización de PEG35 en la preservación oxigenada en frío.

La preservación estática en frio es el método más utilizado en el trasplante de órganos. No obstante, existen nuevas estrategias de preservación dinámica, como es la máquina de perfusión para mantener una perfusión hipotérmica oxigenada (HOPE) al injerto. Esta estrategia combina, por un lado, los beneficios de la preservación en frio, que ha demostrado tener efectos beneficiosos en la isquemia hepática [101], con el aporte de oxígeno al órgano mediante un sistema de perfusión, de manera que se mantiene el metabolismo aeróbico [174]. En la clínica, la preservación estática seguida de un corto periodo de HOPE previamente al trasplante mejora las patologías relacionadas con el daño por isquemia reperfusión después del trasplante, como es el riesgo a colangiopatías [175,176]. El mecanismo protector de HOPE se basa en que el aporte de oxígeno y el frio mantienen el metabolismo aeróbico hipotérmico, un metabolismo lento pero no truncado como en la preservación estática, y por tanto evita la acumulación de succinato y previene la depleción del ATP, de manera que previene el estrés oxidativo y la consecuente inflamación tras la reperfusión.

Los medios de preservación durante HOPE son los mismos que los utilizados en la preservación estática. La composición de las soluciones de perfusión para la perfusión oxigenada hipotérmica hepática es idéntica a la utilizada para SCS. Como se mencionó anteriormente, el principal inconveniente de las soluciones que contienen HES (como Belzer MPS) es su alta viscosidad, que puede conducir a un esfuerzo cortante sinusoidal. Para superar estas deficiencias, se desarrolló una nueva solución que contiene PEG35 en lugar de HES, llamada Polysol [177,178] . Como agente oncótico, PEG35 ejerce una presión oncótica similar a HES pero con una viscosidad relativamente más baja y, en consecuencia, menos tensión de cizallamiento en el sinusoide hepático. Se observó una mejor función hepática y menos daño hepático usando la solución Polysol en comparación con Belzer MPS [177,178]. Además, tras 24 h de MP usando Polysol, se mostró una mejor conservación en comparación con la misma solución pero con HES en lugar de PEG (Polysol-HES), destacando el papel protector de PEG. Un PEG de menor peso molecular, PEG20, complementado con una solución de Celsior ofreció una mejor protección de los riñones de cerdo recuperados después de la muerte cardíaca usando MP, en comparación con Celsior sin complemento o con Belzer MPS [179]. Schlegel y colaboradores [127] demostraron que la limpieza o reparación endotelial del glicocálix representa un importante mecanismo de protección conferido al hígado por HOPE. El glicocálix se puede escindir mediante la escisión enzimática de las proteínas centrales de proteoglicanos o el estrés oxidativo directo de las ROS, como consecuencia de la lesión por IR, lo que induce permeabilidad endotelial y edema [180]. Como se ha

mencionado anteriormente, López y colaboradores [85] mostraron pruebas convincentes de los beneficios de la solución IGL-1 en hígados esteatósicos, resaltados por la importancia de la protección del glicocálix durante SCS. El PEG35, presente en la solución IGL-1, previene la inflamación celular y el daño del endotelio vascular mediante la estabilización de las membranas lipídicas y la reducción de la permeabilidad de la membrana.

Otro aspecto a tener en cuenta es la tensión de cizallamiento o *shear stress*, que se define como el componente de la tensión que está en el mismo plano que la superficie de un material, lo que origina tensión en la superficie. Aplicado a la reperfusión, es un aspecto importante dado que depende del flujo y puede provocar cambios celulares, principalmente en el glicocálix. En ese sentido, es necesario profundizar en la investigación de López y colaboradores [85] durante el daño por IR hepática y su conexión con PEG35. La aplicación de HOPE podría ser una estrategia válida para investigar la tensión de cizallamiento inherente a IRI y su efecto sobre la integridad de glicocálix utilizando un perfundido que contiene PEG35. Este enfoque terapéutico de protección del glicocálix podría mejorar potencialmente la viabilidad de los órganos de los injertos marginales y disminuir la gravedad de la IR.

La utilización de PEG35 ha demostrado ser beneficiosa en la preservación estática en frio en hígados esteatósicos de rata. En nuestros resultados, la preservación estática en frio con el medio IGL-2 ha demostrado una mejor preservación que las soluciones utilizadas de forma clásica, gracias a la presencia de PEG35 y GSH. Esta protección se ha mostrado a nivel mitocondrial, en que consigue mantener los niveles de ATP y disminuir la acumulación de succinato; a nivel oxidativo, con menores niveles de marcadores de estrés, como MDA, AOPP, 4-HNE proteínas carboniladas, así como en la respuesta antioxidante mediante activación de enzimas detoxificadoras y el incremento de GSH/GSSG, mediada por Nrf2; a nivel del retículo endoplasmático, menores niveles de estrés, apoptosis y piroptosis; todo esto contribuyendo a niveles inferiores de inflamación. Por estos motivos se propone la utilización de PEG35 no solo en la preservación estática sino también en la preservación oxigenada hipotérmica, tal y como se detalla en los capítulos V y VI.

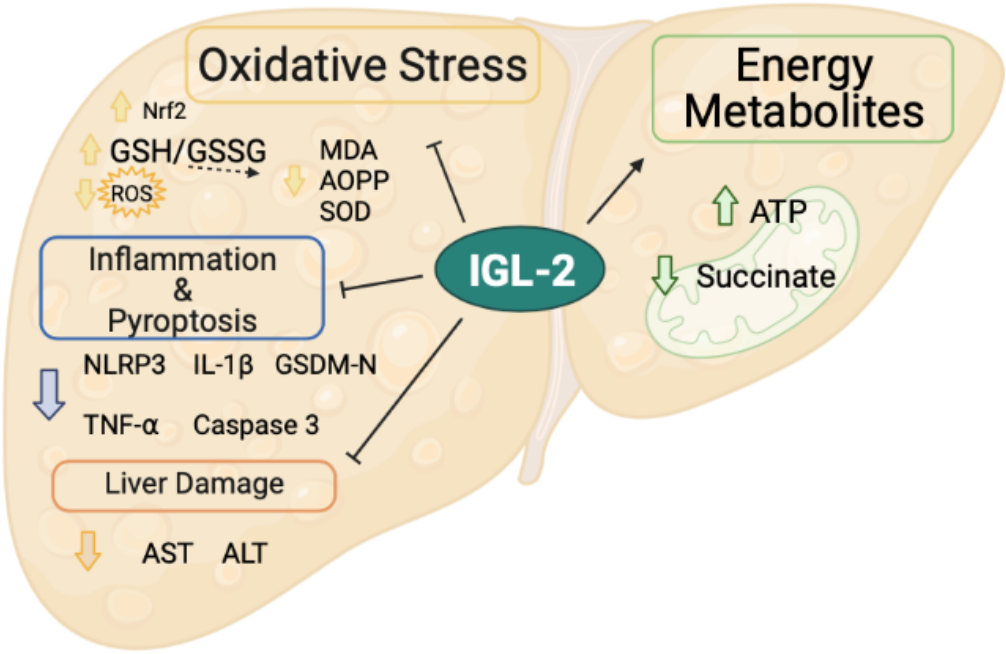

Figura 7. Efecto protector de la solución IGL-2 en la preservación de hígados grasos. IGL-2 a resultado ser protectora a nivel mitocondrial, de manera que una menor producción de ROS disminuye el estrés oxidativo, la inflamación y apoptosis y, finalmente, el daño en el hígado.

Hasta el momento, el criterio de selección de hígados descarta los hígados esteatósicos para el trasplante debido a la baja viabilidad. La estrategia combinada de preservación estática con PEG35 con la perfusión oxigenada hipotérmica utilizando este mismo medio podría ampliar el criterio de selección de órganos, aumentando el número de hígados viables disponibles, lo cual disminuiría las listas de esperas y además permitiría ampliar los criterios para entrar en ella.

CONCLUSIONES

- El Poletilenglicol-35 protege en el daño por isquemia en la preservación estática en frio (SCS) de hígados esteatósicos, disminuyendo el daño hepático (AST, ALT, GLDH) y mejorando la capacidad de reperfusión (NO), así como mejorando la integridad mitocondrial, aumentando la expresión de las enzimas protectoras ALDH2 y UCP2, preservando el ATP y promoviendo la mitofagia protectora (PINK1, LC3, Beclina) cuando son preservados en solución IGL-2, mejorando los resultados respecto IGL-0 e IGL-2.

- La preservación del injerto hepático en frio en el medio IGL-2 previene del estrés oxidativo y mejora la defensa antioxidante gracias al aumento de PEG35 y GSH en la solución respecto a las soluciones IGL-0 e IGL-1, activa la respuesta antioxidante a través de Nrf2, reduce la oxidación de lípidos (TBARS, y 4HNE) y proteínas (AOPP y DNP), y promueve la defensa antioxidante a través de la síntesis de GSH.

- El medio IGL-2 previene el estrés del retículo endoplasmático en la preservación estática respecto a las soluciones clásicas UW y HTK, disminuye la expresión de GRP78, PPERK, de manera que previene de la UPR.

- La solución de preservación IGL-2 reduce el daño por DAMPS (HMGB1 y Hasp70) y por tanto la activación de los PRR (TLR4 e inflamosomas), disminuye la respuesta inflamatoria (interleuquinas y NF-kB), previene la apoptosis (Caspasas) y la Piroptosis (GSDM) durante la SCS en comparación con las soluciones UW y HTK.

- El medio IGL-2 es el mejor candidato para la preservación dinámica HOPE como tratamiento previo al trasplante hepático gracias a la presencia de PEG35 y GSH.

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