Casa abierta al tiempo

UNIVERSIDAD AUTÓNOMA METROPOLITANA

UNIDAD IZTAPALAPA

DIVISIÓN DE CIENCIAS BIOLÓGICAS Y DE LA SALUD

MAESTRÍA EN BIOLOGÍA EXPERIMENTAL

Caracterización de distintas dietas esteatogénicas en el inicio del daño hepático con potencial carcinogénico

TESIS

PARA OBTENER EL GRADO DE MAESTRÍA EN

Biología Experimental

Ortiz Pedraza Yunuen Ismerai

DIRECTOR

Dr. Luis Enrique Gómez Quiroz

CO-DIRECTOR

Dr. Cedric Coulouarn

ASESORA

Dra. Mina Konigsberg Fainstein

CDMX.,Ciudad de México, 05 de Septiembre del 2018

Casa abierta al tiempo

UNIVERSIDAD AUTÓNOMA METROPOLITANA

UNIDAD IZTAPALAPA

DIVISION OF BIOLOGICAL AND HEALTH SCIENCES

MASTER'S IN EXPERIMENTAL BIOLOGY

Characterization of different steatosis-induced diets in the initiation of liver damage with potential carcinogenic effects

THESIS

TO OBTAIN THE MASTER DEGREE OF

Experimental Biology

Ortiz Pedraza Yunuen Ismerai

DIRECTOR

Luis Enrique Gomez Quiroz, PhD

CO-DIRECTOR

Cedric Coulouarn, PhD

ADVISER

Mina Konigsberg Fainstein, PhD

Mexico City, September 5th, 2018

Este trabajo fue realizado en el Laboratorio de Fisiología Celular, en el departamento de Ciencias de la Salud de la Universidad Autónoma Metropolitana Unidad Iztapalapa.

“El Programa de Maestría en Biología Experimental de la Universidad Autónoma Metropolitana pertenece al Programa Nacional de Posgrados de Calidad (PNPC) del CONACYT, registro 001481, en el Nivel Consolidado y cuenta con apoyo del mismo Consejo, clave DAFCYT-2003IMPTNNN0020”.

El proyecto fue apoyado por CONACYT en el fondo Fronteras de la Ciencia #1320, otorgado al Dr. Luis Enrique Gómez Quiroz contó con apoyo de SEP-PRODEP 913026–14612111. Agradezco la beca otorgada por CONACyT durante el periodo de duración del programa de Maestría, así como el apoyo con Beca Mixta o de Movilidad para realizar la estancia académica en la Universidad de California en San Francisco (UCSF), en el Laboratorio de la Dra. Xin Chen.

Los miembros del jurado por la División de Ciencias Biológicas y de la Salud, Universidad Autónoma Metropolitana, abajo firmantes, aprobando la tesis titulada: **"Caracterización de distintas dietas esteatogénicas en el inicio del daño hepático con potencial carcinogénico"**, con fecha 05 de septiembre del 2018.

MIEMBROS DEL JURADO

PRESIDENTE
Dra. Verónica Souza Arroyo

Universidad Autónoma Metropolitana-I,
Laboratorio en Medicina Experimental
Instituto de Biomédicas, UNAM/INCICH

SECRETARIA
Dra. Natalia Nuño Lámbarri

Investigador básico de la Unidad
de Investigación Transnacional,
Clínica y Fundación Médica Sur.

VOCAL
Dr. Javier Esteban Jiménez Salazar

Departamento de Ciencias de la Salud
Universidad Autónoma Metropolitana

VOCAL
Dra. María del Refugio Denise Clavijo Cornejo
Investigadora en Ciencias Médicas
Instituto Nacional de Rehabilitación
Luis Guillermo Ibarra Ibarra

II

COMITÉ TUTORAL

DIRECTOR

Luis Enrique Gómez Quiroz, PhD

Profesor "C"

Nivel de Investigador Nacional III, SNI

Universidad Autónoma Metropolitana-Iztapalapa

Laboratorio en Medicina Experimental

Instituto de Biomédicas, UNAM/INCICH.

CODIRECTOR

Cédric Couloarn, PhD

Investigador Nacional Nivel III, SNI

INSERM, UMR991, Metabolismo lipídico y cáncer

Rennes, Francia

ASESOR INTERNO

Mina Konigsberg Fainstein, PhD

Profesor “C”

Nivel de Investigador Nacional III, SNI

Laboratio de Bioenergética y Envejecimeinto Celular

Departamento de Ciencias de la Salud

AGRADECIMIENTOS

DEDICATORIA

RESUMEN

El Carcinoma Hepatocelular (HCC) es considerado por ser el un tumor hepático primario, posicionándose como la segunda causa de muerte en el mundo. Esta estrechamente relacionada con el Hígado graso no alcohólico (por sus siglas en inglés, NAFLD), Hígado graso alcohólico, virus de Hepatitis B y C.

NAFLD tiene la característica de presenciar una acumulación exacerbada de lípidos (denominado “Esteatosis hepática”) en los hepatocitos y esto permitiría el progreso de esteatohepatitis (por sus siglas en inglés, NASH), fibrosis, cirrosis y, finalmente HCC.

Si bien, la sobrecarga de lípidos en el hígado es un aspecto característico de NAFLD, por lo que la movilización de los lípidos es determinante e indispensable para la iniciación de la regeneración del hígado y por consiguiente en hepatocarcinogenesis.

Datos de nuestro grupo y otros grupos de investigación han demostrado que la sobrecarga de colesterol tiende a generar más daño que otro tipo de lípido.

El presente trabajo tiene como objetivo saber cuál es la relevancia de la sobrecarga diferencial de lípidos en hígado en la iniciación de la hepatocarcinogénesis.

Se usaron ratones macho con 14 días de nacimiento de la cepa C57BL6, fueron alimentados con dieta estándar purina 5001, dieta hipercolesterolémica, dieta alta en grasa y dieta western por 30 días, y fueron tratados con un agente tóxico N-nitrosodietilamine, por sus siglas en inglés DEN, en una sola dosis (10ug/ul) para inducir daño al DNA e iniciar con el proceso de carcinogénesis.

Datos de nuestro grupo de investigación han concluido que la sobrecarga de colesterol en dieta es suficiente para generar daño en la reparación al DNA, debido

a que promueve la acumulación de mutaciones que pudieran permitir el desarrollo de cáncer. Por lo tanto, la sobrecarga de colesterol hepático es considerado como un factor de riesgo para desarrollar cáncer de hígado.

Los siguientes datos sugieren que un factor importante en la carcinogénesis es la concentración aberrante de lípidos así como un desbalance en los procesos fisiológicos y celulares; por lo que se puede apreciar claramente que el colesterol es el responsable de dicho desbalance en el hígado.

Por lo que dichas dietas esteatogénicas (HCD, HFD y WD) promueven un desbalance en el estado redox, nuestros datos lo confirman con el incremento de colesterol en hígado o bien, una elevada oxidación de proteínas, daño presentado en el ADN (comprobado a través de la actividad de p-H2AX y ensayos cometas, figuras 8-11) y una baja actividad de caspasa 3, como se muestra en la dieta western, con y sin DEN, con un contenido de 2.071% de colesterol. Por lo que concluimos que el colesterol tiene un papel importante en la supervivencia celular y es un fuerte promotor para el establecimiento de HCC.

INTRODUCCIÓN

EL HÍGADO

El hígado es un órgano complejo que contiene más de 500 funciones diferentes, como el metabolismo de bilirrubina, porfirina, ácido biliar, hormonas, vitaminas, aminoácidos, proteínas, y lípidos y lipoproteínas; biotransformación y función de desintoxicación, degradación del alcohol y equilibrio ácido-base (E. Kuntz, 2002).

Cuan hay presencia de daño crónico en el hígado, esto podría conducir a una inflamación al reclutar células inflamatorias que eventualmente proporcionen una producción mejorada de especies reactivas de oxígeno (ROS) (S. Reuter, 2010). Sin embargo, se ha demostrado que la sobrecarga de lípidos en el hígado aumenta la producción de ROS e induce daño por estrés oxidante (Domínguez-Pérez M., 2016; M. Marı, 2006) (Dominique., 2007; L. Diesen D. , 2011; López-Islas A., 2016) (Nuño-Lámbarri N., 2016).

HÍGADO GRASO NO ALCOHÓLICO

El hígado graso no alcoholico, por sus siglas en inglés “NAFLD”, es la enfermedad hepática más común en el mundo y puede afectar entre el 25 al 35% de la población en general (Zelber-Sagi, Ratziu y Oren, 2011). Se caracteriza por un aumento en la deposición de lípidos en ausencia de consumo de alcohol, en al menos un 5% de los hepatocitos, que progresivamente alcanza un estado inflamatorio conocido como esteatohepatitis no alcohólica (EHNA), y puede progresar a fibrosis, cirrosis y, finalmente HCC (Andersen, 2015) (KJ Thompson, 2013). Grupos de investigación han reportado que el 10% de los pacientes con hígado graso no alcohólico desarrollan EHNA, y del 8 a 26% de estos casos,

pueden progresar a cirrosis, con un alto riesgo de desarrollar HCC (A. Wree, 2011) (L. E. Gomez-Quiroz, 2016).

Se están investigando los mecanismos moleculares sistémicos y hepáticos implicados en la hepatocarcinogénesis inducida por la obesidad y NAFLD, así como los posibles marcadores tempranos de HCC (Marengo et al., 2016); sin embargo, actualmente no hay evidencia convincente sobre el mecanismo de los lípidos implicados en el daño hepático para dar pie a la iniciación de HCC.

CARCINOMA HEPATOCELULAR

El Carcinoma hepatocelular (HCC) es el tumor maligno primario en hígado y es aceptado como una de las principales enfermedades malignas actualmente (Kew, 2014). Es la tercera causa más común de cáncer en el mundo, , por lo que se ha estimado que del 4 al 22% de los casos de HCC pueden asociarse a NAFLD (A. Tessitore, 2016).

HCC es un proceso multifásico debido a la interacción del ADN con carcinógenos (como N-nitrosodietilamina, DEN), daño oxidativo o radicales libres producidos durante la biotransformación o inflamación, y esto podría inducir un daño al ADN a, así como lteraciones en las vías de señalización relacionadas a la reparación del daño al DNA (Dufour., 2016) (Mazzocca A., 2016).

MODELO EXPERIMENTAL DE HEPATOCARCINOGÉNESIS

Uno de los principales modelos en animales para la inducción de hepatocarcinogénesis es en ratones es con el uso de N-nitrosodietilamina, por sus siglás en inglés DEN (YaryginK.N) (Khairy MA Zoheir, 2015), este agente tóxico puede inducir tumores específicos según la especie, HCC es el tumor más

frecuente observado en este modelo, pero también puede inducir colangiocarcinoma o incluso ambos al mismo tiempo. Se ha observado que el efecto de DEN depende de la dosis (Zoheir et al., 2015), incluso, la Agencia Internacional para la Investigación del Cáncer (IARC) informó que después de p.o. exposición, los ratones desarrollan tumores estomacales y esofágicos (L. Vena, 1996)

Por lo que es importante resaltar el aspecto de la Biotransformación del DEN en hígado, ya que hay muchos pasos ello; el primer paso es la α-hidroxilación mediada por citocromo (CYP) P450, que produce una α-hidroxilanitrosamina. El CYP2E1 inducible por etanol y otras formas de P450 podrían hidroxilar NDEA. NDEA puede formar aductos de ADN en hígado y favorecer mutaciones. Las isoenzimas CYP son responsables de la desintoxicación de NDEA, lo que hace que la biotransformación sea compleja. La biotransformación DEN puede proporciona incremento significativo en la producción de ROS, además de inducir daño al ADN (L. Vena, 1996).

El siguiente paso es la alquilación y reparación; después de que NDEA se bioactiva a un ion electrofílico de etildiazonio, se somete a reacción con nucleófilos, incluidas bases de ADN, para formar aductos. La formación de aducto depende de cada carga negativa en cada átomo en el ADN, pero está impedida por enlaces de hidrógeno de doble cadena. Existen diferentes mecanismos de reparación para los aductos. AT repara O6-etildeoxiguanosina (O6-EtdG) mediante la transferencia del grupo alquilo a un sitio aceptor de cisteína en la proteína. La actividad de esta enzima de reparación, O6-EtdG, es alta en hígado. Si las bases de ADN están etiladas y no se reparan, será mutagénico si el aducto puede interrumpir el emparejamiento de bases. Los aductos O6-EtdG y O4-EtdT son más mutagénicos

porque potencialmente se codifican erróneamente y se forman en las cantidades más grandes (L. Vena, 1996).

RESPUESTA DE REPARACIÓN AL ADN

La respuesta de daño del ADN (DDR) es el orquestador para detectar y reparar el daño del ADN con detención cuando el ciclo celular está en marcha, para garantizar el mantenimiento de la estabilidad genómica (Bottai G., 2014). La vía de DDR puede limitar el desarrollo del tumor en sus etapas iniciales al actuar como una barrera para la proliferación de células aberrantes.

En muchos tumores, muestran pérdida funcional o desregulación de las proteínas clave implicadas en DDR y la regulación del ciclo celular, como p53, ATM, BRCA1/2. La desregulación de las vías de DDR también contribuye al desarrollo de la inestabilidad genómica; siendo una característica de cánceres en el ser humano que pueden acelerar las alteraciones genéticas, que impulsan el desarrollo de tumores (Wanga H., 2015).

Sin embargo, la activación de ATM puede promover la fosforilación de los blancos, facilitando las respuestas celulares al daño. El blanco de ATM es la proteína histona H2AX, la forma fosforilada de H2AX (γH2AX), es la plataforma para el reclutamiento de factores DDR adicionales, mejorando las vías de señalización (A., 2005). γH2AX recluta MDC1, que recluta moléculas ATM en las regiones que rodean a los DSBs, por esta razón hay una amplificación de la señalización ATM. Entonces, BRCA1 se recluta al sitio DSB uniendo cadenas de ubiquitina a través de un complejo de proteína que contiene Rap80 y Abraxas (Wanga H., 2015).

Por otra parte, ATR es una de las cinasas centrales implicadas en DDR, se activa mediante estructuras de ADN monocatenarias. ATR actúa a través de los objetivos siguientes para promover la reparación del ADN, estabilización y el reinicio de las horquillas de replicación estancadas y la detención transitoria del ciclo celular. ATR juega un papel importante en la aplicación de la progresión de la fase S y en respuesta al daño del ADN. Además, es un mediador principal del punto de control del ciclo G2/M para evitar la entrada prematura de células en la mitosis, antes de que se complete la replicación del ADN o en presencia de daño en el ADN (M., 2014).

CONCENTRACIONES ALTAS DE LÍPIDOS EN DIETAS EXPERIMENTALES

En el presente estudio, utilizamos la dieta estándar (CW), como control, y diferentes dietas experimentales inducidas por esteatosis (Tabla 1), como la dieta hipercolesterolémica (HCD), dieta alta en grasas (HFD) y dieta occidental (WD); con el objetivo de caracterizar si existen algunas diferencias en el daño inducido por DEN bajo el consumo de estas dietas.

DIETA ALTA EN GRASAS (HFD)

La diet HFD puede inducir la lipoperoxidación y contribuye al daño del ADN, es considerado un factor de riesgo para HCC. Sin embargo, estudios recientes sugieren que HFD podría retrasar el desarrollo de cáncer en varios órganos como el de mama, próstata e hígado (Xiao-Yan Duan, 2014)

DIETA OCCIDENTAL (WD)

Este tipo de dieta se caracteriza por contener un alto porcentaje de grasa, fructosa y colesterol; se asemeja al estilo de comida rápida que han sido implicadas en la patogénesis de NAFLD en humanos. Este tipo de dieta puede promover esteatosis hepática, aumento del peso de hígado y un alto contenido de TAG en hígado (P. Jegatheesan, 2016).

DIETA HIPERCOLESTEROLÉMICA (HCD)

Nuestro grupo de investigación ha abordado ampliamente los efectos de la sobrecarga de colesterol en el hígado en ratones que fueron alimentados con una dieta rica en colesterol (2% de colesterol), y reportamos que el alto consumo de colesterol causaba la aparición de tumores más agresivos en un modelo de carcinogénesis química con DEN, en ratones de la cepa C57Bl6 alimentados durante ocho meses con una dieta rica en colesterol (HC 2% con 0,5% de colato de sodio).

Mismos datos revelan que el hígado presentaba disfunción mitocondrial, estrés oxidante y daño en el ADN (Enriquez-Cortina et al.). cuando hay ingesta excesiva de colesterol, la acumulación de lípidos en los hepatocitos puede causar daño hepático, porque el hígado sintetiza más triglicéridos y estos triglicéridos no pueden exportarse de manera normal, por lo tanto los triglicéridos se acumulan en los hepatocitos causando NAFLD (Karagozian, Derdak, y Baffy, 2014).

Y de acuerdo a los resultados y de otros grupos de investigación, se llega a la conclusión que el colesterol contribuye a ser una característica importante en el desarrollo del cáncer. La acumulación y oxidación del colesterol promueve

respuestas inflamatorias y se considera un factor de riesgo importante para el inicio de la carcinogénesis (Kristine Pelton, 2012 2012).

JUSTIFICACIÓN

El Instituto Nacional del Cáncer (NCI/NIH) en el "Informe anual a la nación sobre el estado del cáncer, 1975-2012" comentó que la prevalencia de HCC ocupó el quinto lugar en hombres y el noveno en mujeres. Asimismo, informó entre 1999-2015 un aumento en la tasa de mortalidad en cáncer de hígado con un porcentaje de 1.6% en hombres y 2.7% en mujeres. Sin embargo, informes recientes respaldan que las tasas de incidencia y mortalidad de HCC están aumentando y esto podría estar relacionado con una alta ingesta de lípidos en la dieta (Cronin K. A., 2018). Los pacientes con NAFLD tienen cuatro veces más riesgo de HCC (María Guadalupe Castro-Martínez y Jesús Cenobio Ramírez-Martínez, 2012). Recientemente, se ha informado que hay entre 4 y 22% de los casos en pacientes con HCC y esto se atribuye al NAFLD (A. Tessitore, 2016).

La ingesta de colesterol puede actuar como un fuerte promotor patógeno en el desarrollo de HCC y ser el principal mediador tóxico en NAFLD, pero los mecanismos por los cuales este lípido actúa como carcinógeno permanecen desconocidos (Marengo et al., 2016) .

En el presente trabajo continué con la manipulación de ratones machos de la cepa C57Bl6 para realizar la generación de hepatocarcinoma celular (HCC) bajo un modelo de mutagénesis química en hígado con esteatohepatitis diferencial (triglicéridos o colesterol) y quería saber si tales sobrecargas lipídicas diferenciales en el hígado podrían comportarse como un potencial promotor del daño al ADN mediante análisis comparativo para encontrar aquellos vías de señalización que participan en la iniciación del tumor.

PREGUNTA DE INVESTIGACIÓN

¿Cuál es el efecto de la sobrecarga diferencial de lípidos en el hígado inducido por diferentes dietas esteatógenas, particularmente en el estado redox y la inestabilidad genómica en un modelo de hepatocarcinogénesis química?

HIPOTESIS

La sobrecarga diferencial de lípidos en el hígado de ratones C57BL6 puede inducir cambios en el estado redox celular que conducirá a la inestabilidad genómica diferencial.

OBJETIVO

Determinar el efecto de la sobrecarga diferencial lipídica en hígado en las etapas iniciales de la hepatocarcinogénesis por un agente químico.

OBJETIVOS PRINCIPALES

1. Evaluar la función hepática en ratones bajo diferentes dietas inducidas por esteatosis.
2. Evaluar si las dietas esteatogénicas tienen un efecto diferencial en las etapas iniciales del proceso de hepatocarcinogénesis.
3. Determinar si la sobrecarga diferencial de lípidos en el hígado tiene un efecto sobre las lesiones premalignas generadas por DEN.
4. Evaluar si la sobrecarga diferencial de lípidos en el hígado tiene un efecto sobre las vías de señalización relacionadas con las enzimas antioxidantes y reparadoras y el daño del ADN en las primeras etapas de la hepatocarcinogénesis química.

RESULTADOS

Uno de los objetivos fue evaluar si las dietas esteaogénicas presentan efectos diferenciales sobre la función hepática a partir de los 30 días con tratamiento, determinamos la proporción de peso cuerpo-hígado mostrando en la figura 3.A que el hígado de los ratones alimentados con las dietas HCD y HCDD mostraron un aumento en el peso del hígado en comparación con los otros grupos, sin embargo los ratones alimentados con dieta HCDD tenían un aumento significante. Sugiriéndonos que al presentar una acumulación de lípidos en el hígado promovería daño en la funcionalidad y en la exportación de lípidos.

Además se midieron marcadores de daño hepático como AST, ALT, LDH; en la cual en la figura 4.A hay niveles séricos de ALT y LDH elevados, tres veces más, en las dietas CWD, HCD, HCDD, HFD, HFDD, WD y WDD con respecto al grupo después de 30 días de tratamiento. Y claramente, la figura 4.B-C muestra una elevada concentración de AST en todas las dietas sin embrago, HFD mostró diferencia significativa respecto al control; percibiendo los mismos efectos de mayor daño en la dieta WD y WDD con niveles elevados de colesterol sérico y glucosa; deduciendo que las dietas esteatogénicas promueven lesiones en el hígado.

En la figura 6 se midió colesterol total en el hígado mediante el método O-phtaldehyde, en donde se mostró claramente un aumento significativo, hasta seis veces, de colesterol en hígado confirmando la presencia de esteatosis en hígado en todas las dietas esteatogénicas.

También se midió el contenido de proteínas carboniladas, considerados como biomarcadores de daño oxidante, en donde hubo un aumento de grupos carbonilos en dieta WD y WDD por lo que esto nos sugiere mayor presencia de daño oxidante.

Por lo que si hay daño oxidante se esperaba que se presentara daño en la reparación de ADN, y para ello se midió el contenido proteico de proteínas relacionadas con reparación al ADN y como se esperó en las dietas esteatogénicas hubo mayor contenido proteico de p-Chk1, p-Chk1, p-H2AX y Aurora A. Además se midió el contenido de p-H2AX por inmunofluorescencia y se mostró un incremento en las mismas dietas (Figura 9). También quisimos corroborar el daño específicamente del ADN a través de ensayos cometa promovida por la sobrecarga de colesterol, datos ya mencionados. Y se aprecia en la figura 10 mayor daño en dieta WD y WDD confirmando datos previos.

Además se midió el contenido proteico de proteínas relacionadas con el ciclo celular (Figura 11), los datos sugieren que las células se han dañado y que la reparación ante daños no es eficiente, siendo una característica de un proceso carcinogénico.

Estos resultados nos llevaron a la incógnita acerca del papel con la muerte celular en hepatocitos con carga diferencial esteatogénica, resultando que a concentraciones elevadas de colesterol (dieta) influye una resistencia ante muerte celular por apoptosis (figura 12).

Y por último, se realizó una correlación entre los niveles de colesterol en hígado y muerte celular por apoptosis a través de la actividad de caspasa 3 (Figura 13) indicando que una dieta alta en colesterol tiende a resistirse ante la muerte celular por apoptosis.

DISCUSIÓN

NAFLD ha adquirido relevancia en la última década como uno de los principales trastornos hepáticos que afectan a un número cada vez mayor de pacientes en todo el mundo (Friedman SL, 2018). Se sabe que la susceptibilidad del daño, debido a un mayor contenido de lípidos, es impulsado por el tipo de lípido en lugar de la cantidad (Montserrat Marí).

En el presente trabajo nos enfocamos en explorar las características del daño de diferentes dietas estetogénicas, particularmente abordando algunos de los determinantes clave en el inicio de un posible proceso mutagénico.

Se informó que solo las dietas esteatogénicas causan un desequilibrio en el estado redox celular, donde una de las principales consecuencias de este cambio en el estado redox es la oxidación de las proteínas, confirmado al correlacionar el aumento en el contenido de colesterol en el hígado (Figura 7) con el aumento en la oxidación de proteínas (Figura 6), donde el grupo WDD tiene la mayor acumulación de colesterol y oxidación de proteínas. Estos cambios en el estado redox provocados por el colesterol son los principales inductores del daño del ADN, como se muestra en el panel western blot (figura 8) y en la inmunofluorescencia de histona H2AX (Figura 9), lo que indica que los grupos experimentales en comparación con el grupo de control activan esta maquinaria de manera más eficiente ante el daño crónico. Confirmando con el ensayo cometa, otro marcador para el daño del ADN (Figura 10), confirma la exacerbación del daño genómico promovido por el alto contenido hepático de colesterol y el DEN, que permite una transformación celular y, por lo tanto, la generación de tumores.

Los resultados indican que la activación de las proteínas de respuesta al daño del ADN puede aumentar durante las primeras etapas de la tumorogénesis. Sin

embargo, la apoptosis en todos los grupos con dietas esteatógenas y DEN disminuye (Figura 12), lo que indica que la supervivencia celular va en aumento.

Por lo que el colesterol es un factor importante en la estabilización de la transformación, debido a que cuanto más concentración de colesterol hay, menos actividad de caspasa 3 se presenta (Figura 13); el colesterol es importante en la supervivencia celular y en el establecimiento del hepatocarcinoma celular.

CONCLUSION

Los datos confirman que el colesterol puede desempeñar un papel relevante en HCC y en la transformación celular, debido al incremento en los niveles de ROS y la disminución de apoptosis conduciendo al daño del ADN y dar inicio a la carcinogénesis. Nuestros hallazgos sugieren que el colesterol es un promotor tumoral al alterar el sistema de reparación del daño del ADN y promoviendo la progresión tumoral; por lo que la hipercolesterolemia debe ser sumamente monitoreada en pacientes con factores de riesgo a HCC.

ABSTRACT

Hepatocellular carcinoma (HCC) is the primary malignant liver tumor, and is the second leading cause of death in the world. It is closely related to non-alcoholic fatty liver disease, (NAFLD), but also to alcohol consumption, and virus infection, such as hepatitis virus B and C.

NAFLD is characterized by an increase in the deposition of lipids (hepatic steatosis) in the hepatocytes that could progress to steatohepatitis (NASH), fibrosis, cirrhosis and, eventually HCC.

The overload of lipids is characteristic of NAFLD, so the mobilization of lipids is determinant and indispensable in the initiation of the regeneration of this tissue and consequently in hepatocarcinogenesis.

Data of our group and other research groups have shown that cholesterol overload, tends to generate more damage than another kind of lipid.

The present work was aimed to figure out the relevance differential lipid overload in the liver in the initiation of the hepatocarcinogenesis.

14 days-old C57BL6 male mice were used in the study. Animals were fed with a high cholesterol diet, Western diet and high fat diet for 30 days, and were treated or not with a single dose of N-nitrosodyetilamine, to induce DNA damage and initiate a carcinogenesis process.

Data show that the presence of high content of cholesterol in the diet is sufficient to promote damage in the mechanisms related to impairment of DNA repair system, it promotes accumulation of mutations that progressively allows the development of cancer. Therefore, the presence of hepatic cholesterol overload is a risk factor for the development of liver cancer.

The following data suggest an important factor in carcinogenesis is the aberrant concentration of lipids as well as an imbalance in the physiological and cellular processes; as is the case with cholesterol, because is responsible for this imbalance in the liver.

These steatogenic diets (HCD, HFD and WD) promote an imbalance in the redox state, our data confirm it with the increase of cholesterol in the liver or, a high oxidation of proteins, DNA damage (verified through the activity of p-H2AX and comet assay, figures 8-11) and a low activity of caspase 3, as shown in the western diet, with and without DEN, with a content of 2,071% cholesterol.
So we conclude that cholesterol plays an important role in cell survival and is a strong promoter for the establishment of HCC.

ABBREVIATIONS

NAFLD: por sus siglas en inglés Non-Alcoholic Liver Disease, Enfermedad del hígado graso no alcohólico.

NASH: por sus siglas en inglés Non-Alcoholic Steatohepatitis, Esteatohepatitis no alcohólica

HCC: por sus siglas en inglés Hepatocellular carcinoma

MDC1: por sus siglas en inglés Mediator of DNA Damage Checkpoint 1, Mediador de daño al DNA checkpoint 1.

ATM: por sus siglas en inglés Ataxia-telangiectasia Mutated

ATR: por sus siglas en inglés Ataxia-telangiectasia and Red3 related

Gamma-H2AX: por sus siglas en inglés, the Histone H2AX, Histona H2AX.

DSBs: por sus siglas en inglés, DNA double-strand break, Rotura de doble cadena de ADN

Rap80: por sus siglas en inglés, protein recruitment to DNA double-strand breaks

Chk2: por sus siglas en inglés, Checkpoint kinase 2.

Chk1: por sus siglas en inglés, Checkpoint kinase 1.

p53: por sus siglas en inglés p53 gen, TP53, Guadian of the genome, guardían del genoma

p21: por sus siglas en inglés, cyclin-dependent kinase inhibitor (K. J. Thompson)CKI).

PCC: por sus siglas en inglés, Protein Carbonyl Content, Contenido de carbonilación de proteinas

Bcl-XL: por sus siglas en inglés BCL2- extra large, proteína relacionada con Bcl-2 de cadena larga.

Bax: por sus siglas en inglés BCL2-Associated X Protein, proteína X asociada a BCL2.

FA: por sus siglas en inglés Fatty Acid, ácido graso.

FFA: por sus siglas en inglés Free Fatty Acid, ácido graso libre.

CW: Dieta Chow

CWD: Dieta Chow con DEN

HCD: Dieta Hipercolesterolémica

HCDD: Dieta Hipercolesterolémica con DEN

HFD: Dieta alta en grasa

HFDD: Dieta alta en grasa con DEN

WD: Dieta Western

WDD: Dieta Western con DEN

DEN: por sus siglas en inglés, N-nitrosodietilamine

ÍNDEX

INDEX OF FIGURES AND TABLES

INTRODUCTION

THE LIVER

The liver is an amazing organ that sustains more than 500 different functions, such as bilirubin, porphyrin, bile acid, hormone, vitamin, amino acid, protein, lipid and lipoprotein metabolism, trace elements and the liver, biotransformation and detoxification function, alcohol degradation and acid-base balance (E. Kuntz, 2002). In humans, the organ is divided in two regions, the left and right lobe, which is separated by the falciform ligament (translucent). The liver is on average between 25 to 30 cm in width, 12 to 20 cm in length and 6 to 10 cm in thickness (E. Kuntz, 2002). The organ receives the blood supply from two sources: 80% comes from the portal vein, which originates in the spleen and intestine, and the remaining 20% is oxygenated blood from the hepatic artery (Sibulesky, 2013). There are many cell types in the liver that were identified based on morphology and activity features, so the liver is composed of parenchymal cells or hepatocytes, and non-parenchymal cells such as Kupffer, Ito or stellate (fat storing cells), Pit and endothelial cells, in addition, cholangiocytes are also remarkable cell implicated in the transport of homeostasis of the bile (Janie L. Baratta, 2009), all these cells are specialized in specific functions, leading to a well-organized organ that sustains all others in the body.

The hepatocytes are the main cell type in the organ. They are polygonal epithelial cells subjected to a continue damage due to the detoxification processes elicited by hepatocytes, for this reason these cells are constantly in proliferation, in order to reestablish hepatocyte population and optimal organ function.

In addition to hepatocytes proliferation, the organ can be healed by hepatic oval cells, which under certain noxious damage stimulus differentiate in both main epithelial liver cells, the hepatocytes and the cholangiocytes (YaryginK.N., 2017), which can be differentiated into hepatocytes and cholangiocytes

THE LIVER AS DETOXIFICATION ORGAN

The liver has no significant problem in driving detoxification process, even in acute damage, for example in an exacerbated alcohol consumption, but when the alcohol intake become chronic, the wound-healing process subjected by the liver enters in a short-cut, increasing and sustaining the wound-healing response, and this could be a serious problem, particularly when DNA has been damaged leading to mutations (L. Sahuquillo, 2011).

Chronic damage of the liver could lead to sustained inflammation, recruiting inflammatory cells that eventually provide an enhanced production of reactive oxygen species (ROS) (S. Reuter, 2010). This mechanism is independent of the etiology, hepatitis B and C virus, alcohol consumption, or lipid overload in the liver, could induce oxidative stress (F. M. Marcello Dallio, 2018)

ROS represent the most important molecules in the pathogenesis of many diseases, not only in the liver but in all the organs (Moreira, 2018; S. Reuter, 2010), certainly, the increment of ROS is strongly association to the initiation and progression of liver diseases, our group published that ROS could display ROS-mediated pro-survival signals (D. Clavijo-Cornejo & Gómez-Quiroz, 2013) leaving clear that ROS act as a kind of second messengers, and the noxious or beneficial effects depends of the amount, the place and the timing of ROS production.

Particularly, the lipid overload in the liver, has been extensively demonstrated that increases the ROS production and induces oxidative stress damage (Domínguez-Pérez M., 2016

; M. Marı´, 2006) (Dominique., 2007; L. Diesen D., 2011; López-Islas A., 2016) (Nuño-Lámbarri N., 2016) lipid-induced ROS have been positioned as a key molecular mechanism in NAFLD.

NON-ALCOHOLIC FATTY LIVER DISEASE

Although, the liver modulates glucose homeostasis and lipid metabolism, when the balance is disrupted can lead to NAFLD, this is relatively easy; in fact, one of the constants in liver damage is steatosis (Zou Y., 2006). NAFLD is the most common liver disease worldwide that can affect between 25 to 35% of the general population (Zelber-Sagi, Ratziu, & Oren, 2011). It is characterized by an increase in the deposition of lipids in absence of alcohol intake, in at least 5% of the hepatocytes, which progressively reaches an inflammatory state to non-alcoholic steatohepatitis (NASH), and could progress to fibrosis, cirrhosis and, eventually, HCC as previously stated (Andersen, 2015) (K. J. Thompson, 2013). It has been published that 10% of the patients with NAFLD develop NASH, and 8–26% of these can progress to cirrhosis, with a concomitant risk to develop HCC (A. Wree, 2011) (L. E. Gomez-Quiroz, 2016)

This disease is strongly associated with obesity and insulin resistance, so it is considered the liver component of the metabolic syndrome. Physiologically, presents an excess of fatty acids (FA), since already formed triglycerides are stored inside the hepatocyte in lipid vesicular structures, promote steatosis (Cortez-Pinto, 2016)

Nowadays, the risk of HCC associated with metabolic factors has been underestimated and the lack of research has delayed adequate treatments (Friedman S. L., 2018). In contrast, the systemic and hepatic molecular mechanisms involved in hepatocarcinogenesis induced by obesity and NAFLD, as well as the potential early markers of HCC, are being investigated (Marengo et al., 2016) however, nowadays, there is no convincing evidence regarding the mechanism of lipid-mediated liver damage in the initiation of HCC.

HEPATOCELLULAR CARCINOMA

HCC is the primary malignant tumor in the liver and is accepted as one of the major malignancies currently in the world (Kew, 2014). It is the second leading cause of death in the world. Also, is the most common form of liver cancer because is closely related to chronic hepatitis B and C virus, non-alcoholic fatty liver disease, alcoholic liver diseases and some hereditary metabolic disorders (Pascale et al., 2016).

HCC is the third most common cause of cancer worldwide, more than half a million cases worldwide are reported annually. The incidence shows gender susceptibility, being male more vulnerable to females in the ratio of 3:1 to 9:1 (Parkin, Bray et al., 2001). It has been estimated that 4 to 22% of HCC cases can be associated to NAFLD (A. Tessitore, 2016).

The cancer incidence rates from 2008 to 2014 decreased by 2.2% per year among men, but remained stable among women. In general, cancer mortality rates between 1999 and 2015 decreased by 1.8% per year among men and by 1.4% per year among women. The incidence rates in men during the most recent 5-year period (2010-2014) decreased in 7 of the 17 most common types of cancer, and death rates (2011-2015) decreased in 11 of the 18 most common types. The

incidence rates in women decreased in 7 of the 18 most common cancers, and death rates decreased in 14 of the 20 most common cancers. Mortality rates decreased, including the lungs and bronchi (men and women), colorectal (men and women), female breast and prostate. Mortality rates increased in liver cancer. This could be due to the fact that the intake of lipids in the diet increased (Cronin et al., 2018).

HCC is a multiphase process due to the interaction of DNA with carcinogens (like N-nitrosodiethylamine, DEN), oxidative damage or free radicals produced during biotransformation or inflammation, could induce DNA damage and alterations in signaling pathways directed to repair the damage (Dufour., 2016) (Mazzocca A., 2016). Damage can lead genomic instability which could lead to chromosome aberrations, oncogenic mutations, inhibition of tumor suppressors, overexpression of oncogenes and deregulation of multiple signaling pathways (N. Aravalli R., 2008) (R. Pascale M., 2016).

The liver has a remarkable regenerative capacity in the face of damage, the repair and regeneration of this organ is perhaps one of the key issue in the susceptibility to carcinogenesis. The carcinogenic process involves the transition from a normal cell to an initiated cell that eventually can raise a malignant tumor (Severi et al. 2010).

Two conditions are needed for cell initiation, the first one is the action of a carcinogenic compound, and the second one an increased capacity of proliferation, both condition are frequently presented in the liver as stated.

The cellular and molecular features that lead to HCC initiation and progression are not completely understood. There is evidence that the accumulation of mutations and genetic changes in preneoplastic hepatocytes causes a malignant transformation and leads to the development of HCC (R. N. Aravalli, 2012)

EXPERIMENTAL MODEL OF HEPATOCARCINOGENESIS

The carcinogenesis is multistep process characterized by have genetic alterations in activated signal transduction pathways and promoted malignant cells. For this reason some genetic alterations are accumulate during hepatocarcinogenesis (Zhao-Shan N., 2016)

One of the main animal model for the induction of hepatocarcinogenesis in mice is the use of the chemical N-nitrosodiethylamine (YaryginK.N.) (Khairy MA Zoheir, 2015), it can induce specific tumors depending the species, HCC is the most frequent tumor observed in this model, but also can induce cholangiocarcinoma or even both at the same time. It has been observed that the effect of DEN depends on the dose (Zoheir et al., 2015), even more, the International Agency for Research on Cancer (IARC) reported that after p.o. exposure, mice developed, in addition to the liver, esophageal and for stomach tumors (L. Vena, 1996).

There are many steps for DEN biotransformation; the first step is Cytochrome (CYP) P450-mediated α-hydroxylation, producing a α-hydroxylnitrosamine. The ethanol-inducible CYP2E1, and others P450 forms could hydroxylate NDEA. NDEA may form DNA adducts in the liver, and favors mutations. The CYP isozymes are responsible for the detoxification of NDEA, which makes biotransformation complex, the DEN biotransformation also provides significant increment in ROS production, with in addition, can also induce DNA damage (L. Vena, 1996).

The next step is the alkylation and repair; after NDEA is bioactivated to an electrophilic ethyldiazonium ion, it undergoes reaction with nucleophiles, including DNA bases, to form adducts. The adduct formation is dependent of each negative charge at each atom in the DNA, but it is impeded by double-stranded hydrogen

bonding. The greatest negative charge is found at cytosine N3. There are different repair mechanisms for adducts. AT repairs O^6-ethyldeoxyguanosine (O^6-EtdG) by the transfer of the alkyl group to a cysteine acceptor site on the protein. The activity of this repair enzyme, O^6-EtdG, is highest in liver. Whether DNA bases are ethylated and not repaired, it will be mutagenic if the adduct can interrupt base pairing. The O^6-EtdG and O^4-EtdT adducts are more mutagenic because they are potentially miscoding and are formed in greatest amounts (L. Vena, 1996).

DNA REPAIR RESPONSE

The DNA damage response (DDR) is the orchestrator of detects and repair DNA damage with arrest when the cell cycle is underway, to ensure maintenance of genomic stability (Bottai G., 2014). The DDR pathway may limit tumor development in its early stages by acting as a barrier for proliferation of aberrant cells. One of its functions is detect the onset of a lesion by activating signaling pathways and cell cycle control points, DNA repair and induction of cell death (M., 2014)

When not repaired properly, such damages may lead to mutations, deletions, insertions or chromosomal rearrangements upon DNA replication or cell division. To maintain the stability of the genome, cells trigger a specific network of cellular responses (Bottai G., 2014). In many tumors show functional loss or deregulation of key proteins involved in the DDR and cell cycle regulation, like p53, ATM, BRCA1/2. Deregulation of DDR pathways also contributes to the development of genomic instability; this is a characteristic of human cancers that can accelerate the genetic alterations, which drive tumors development (Wanga H., 2015)

Ataxia-telangiectasia Mutated (ATM) and Ataxia-telangiectasia and Red3 related (ATR) are members of the phosphatidylinositol 3-kinase-related kinase family (PIKK) of serine/threonine protein kinases (A., 2005).

In anthological conditions, ATR in the N-terminus also contains the binding site for ATRIP (ATR-interacting protein), that regulates the localization of ATR to site of DNA damage. ATM plays a critical role in DNA damage signaling originating at DNA double strand breaks (DSBs), whereas ATR responds to single-stranded DNA (ssDNA) regions are the most deleterious. ATM is one of the central kinases involved in cellular response to DSBs, which may arise (Wanga H., 2015).

ATM ACTIVATION AND DOWNSTREAM SIGNALING

In anthological conditions, the activation of ATM kinase can promote phosphorylation of targets, facilitating cellular responses to the damage. ATM target is histone protein H2AX. The phosphorylated form of H2AX, γH2AX, is the platform for recruitment of additional DDR factors and enhancement of signaling pathways (A., 2005). γH2AX recruits MDC1, which recruits ATM molecules to regions surrounding DSBs, for this reason there is an amplification of ATM signaling. Then BRCA1 is recruited to DSB site by binding ubiquitin chains via a protein complex containing Rap80 and Abraxas (Wanga H., 2015).

Activated ATM also phosphorylates substrates like NBS1, Chk2, Brca1, and p53, which are related in activation of cell cycle checkpoints. ATM-dependent phosphorylation stabilizes p53, which up-regulates p21 expression induces the G1 arrest, while Chk2 and Brca1 appear to be involved in S and G2/M checkpoints. (A., 2005)

ATR ACTIVATION AND DOWNSTREAM SIGNALING

ATR is one of the central kinases involved in DDR, is activated by single stranded DNA structures. ATR acts via downstream targets to promote DNA repair, stabilization and restart of stalled replication forks and transient cell cycle arrest. The recruitment of ATR/ATRIP complexes to these sites of replication stress and DNA damage is mediated by direct interaction of ATRIP with ssDNA-bound RPA (A., 2005).

ATR plays an important role in enforcement of the intra-S-phase progression and in response to DNA damage. Also, is a principal mediator of the G2/M cycle checkpoint to prevent the premature entry to cells into mitosis, before DNA replication is completed or in the presence of DNA damage (M., 2014).

DIETS

The consumption of compound with potential carcinogenic effects is a fundamental issue that should be addressed by government. Perhaps, this could be obvious in the case of alcohol heavy drinking, or the use of illegal drugs, but it is poorly surveilled the regular diet ingested by general population.

A lipid-rich diet plays an important role in NAFLD development (Angela M Zivkovic, 2007), and we have proved that cholesterol-rich diet promotes HCC (REF CRISTINA).

There is a general belief that changes in lifestyles, like modifications on diet, could lead to decrease the incidence rates of cancer (K. J. Thompson, 2013), definitively, this is true, as previously our group has reported (REF).

The type and amount of dietary components present during the initiation stage may have modulatory effects on hepatocarcinogenesis, particularly in those that require metabolic activation (K. J. Thompson, 2013).

This work was focused to understand if diets under different lipid formulation, could present a differentiated process of damage, particularly in the DNA, and if this could be relevant in cancer initiation. Our research team has previous evidence that supports that cholesterol induced ROS that eventually could lead to mutations (Domínguez-Pérez M., 2016; M. Marı´, 2006; Nuño-Lámbarri N., 2016).

HIGH LIPIDS EXPERIMENTAL DIETS

In the present study, we used the standard chow diet (CW), as control, and different experimental steatosis-induced diets (Table 1), such as Hypercholesterolemic diet (HCD), High Fat diet (HFD) and Western diet (WD); with the objective to characterize if there are some differences in the damage induced by DEN under the consumption of these diets.

HIGH FAT DIET (HFD)

Some reports have concluded that high glycemic indexes can induce steatohepatitis in rodents (C. Gaemers I., 2011; Zheng-Jie X., 2010) (Bortolotti, 2008; Zou Y., 2006). A report showed that short-term HFD (72 hours) was enough to induce

triglyceride accumulation in the liver along with the development of hepatic insulin resistance (Bortolotti, 2008).

As HFD can induce lipid peroxidation and contribute to DNA damage, it is considered a risk factor for HCC. However, some recent studies have interestingly suggested that HFD could delay the development of cancers in several organs such as breast, prostate, and liver (Xiao-Yan Duan, 2014)

Studies in animals and humans suggest that carbohydrates exert a more deleterious effect than fat (C. Oliveira L.S., 2014; K., 2017; Messier C., 2007

)

Carbohydrates, specifically simple carbohydrates, stimulate the accumulation of liver fat and oxidative stress. The HFD also increases hepatic triglyceride levels in humans as in rodents and triggers inflammation (Bortolotti, 2008)

Recent studies have indicated that nutrition status, as defined by such parameters as serum triglyceride, High Density Lipoprotein Cholesterol (HDL-C) and Low Density Lipoprotein Cholesterol (LDL-C), has profound impact on the initiation and progression of hepatic carcinogenesis (Xiao-Yan Duan, 2014).

WESTERN DIET (WD)

This kind of diet is characterized by containing a high percent of fat, fructose and cholesterol; it was mimics fast food style diets that have been implicated in NAFLD pathogenesis in humans.

People who eat frequently this type of diet (Zelber-Sagi et al.) and low intake of fresh fruits and vegetables, contains processed foods and is high in saturated and trans fats.

One study had demonstrated in many animals that intake western dietary were correlate with an increased levels of inflammation (A. Schultz, 2014). This type of diet can promote hepatic steatosis, increased liver weight and a high TAG content in liver. One characteristic of WD is the increase of visceral fat mass over time (P. Jegatheesan, 2016).

HIGH CHOLESTEROL DIET (HCD)

Dietary cholesterol is transported from gut to the liver, and from there is transported to other tissues. Cholesterol in extrahepatic tissues can be packed into HDL-C, a lipoprotein, to come back to the liver, this process is called Reverse Cholesterol Transport (Zhishi Yang, 2018).

Our group has extensively addressed the effects of cholesterol overload in the liver in mice that were fed with a high percent of cholesterol (2% cholesterol) in the diet, and we reported the high intake of cholesterol caused the appearance of more aggressive tumors in a model of chemical carcinogenesis with DEN, in mice of the C57Bl6 strain fed for eight months with a high cholesterol diet (HC 2% with 0.5% sodium cholate). Data revealed that liver presented mitochondrial dysfunction, oxidative stress and DNA damage (Enriquez-Cortina et al.).

Some studies have indicated that dietary cholesterol has an important role in the progression of liver fibrosis (T. Teratani, 2012), when there are excessive intake of cholesterol, the accumulation of lipids in hepatocytes can cause liver injury, because the liver synthesizes more triglycerides and this triglycerides cannot export them, consequently the triglycerides are accumulate in the hepatocytes causing NAFLD (Karagozian, Derdak, & Baffy, 2014).

Another report shows the importance of the cholesterol, besides the increase of this kind of lipid contributes to sensitize the hepatocytes to cytokine signaling, and leads to mitochondrial glutathione (mGSH) depletion contributing to hepatocyte cell death by apoptosis and necrosis (Garc´ıa-Ruiz C., 2016) (YaryginK.N., 2017).

THE TWO FACES OF CHOLESTEROL

The cholesterol is a fascinating molecule that is essential for life. It is a complex flat molecule with a great proportion of hydrophobic component, and just a small, but relevant polar head, a hydroxyl group, site where esterification occurs. Cholesterol is necessary for an efficient cellular function because it is required in many processes and for cellular biology. Cholesterol is a fundamental constituent of plasma membrane conferring fluidity, playing key role in lipid raft functionality and signal transduction, the endomembrane are poor in cholesterol, mitochondria load around 1-2% of cholesterol, and the overload of cholesterol lead to oxidative stress, as previously stated (Montserrat Marí). In addition, cholesterol is the precursor of bile acids, which are necessary for a proper digestion in the duodenum, and it is also precursor of many other biomolecules such as hormones (T. Teratani), taking in consideration these features, it is clear that cholesterol is essential for the life.

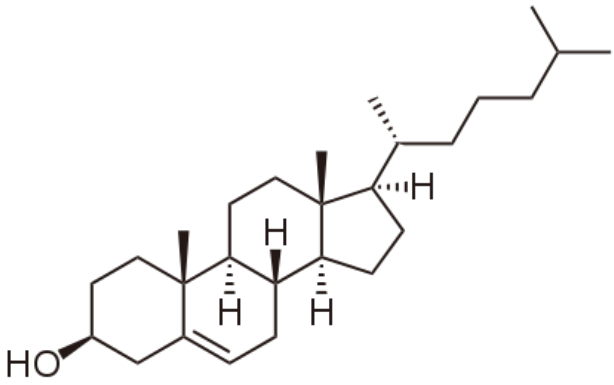

Ilustración 1. Cholesterol molecule structure

But, an increase or decrease of this complex lipid at cellular level could lead to serious problems.

It has been shown that if the cholesterol content of the cell membrane decreases, the activation of growth factors deteriorates and, therefore, the arrest of the cell cycle is triggered. CD44 is an adhesion molecule expressed by cancer cells and is also associated with lipid rafts, suggesting that cholesterol imbalance directly contributes to the progression of metastasis (Murai, 2012).

The cholesterol contributes to be an important feature in the development of cancer. The accumulation and oxidation of cholesterol promotes inflammatory responses and is considered an important risk factor for events like initiation of carcinogenesis (Kristine Pelton, 2012 2012).

A feature of NAFLD is the alteration of the lipid composition that can affect functioning of intracellular membranes as seen with free cholesterol loading in the mitochondria (A. Federico, 2012), these results show that cholesterol can perturbs the mitochondrial functions and promotes changes in the mitochondrial dynamic (Garc´ıa-Ruiz C., 2016).

JUSTIFICATION

The National Cancer Institute (NCI / NIH) reported in "Annual report to the nation on the status of cancer, 1975-2012" that the prevalence of HCC was ranked fifth in men and ninth in women. Likewise, it reported between 1999-2015 an increase in the mortality rate in liver cancer with a percentage of 1.6% in men and 2.7% in women. However, recent reports support that the incidence and mortality rates of HCC are increasing and this could be related to a high intake of lipids in the diet (Cronin K. A., 2018). Patients with NAFLD have increase in four times the risk of HCC (María Guadalupe Castro-Martínez & Jesús Cenobio Ramírez-Martínez, 2012). Recently, it has been reported that there is between 4 to 22% of cases in patients with HCC and this is attributed to the NAFLD (A. Tessitore, 2016)

It is well known that it is the kind of lipid rather than the amount of it, what marks susceptibility to liver damage (Mari et al. 2006).

Mexican diet is particularly elevated in cholesterol content, approximately 10 times more than Mediterranean diet (María Concepción Gutiérrez Ruiz & Natalia Nuño Lámbarri, 2012). Although the impact of high fat diets or carbohydrate-rich ones have been characterized in the development of NAFLD, the effects on the health of the liver of a diet high in cholesterol have been neglected.

Lipid overload is associated with a poor prognosis in HCC; gene expression of lipogenesis-related proteins or enzymes shows a subtype of HCC with aggressive phenotype and poor prognosis (Calvisi et al., 2011) (Kaposi-Novak et al., 2006). In particular, cholesterol intake may act as a strong pathogenic promoter in the development of liver cancer as well as being the principal toxic mediator in NAFLD,

but the mechanisms by which this lipid acts as a carcinogen remains unknown (Marengo et al., 2016).

The effect of a single dose of the carcinogenic agent diethylnitrosamine (DEN, 10ug/kg) and a diet for eight months with a high cholesterol content, resulted in animals having more tumors and larger size and hypervascularization, effects that we did not see in the animals that only received the DEN and standard balanced diet (Enriquez-Cortina et al.)

In the present work I continued with the manipulation of male mice of the strain C57Bl6 in order to carried out the generation of cellular hepatocarcinoma (HCC) under a model of chemical mutagenesis in the liver with differential steatohepatitis (triglycerides or cholesterol) and I wanted to know whether such differential lipid overloads in the liver could behave as a potential promoter of DNA damage by comparative analysis to find those signaling pathways that are involved in tumor initiation.

RESEARCH QUESTION

Which is the effect of differential lipid overload in the liver induced by different steatogenic diets, particularly on the redox state and genomic instability in a model of chemical hepatocarcinogenesis?

HYPOTHESIS

The differential overload of lipids in the liver of C57BL6 mice may induce changes in the cellular redox state that will lead to differential genomic instability.

AIM

To determine the effect of differential lipid overload in the liver at the initial stages of the hepatocarcinogenic process by a chemical agent.

SPECIFIC AIMS

1. To evaluate the hepatic function in mice under different steatosis-induced diets.
2. To evaluate whether the steatogenic diets have a differential effect on the initial stages of the hepatocarcinogenesis process.
3. To determine if differential lipid overload in the liver has an effect on premalignant lesions generated by DEN.
4. To evaluate whether differential lipid overload in the liver have an effect on signaling pathways related to antioxidant and repair enzymes and DNA damage in the early stages of chemical hepatocarcinogenesis.

MATERIALS AND METHODS

All chemicals and reagents were from Sigma-Aldrich (Saint Louse MO, USA), otherwise is indicated.

EXPERIMENTAL DESIGN

C57BL/6 male mice were purchased from Jackson Laboratory (Bar Harbor, Maine, USA) and were maintained in pathogen-free conditions with controlled temperature and humidity on a 12 h light-dark cycle in the animal facility at the Universidad Autonoma Metropolitana. The experimental protocols used were approved and performed in accordance with the Animal Care Committee of the Universidad

Autonoma Metropolitana and the NIH Guide for the Care and Use of Laboratory Animals.

Forty animals were randomly separated in groups of 5 mice and they were fed with each diet used in the study:

Group A Chow diet with isotonic saline solution;

Group B Chow diet and treated with DEN;

Group C HC diet with isotonic saline solution;

Group D composed of male mice fed HC diet and treated with DEN;

Group E High Fat diet with isotonic saline solution;

Group F High Fat diet and treated with DEN;

Group G Western diet with isotonic saline solution;

Group H Western diet and treated with DEN.

Animals under DEN treatment were injected by intraperitoneal injection (i.p.) 10ug/kg with DEN. Not treated animals were injected with saline solution. Animals were euthanized at 30 days after DEN injection.

EXPERIMENTAL DIETS

The figure 2 shows the composition of all the diets that we used in the present work in terms of lipids, carbohydrates, protein and another components of each differential steatogenic diet. Animals were provided with diets and drinking water ad libitum.

The composition of each diet, according to the technical sheet was as follow.

STANDARD REGULAR CHOW DIET (PURINA 5001)

This type of diet was characterized by content 23.3% of carbohydrate, 17.10% of protein, 5% of protein and 0.012% of cholesterol; some amino acids like 1.110% of alanine, 1.08% of arginine, 1.74% of aspartic acid, 0.28% of cysteine, 3.79% of glutamic acid, 0.85% of glycine, 0.57% of histidine, 1.02% of isoleucine, 2.10% of leucine, 1.10% of lysine, 0.75% of methionine, 1.06 % of phenylalanine, 1.67% of proline, 1.13% of serine, 0.84% of threonine, 0.22% of tryptophan, 0.81% of tyrosine and 1.15% of valine; some fatty acids like 1.47% of linoleic acid, 0.14% of linolenic acid, 0.82% of total saturated and 1.06% of total monosaturated; some vitamins like 0.4mg of biotin, 31.9mg of riboflavin, 31.4mg of thiamin, 20mg of Vitamin B12(IU/Kg), 5.757 mg of Vitamin D3(IU/Kg), 136 mg of Vitamin E(IU/Kg).

HYPERCHOLETEROLEMIC DIET (HC)

HC diet was manufactured on the basis of the standard regular rodent diet (Chow # 5001 from Purina) it was characterized by content 23.3% of carbohydrate, 17.10% of protein, 5% of protein; some amino acids like 1.110% of alanine, 1.08% of arginine, 1.74% of aspartic acid, 0.28% of cysteine, 3.79% of glutamic acid, 0.85% of glycine, 0.57% of histidine, 1.02% of isoleucine, 2.10% of leucine, 1.10% of lysine, 0.75% of methionine, 1.06 % of phenylalanine, 1.67% of proline, 1.13% of serine, 0.84% of threonine, 0.22% of tryptophan, 0.81% of tyrosine and 1.15% of valine; some fatty acids like 1.47% of linoleic acid, 0.14% of linolenic acid, 0.82% of total saturated and 1.06% of total monosaturated; some vitamins like 0.4mg of biotin, 31.9mg of riboflavin, 31.4mg of thiamin, 20mg of Vitamin B12(IU/Kg), 5.757 mg of Vitamin D3(IU/Kg), 136 mg of Vitamin E(IU/Kg). This kind of diet was different than CW diet because it was supplemented with 1% of cholesterol.

HIGH FAT DIET (HFD)

This type of diet was characterized by content 27% of protein grams, 46% of carbohydrate grams, 1.958% of cholesterol, 27% of fat grams, 200 gm of Casein, 3 gm of L-Cystine, 72.8 gm of Corn Starch, 100 gm of Maltodextrin 10, 172.8 gm of Sucrose, 50 gm of Cellulose (BW200), 25 gm of Soybean Oil, 177.5 gm of Lard, 10 gm of Mineral Mix S10026, 13 gm of DiCalcium Phosphate, 5.5 gm of Calcium Carbonate, 16.5 gm of Patassium Citrate, 10 gm of Vitamin Mix V10001, 2 gm of Choline Bitartrate, 0.05 gm of FD&C Red Dye #40.

WESTERN DIET (WD)

Western diet characterized by content 22% of protein, 48% of carbohydrates and 30% of Fat, 2.071% of cholesterol; some minerals like 0.90% of calcium, 0.63% of phosphorus, 0.19% of sodium, 0.21% of magnesium, 0.97 of potassium; some fatty acids like 0.02% of C14:0, 0.45% of C16:0, 0.02% of C16: 1, 0.19% of C18:0, 1.07% of C18:1, 2.12% of C18:2, 0.26% of C18:3, 0.02% of C20:0; some amino acids like 1.71% of lysine, 0.73% of methionine, 0.82% of Met+Cys, 0.93% of threonine, 0.27 of tryptophan, 0.76% of arginine, 0.66% of histidine, 1.42% of valine, 1.09% of isoleucine, 2.05% of leucine, 1.11% of phenylalanine, 2.22% of phe+tyr, 0.43% of glycine, 4.69% of glutamic acid, 1.55% of aspartic acid, 2.39% of proline, 0.68% of alanine and 1.24% of serine.

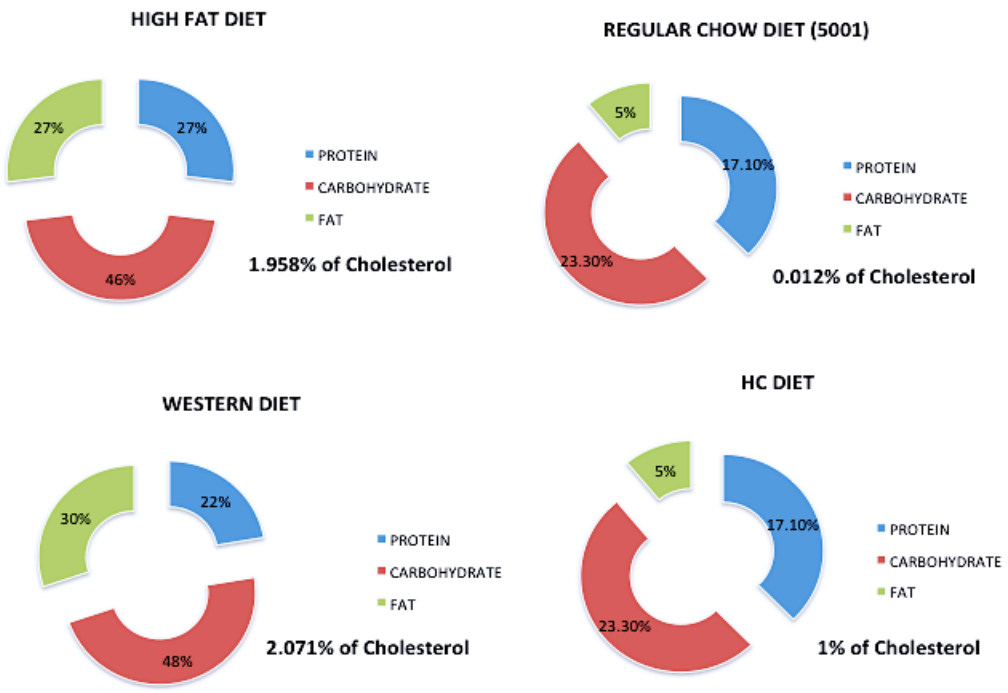

Ilustración 2. Composition of the experimental diets used in the study. Composition of Chow, Hypercholesterolemic, High Fat and Western diet used in this experimental model.

HEPATOCARCINOGENESIS MODEL (N-NITROSODIETHYLAMINE)

In order to investigate the changes that occur in the different steatogenic diets, 15-day old male mice were injected with DEN (N-Nitrosodiethylamine) two days after the diets were provided ad libitum. They were sacrificed at 30 day.

Animals were anesthetized by the intraperitoneal route with Avertin (2,2,2-tribromoethanol, Sigma, USA), the abdominal surface was cleaned with 70% ethanol and an incision was made in the abdomen to expose the liver; the next step were obtaining liver tissue and blood. The livers were respected, weighed and photographed.

LIVER FUNCTION TEST

Blood samples were obtained from the orbital venous plexus under Avertin (2-2-2 tribromoethanol) anesthesia before sacrifice. Serum activity of aspartate aminotransferase (AST), alanine transferase (ALT), and alkaline phosphatase (ALP) were determined by automated method using Reflovet Plus (Roche, Mannheim, Germany).

CHOLESTEROL DETERMINATION

For total cholesterol determination, 10 mg of liver was saponified with alcoholic KOH in a 60 °C heating block for 30 min. After the mixture had cooled, 3 ml of hexane and 600 μl of distilled water was added and shaken to ensure complete mixing. Appropriate aliquots of the hexane layer were evaporated under nitrogen with vacuum (Speed Vac, Savant, Cranbury, NJ US) and used for cholesterol measurement with O-phthalaldehyde dissolved in acetic acid (0.5mg/ml). After that sulfuric acid (1ml) was added and then read at 550 in spectrophotometer (Rudel & Morris, 1973).

GLYCEMIA

Blood glucose concentration was determined by Accu-Chek Performa meter to quantitatively measure glucose in blood, following manufacturer´s instructions.

PROTEIN QUANTIFICATION

The protein content was determined by using the bicinchoninic acid method (BCA, Pierce, Thermo Fisher Scientific.), following the manufacturer's instructions.

WESTERN BLOTTING

Whole liver homogenate was prepared in ice-cold PBS using dounce homogenizer. The cellular pellet was suspended in 200 µl of M-PER (Pierce Chemical, Rockford, IL); lysis buffer was supplemented with proteases and phosphatases inhibitors (Roche). Lysis was performed on ice for 15 min, and cell debris was removed by centrifugation at 13,000xg at 4°C for 10 min, supernatant was recovered (Hernandez et al., 2015). Total protein concentration was determined using a bicinchoninic acid (BCA) kit (Pierce Thermo Scientific, Rockford, IL), according to the manufacturer's instructions. Proteins were separated by electrophoresis using 10% gradient Duramide precast gels (Cambrex, Rockland, ME) and transferred to a polyvinylidene difluoride (PVDF) membranes (Invitrogen, Carlsbad, CA) (Clavijo-Cornejo et al., 2013). Immunodetection was performed using antibodies listed in Table 1. The membranes were revealed with 1 mL of luminescent substrate (SuperSignal® West Pico Substrate, Pierce), the bands were quantified by densitometry using the photoducumentador Gel logic 1500 (Kodak).

Antibody	Brand	Catalogue number	Second Antibody
Bax	Abcam	Ab5714	Mouse
Cdk 4	Santa Cruz	sc- 749	Rabbit
Cdk 6	Santa Cruz	sc- 7181	Rabbit
Cdk 2	Santa Cruz	sc- 748	Rabbit
Cyclin D1	Santa Cruz	sc- 753	Rabbit
Cyclin A	Santa Cruz	sc- 271682	Mouse
P21	Santa Cruz	sc- 6246	Mouse
Bcl-xl	Santa Cruz	sc- 8392	Mouse
Aurora A	Cell Signaling	4718s	Rabbit
p-Chk1	Cell Signaling	2348	Rabbit
p-Chk2	Cell Signaling	2197	Rabbit
p-H2AX	Abcam	Ab11174	Rabbit

Tabla 1. Specific antibodies used in Western Blot. Specific antibodies were related to DNA damage (p-Chk1, p-Chk2, Aurora A, p-H2AX); survival (Bcl-xL, Bax) and cell cycle (Cyclin A, cyclin D1, cdk2, cdk4, cdk6, p21) proteins.

IMMUNOFLUORESCENCE

The tissues were seeded in collagen-treated Lab-Tek chambers, after the intake with different steatogenic diets and the exposure with DEN the cells were fixed with 10% neutral formalin in PBS for 10 minutes at room temperature and were blocked with a solution of 5% bovine serum albumin (BSA) and 3% Triton X-100 for 30 minutes. Then the tissues were washed with PBS-tween for 30

minutes. A specific antibody was added to gene related to DNA repair response (p-H2AX, Table 1), because p-H2AX is a hallmark of DNA repair response and was associated with malignancy and poor prognosis of HCC. The antibody was incubated overnight at 4ºC in humid chamber. Then the tissues were washed with PBS-Tween for 5 minutes and followed by incubation with the corresponding secondary antibody conjugated to Alexa for 1h at room temperature in the dark. The tissues were washed again with PBS-Tween and incubated with DAPI for 5 minutes. Finally, the tissues were examined using the Carl Zeiss LSM 780 NLO confocal microscope (Carl Zeiss, Inc., USA).

PROTEIN CARBONYL CONTENT

The protein carbonyls of the samples were measure using the Cell Biolabs Oxiselect Protein Carbonyl fluorometric, following the manufacturer´s instructions. The cells were centrifugate at 1,500-x g for 5 minutes at 4ºC and it were resuspended in cold 1X Sample Diluent followed with 1X Protein Carbonyl Fluorophore solution. The Fluorophore binds to the protein carbonyl group in a 1:1 ratio. Proteins are then TCA precipitated and free Fluorophore is removed by washing the protein pellet with acetone for three times. The samples were dried out thoroughly for 1 hour. After were dissolving with Protein Solubilization Solution and added diluted Assay Diluent to each tube. In each well, 10 µg of protein tissue was loaded and the final absorbance was read at 485/538nm filter set. The protein carbonyl levels (nmol/mg) were calibrated against oxidized protein standards provided.

COMET ASSAY

This method is responsible for measuring the DNA damage on samples. The tissue preparation consisted in chop tissue into large pieces and it was suspended with ice-cold 1X PBS, then the pellet of cells (75 uL) were combined with Agarose (125ul), since it was added in slides. This mix were treated with lysis buffer (for 30 minutes at 4ºC) and alkaline solution (for 20 minutes at room temperature) in the dark, before the samples were electrophoresed (SCGE) in a horizontal chamber to separate intact DNA from damaged fragments, producing a classic "comet tail" shape. The power supply was adjusted to 1 volt per cm (electrode measured to the electrode) for 20 minutes in a cold temperature. There were viewed slides by epifluorescence microscopy (maximum excitation and emission are respectively 494nm/521nm) by means of staining with Diamond Nucleic Acid Dye, (Promega Inc).

CASPASE 3 ACTIVITY

Caspase 3 activity was quantified using the caspase 3 synthetic fluorogenic tetrapeptide substrate (MW: 675 Daltons, purity ≥98%) Ac-DEVD-AMC (BD Pharmingen) using fresh liver tissue of each sample. Is used to identify and quantify the activity of capase 3 in cell lysates apoptotic. Caspase 3 cuts the tetrapeptide between D and AMC, releasing the fluorescent AMC to be quantified in cell lysates by ultraviolet (UV) spectrofluorometry (380nm excitation and 420 - 460 nm emission). We measure by Cytation™ 5 Cell Imaging Multi-Mode Reader combines automated digital widefield microscopy with conventional multi-mode microplate.

STATISTICAL ANALYSIS

The results are presented as the average of at least three independent experiments carried out by triplicate. For the comparison of means of different groups, we performed a one-way ANOVA (and nonparametric test), and Pearson correlation. P values < 0.05 were considered statically significant.

RESULTS

LIPID OVERLOAD DUE TO HIGH FAT DIETS INDUCED LIVER DYSFUNCTION.

First, with the purpose to evaluated if the steaogenic diets present differential effects on hepatic function from 30 days of intake of the different diet already mentioned, we determined the liver to body weight ratio extirpating the organ and weighed. The data showed in figure 3.A the importance of intake a high concentration of cholesterol in the liver. The liver of the mice fed with the HCD and HCDD diets showed an increase in liver weight than mice fed with CW, CWD, HFD, HFDD, WD and WDD groups after 4 weeks of experimental period, but the mice fed with HCDD diet had a significant increase. These data suggest that the liver presents high accumulation of lipids, which can promote damage in the functionality and for this reason is not possible for the liver to export it like normally. Besides, in figure 3.B, the macroscopic observation showed a pale color (what is characteristic of steatosis) in mice with intake of HCD, with and without DEN, compared with the other diets; besides, these mice presented a gallbladder atrophy and lithiasis. Nevertheless, the mice fed of standard diet (CW), showed a characteristic reddish coloration. Thus, the increase in the size and weight of the liver did not modify the body weight.

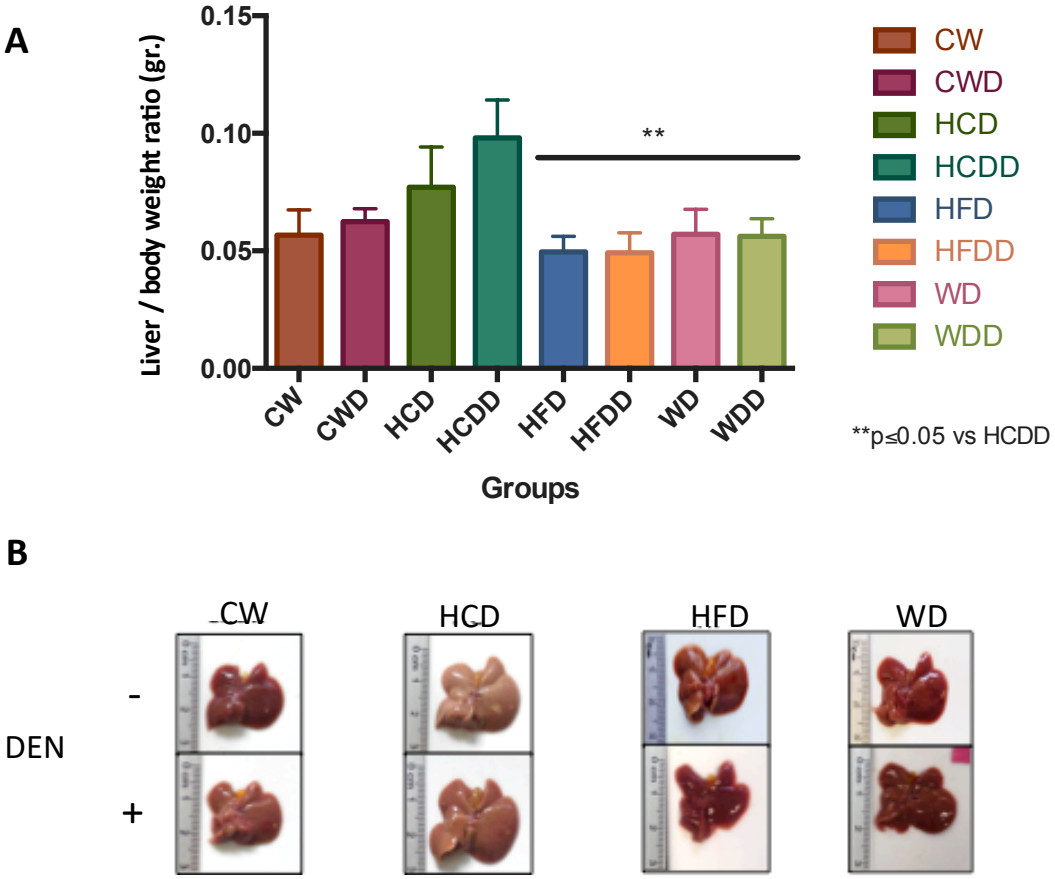

Ilustración 3. Lipid Overload Due To High Fat Diets Induced Liver Dysfunction and Increase of Liver Weight. Determination of liver to body weight ratio **(A).** The macroscopic inspection of different steatogenic diets in the liver of 30 days of treatment **(B).** Each experiment was evaluated with all the aforementioned diets. The images are representative of at least five independent experiments. Values are mean ± standard deviation of five mice. **P <0.05. Vs CW* can indicate us clinical aspects about the hepatic injury (characteristic steatotic).

THE ACCUMULATION OF LIPIDS WERE INDUCED BY A HIGH INTAKE OF CHOLESTEROL

In order to determine the impact of experimental diets in the liver, we quantified the serum concentration of markers of liver damage such as transaminases AST, ALT

and LDH. In the figure 4.A, the data shown the ALT serum levels of CWD, HCD, HCDD, HFD, HFDD, WD and WDD show an elevation of three times the activity of ALT and LDH regarding to control group after 30 days of treatment. While the figure 4.B-C shows high AST activity levels in all the steatogenic diets, but with the HFD diet showed a high significant difference compared with the control group; this suggest the importance of all steatogenic diets can promote injury in the liver compared with the control after 30 days of treatments.

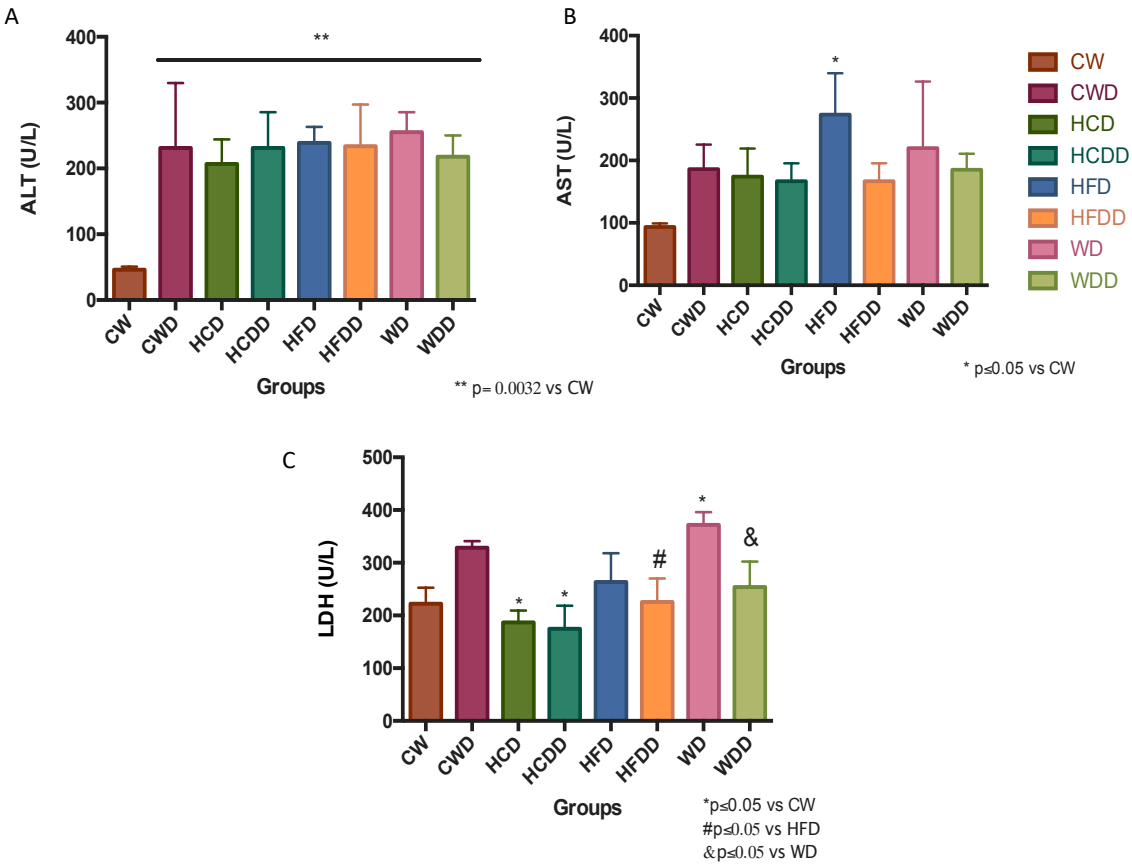

Ilustración 4. Cholesterol Overload Promotes Liver Damage. The activity of **(A)** Alanine aminotransferase (ALT), **(B)** Aspartate transaminase (AST) and **(C)** Lactate dehydrogenase (LDH) in liver tissue. Data are shown as mean ± standard deviation. **P ≤0.05 vs CW; #P ≤0.05* vs HFD; &P *≤0.05 vs WD.* Each column represents the mean ± SEM of at least five independent experiments. The values were determined by one-way ANOVA. **P ≤ 0.05 vs. CW; #P≤ 0.05 vs. HFD; &P ≤ 0.05 vs. WD*.

To confirm the presence of steatosis and changes in normal liver processes, the serum cholesterol and glucose concentration was evaluated of each group. In the table 2 we measured the levels of serum cholesterol, these data suggest that in HFD and WD diets, with and without DEN, the levels were two times higher than CW, CWD, HCD and HCDD after 30 days of intake (Table 2).

Also, in the figure 5, we measured the fasting blood glucose. Changes in blood glucose levels were examined next. The levels were higher in WD and WDD diet compared to the control group. Strikingly, whereas the blood glucose concentration in HCD, HCDD, HFD and HFDD groups decreased two-thirds with significant difference compared to the chow concentration 200 mg/dL.

DIETS	T-Cho
CW	<50 mg/dl
CWD	40 mg/dl
HCD	<50 mg/dl
HCDD	51 mg/dl
HFD*	99 mg/dl
HFDD*	98 mg/dl
WD*	86 mg/dl
WDD*	<50 mg/dl

Tabla 2. Cholesterolemia Determination. Measures of total cholesterol in serum with the different steatogenic diets at 30 days of treatments in liver tissue. These data were determined by the use of specific test strips by automated method using Reflovet Plus (Roche, Mannheim, Germany). Each data represents the mean ± SEM of at least five independent experiments *$P \leq 0.05$ *vs CW.*

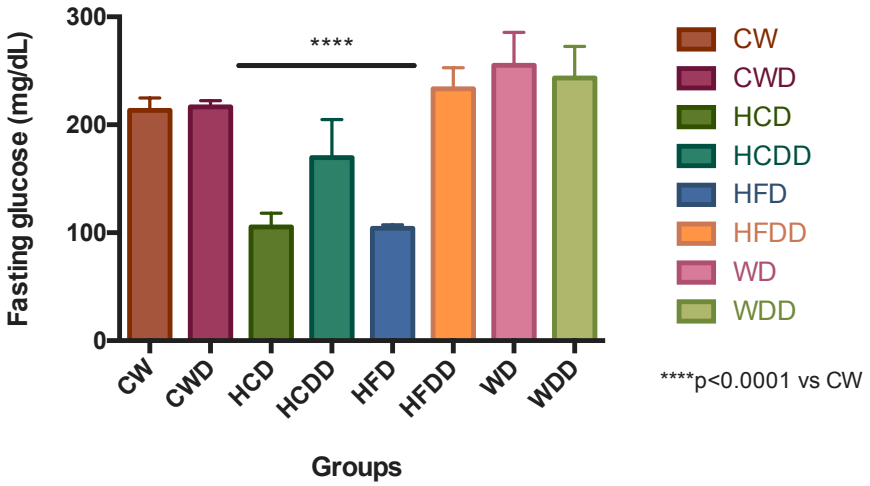

Ilustración 5. Glycemia In Mice Under Different Treatments. Fasting glucose was significantly higher in HCD, HCDD and HFD vs CW, control group. Each column represents the mean ± SEM of at least five independent experiments *, $p \leq 0.0001$ vs CW.

HIGH CHOLESTEROL INTAKE INDUCES STEATOSIS.

In order to figure out if the different experimental diets, for 30 days of treatment, induce cholesterol overload specifically in liver of the male mice in the C57BL6 strain, we proceeded to measure this kind of lipid in the liver tissue by the O-phthaldehyde method. The figure 6 shows that all diets had a significant increase of up to six times in the total cholesterol content, specifically in liver, compared with CW control group, being consistent with previous data. These data confirm the liver steatosis.

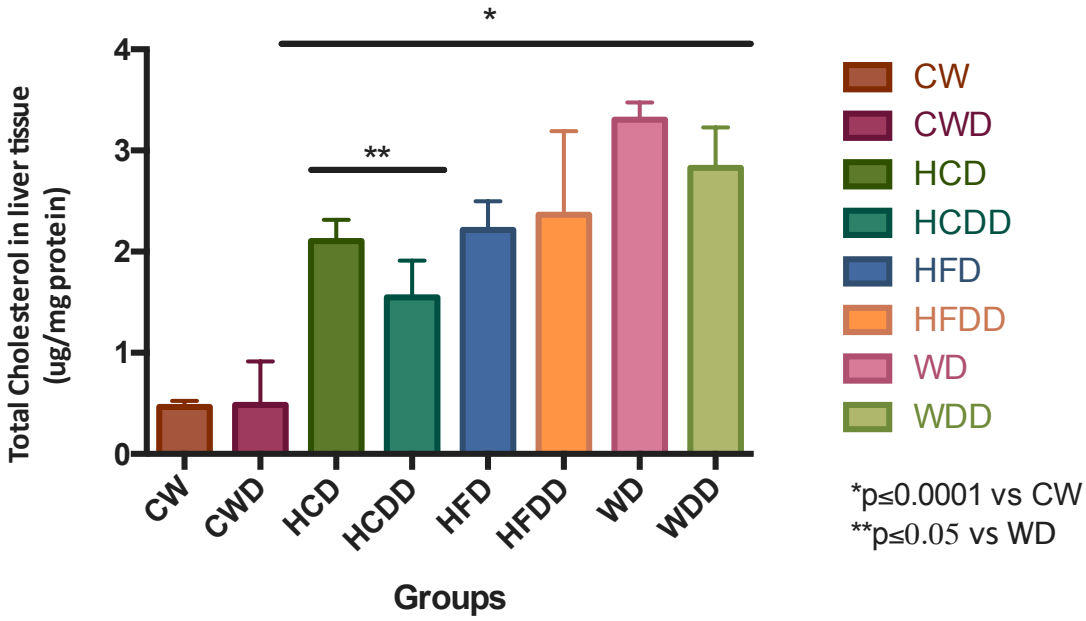

Ilustración 6. High Content of Cholesterol In Liver Tissue. Cholesterol was measured as indicated in materials and methods by using the O-phthaldehyde (OPA) method. Each column represents the mean ± SEM of at least five independent experiments *, $p \leq 0.0001$ vs CW; **$p \leq 0.05$ vs WD.

OXIDATIVE STRESS CAN BE PROMOTED BY CHOLESTEROL OVERLOAD.

Protein carbonyl content (PCC) is one of the most widely used oxidative markers and increased levels of protein carbonyls have been reported in patients with diverse disease. To measure protein oxidation, we utilized a fluorescence-based protein carbonyl assay. Data show WD and WDD induce more formation of carbonylated groups in proteins, indicating protein oxidative modification in these diets, WD induced more effects. A comparison between diets and those with DEN show a high increase of PCC in treatments under DEN, indicating that DEN enhaces the oxidative damage.

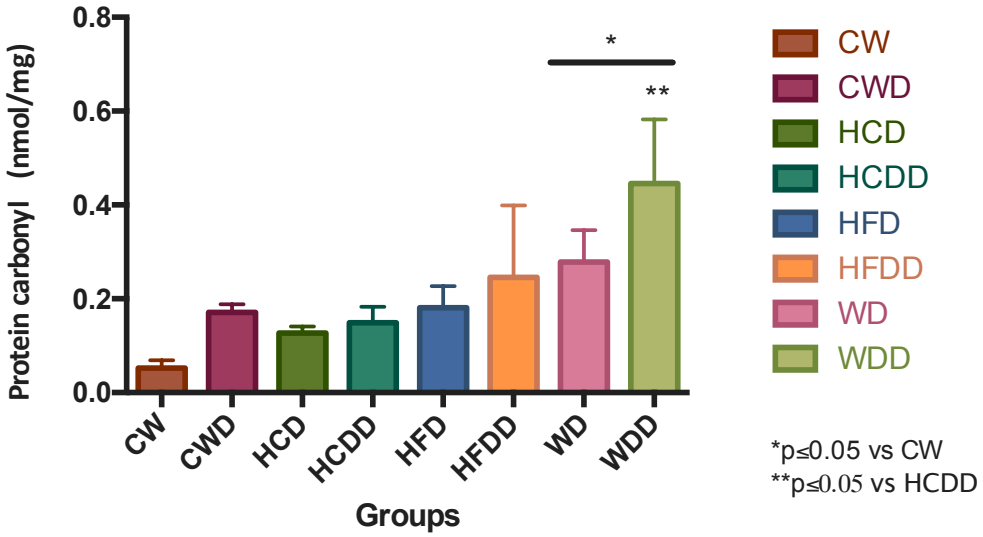

Ilustración 7. Antioxidant Response Can Be Promoted By Cholesterol Overload. Evaluation of the protein carbonyl (CO) groups for liver tissue after 30 days of treatment, the tissue was sonicated in 25mM HEPES. Correlation between carbonyl concentrations, expressed as nmol/mg protein in plasma samples measured by colorimetric methods. Each column represents the mean ± SEM of at least five independent experiments *, $p \leq 0.05$ vs CW; ** $p \leq 0.05$ vs HCDD.

HIGH LIPIDS EXPERIMENTAL DIETS PROMOTE THE DNA REPAIR.

The protein content of enzymes related with the DNA repair response were measure and we found that all the proteins related with this signaling pathway were increase such as p-chk2, p-chk1, p-H2AX and Aurora A, with 30 days of treatment in the mice that were fed with HCD, HCDD, HFDD, WD and WDD compared with the control group (Figure 8). However, in CWD and HFD diets showed a lower protein content of this DNA repair damage on 30 days after the treatments.

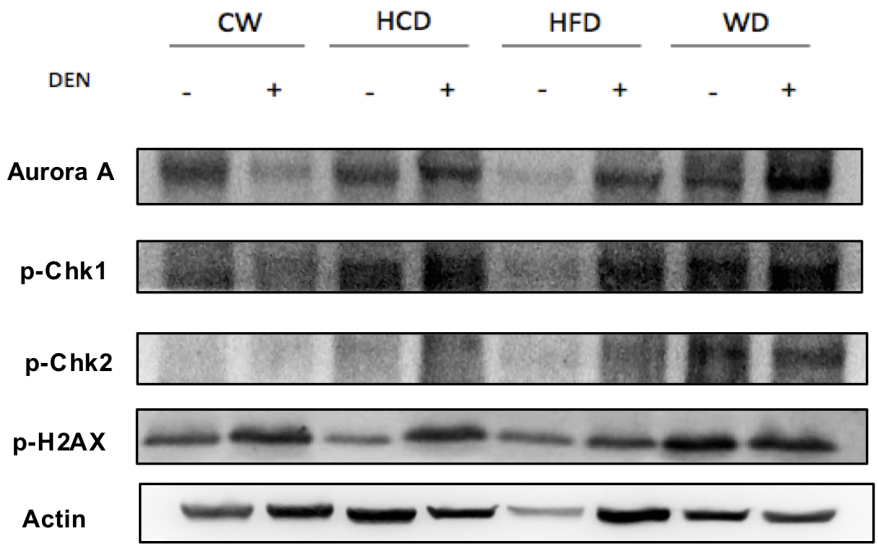

Ilustración 8. Expression Of DNA Repair Response Proteins Caused By Cholesterol Overload. The total protein was isolated from liver tissue of mice fed with the diets already mentioned and taken performed the analysis by Western blot as specified in material and methods. The image is representative of at least three independent experiments. Actin was used as loading control.

To confirm these results, we determined the content of the histone 2AX (p-H2AX) by immunofluorescence, a marker of DNA damage. In the figure 9 the data showed an increment of H2AX phosphorylation in CWD, HCDD, WD and WDD diets compared with the other groups and confirming more DNA damage than the other diets.

Interestingly, data demonstrated an additive effect of the carcinogen DEN plus in the HCDD and WDD was observed in the content of pH2AX.

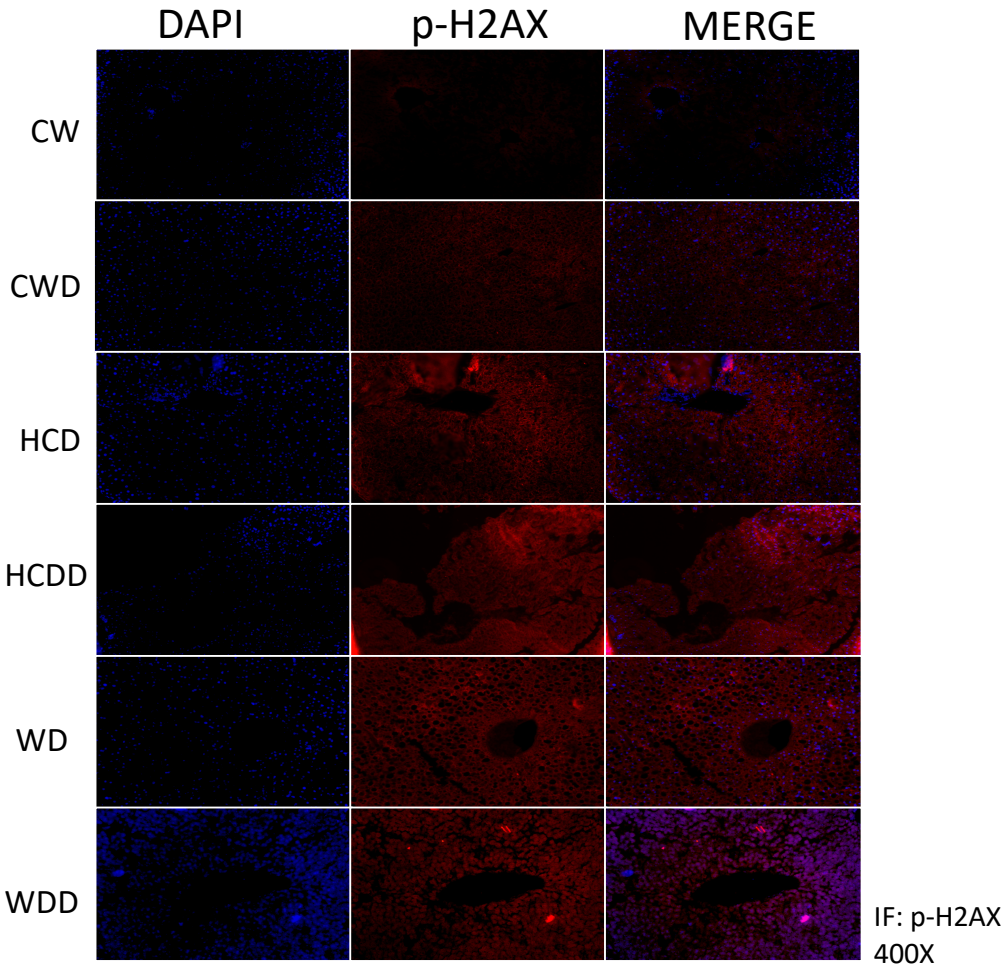

Ilustración 9. High Content Of Cholesterol Induces The Activation Of The Histone 2AX. Samples of 30 days of treatment. Hepatic sections (5um) embedded in paraffin were treated with anti pH2AX. Representative images of at least three different animals.

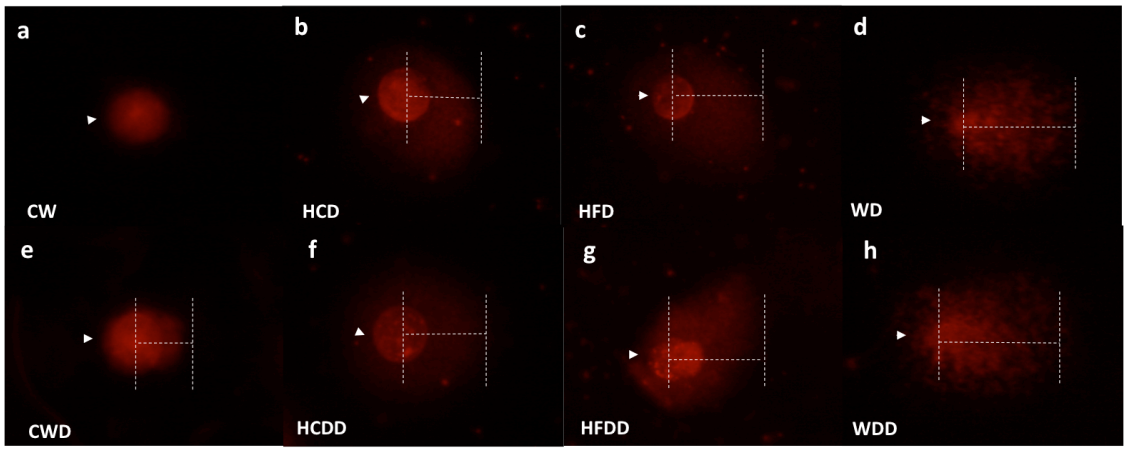

Ilustración 10. Evaluation Of DNA Damage By Comet Assay. Arrowhead: head of the comet; dotted lines: length of the comet tail. a) Without damage; e) low damage; b), c), f), g) moderate damage; d), h) high damage. 400X. Images are representative for at least three independent experiments.

According to the data obtained, is important to investigate what about with the DNA damage respect with the injury promoted by cholesterol overload. In figure 10, the data show, that there are more damage in the WD with or without DEN, compared with the others diets and the control group. We considered, three levels of damage: low, moderate and high damage.

Chow diet did not present DNA damage (Figure 10.a) compared with CWD; it can be mentioned that there is an additive damage caused by exposure to DEN, however, the damage presented is lower than the other diets (Figure 10.e)

The HCD and HCDD diet presented moderate damage, although HCDD presented greater damage than HCD, this was not representative (Figure 10.b, f, respectively)

Likewise, HFD and HFDD presented moderate damage (Figure 10.c, g, respectively), again, the exposure to DEN produced greater damage in the DNA, however, this was not representative in comparison to that produced by the diet itself.

Exposure to high levels of cholesterol in the WD diet promoted high levels of DNA damage (Figure 10.d), the additive damage caused by DEN in the WDD diet was not representative (Figure 10.h).

The intake of a high diet with lipids modifies the expression of enzymes related in cell cycle regulation.

We measured the protein content of each protein related with the cell cycle, as CDK2, CDK4, CDK6 and cyclin D1 involved in the transition of the G1 / S phase in the cell cycle, but we know that p21 blocks the cell cycle in this transition, joining the CDK4/cyclin D and CDK2/cyclin E complexes responsible for this transition. Our data suggest in WD and WDD diets a differential expression of these proteins, because we noticed an increase of CDK2, CDK4, CDK6 and cyclin D1, a decrease of the protein content of p21 after 30 days of treatment (Figure 11). Data suggest that the cells have been damaged and the repaired program is not efficient, even more the cell replication is ongoing, all these concerns are clear characteristics of a carcinogenic process.

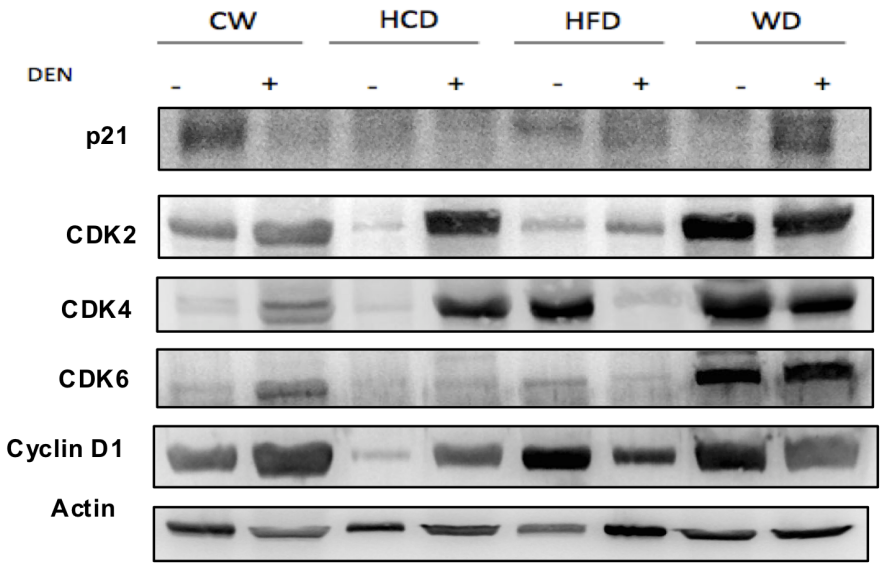

Ilustración 11.The Intake Of a High Diet With Lipids Modifies The Expression Of Enzymes Related In Cell Regulation. Western blot analysis of the main cell cycle regulator proteins p21, CDK2, CDK4, CDK6, Cyclin A and Cyclin D1. Images are representative of at least three independent experiments. Actin was used as housekeeping loading control. Images are representative for at least three independent experiments.

RESISTANCE TO CELL DEATH DUE TO CHOLESTEROL OVERLOAD.

The caspase-3 activity was measured by the caspase 3 synthetic fluorogenic tetrapeptide substrate. Results show in WD diet, with or without DEN, generated greater caspase-3 activity compared to control diet and all the remaining steatogenic diets (HCD and HFD) with or without DEN (Figure 11).

In addition, these results were confirmed by Western blot to measure the protein content of proteins related to apoptosis (Bax, BcL-xl). And finally, these data suggest that high concentration of cholesterol in the diet can confer greater resistance to cell death by apoptosis (Figure 12).

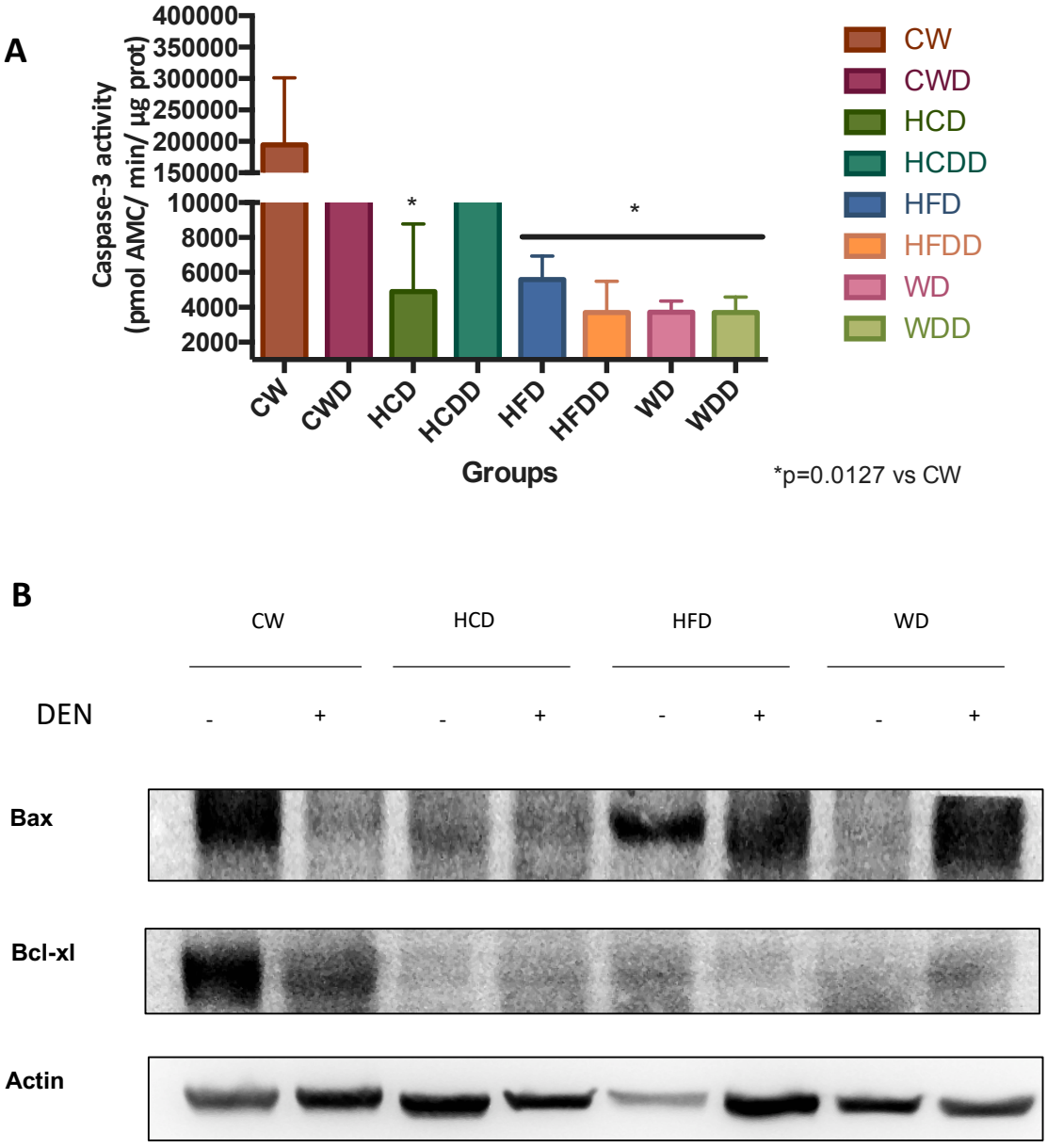

Ilustración 12. Cholesterol Overload Confers Resistance To Apoptosis. Caspase-3 activity **(A),** and Western blot analysis of Bax and Bcl-xl **(B).** Images are representative of at least three independent experiments. Actin was used as housekeeping loading control. Each column represents the mean ± SEM of at least three independent experiments *, $p \leq 0.0127$ vs. CW

And the last correlation that is important to mention is a correlation between cholesterol levels in the liver, that were measure by O-phthaldehyde assay, and the presence of cell death by caspase-3 activity. These results indicate how important is the concentration of the cholesterol in the cell regard to the cell death. The data shown a diet rich in cholesterol has a lower cell death due to apoptosis than a diet low in cholesterol (Figure 13).

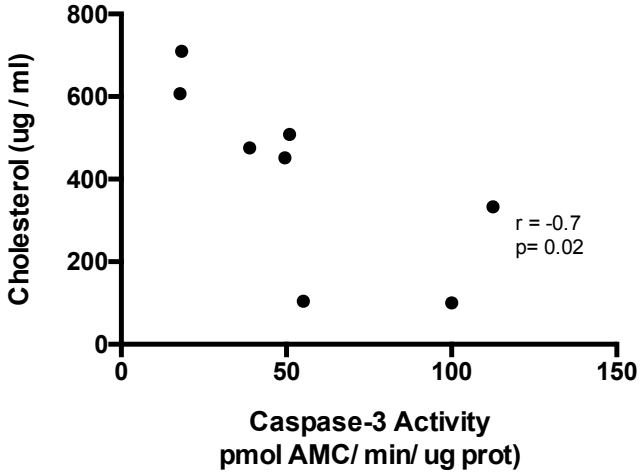

Ilustración 13. Cholesterol Strongly Potentiates Resistance To Cell Death. Correlation between the data of O-phthaldehyde and Caspase-3 activity expressed as ug/ml and pmol AMC/min/ ug prot. Tissue after 30 days of treatment can demonstrate the importance of the cholesterol like a stronger promoter of survival. Pearson correlation *P value < 0.02.*

DISCUSSION

NAFLD has been gaining relevance in the last decade as one of the main liver disorder affecting an increasing number of patients worldwide (Friedman S. L., 2018), It is known that the susceptibility of damage, due to an increased content of lipid, is driven by the kind of lipid rather than the amount (Montserrat Marí).

Although recently our group published that the consumption of a high cholesterol diet leads to HCC, in presence of DEN, and that these tumor exhibited characteristics of highly aggressiveness (Enriquez-Cortina et al.), the knowledge of the phenomena at the beginning of the process and what about the distinction by using different high lipids diets remained unaddressed.

In the present work we were focused to explore the characteristics of damage of different steatogenic diets, particularly addressing some of the key determinants in the initiation of a possible mutagenic process.

Our results strongly suggest that an aberrant cellular lipid concentration in the liver is an important factor in the first steps of the hepatic carcinogenesis, due to the unbalance in the cellular and physiological processes as show in the figure 3, the macroscopic liver elements present a remarkable change both in the coloration and size, as can be observed in the group HCD, which presents the typical characteristics of NAFLD, also in the groups HFD and WD these same changes are appreciated but in a more discreet way.

What could indicate that cholesterol is the main responsible for the imbalance in this organ? Although from a macroscopic point of view these changes are more noticeable in the HC group, different parameters of cell damage in the liver are not

altered, as shown in figure 4 where the levels of both ALT and AST are not altered when the comparison between groups is made.

Previously, it was reported that only the steatogenic diets cause an imbalance in the cellular redox state, where one of the main consequences of this change in the redox state is the oxidation of proteins, it is confirmed by correlating the increase in the content of cholesterol in the liver (Figure 7) with the increase in the oxidation of proteins (Figure 6), where the WDD group has the highest accumulation of cholesterol and protein oxidation. These changes in the redox state caused by cholesterol are the main inducers of DNA damage, as shown both in the western blot panel (figure 8) and in the histone H2AX immunofluorescence (Figure 9) where both the DEN treated or not groups, presented an increase in the protein content related to DNA repair, which indicates that Experimental groups in comparison with the control group activate this machinery more efficiently in the face of chronic damage. Even more, comet assay, another excellent marker for DNA damage (Figure 10), confirms the exacerbation of the genomic damage promoted by the high cholesterol liver content and DEN treatment, which allows a cell transformation and therefore the generation of tumors.

The extensive DNA damage triggers a mechanism named DNA damage response, which detects and repairs DNA by inducing cell cycle arrest to guarantee that only cells in good conditions can progress and proliferate, as show in Figure 11, where the groups that presented the greatest DNA damage, the proteins that regulate the cell cycle in a negative way are increased and the proteins. That indicates that activation of DNA damage response proteins can be increased during early stages of tumorigenesis. However in terms of carcinogenesis this scenario is good, because the apoptosis in all groups with a steatogenic diets and DEN is diminish

(Figure 12), which indicates that cell survival is increased.

The increase of cholesterol concentration correlates with the caspase-3 activity diminish, this data suggest strongly that the cholesterol is a factor really important in the transformation stabilization, due to the fact that the more cholesterol less activity of caspase 3 (Figure 13), this indicates that the cholesterol is of great importance in the cellular survival and later in the establishment of a cellular hepatocarcinoma.

CONCLUSION

Data confirm that cholesterol can play a relevant role not only in HCC progression but in cell transformation, by a mechanism related to the increment of ROS levels and apoptosis decrement at early times that lead to DNA damage and initiation of carcinogenesis. Our findings are positioning cholesterol cellular overload in the liver as a tumor promoter, inducing oxidative stress, impairing DNA damage repair system and promoting and accelerated tumor progression Hypercholesterolemia should be closely monitored in those patients with HCC risk factors.

REFERENCES

A., E. J. E. a. B. S. (2005). Genomic instability and cancer: Networks involved in response to DNA damage. *ELSEVIER*.

A. Federico, E. D. A., F. Borriello, G. Barra, A. G. Gravina, M. Romano, R. De Palma. (2012). Fat: A matter of disturbance for the immune system

. *World Journal Gastroenterology, 16*(38).

A. Schultz, D. A. C. M., I. Bringhenti, S. Barbosa-da- Silva, T. da Silva Faria and V. Souza-Mello. (2014). Nonalcoholic Steatohepatitis: Lessons from Different Diet-induced Animal Models. *Journal of Diabetes, Metabolic Disorders & Control, 1*(3).

A. Tessitore, G. C., F. Del Vecchio, A. Gaggiano, D. Verzella, M. Fischietti, V. Mastroiaco, A. Vetuschi, R. Sferra, R. Barnabei, D. Capece, F. Zazzeroni and E. Alesse. (2016). MicroRNA expression analysis in high fat diet-induced NAFLD-NASH-HCC progression: study on C57BL/6J mice. *BMC Cancer*.

A. Wree, A. K., G. Gerken and A. Canbay. (2011). Obesity Affects the Liver – The Link between Adipocytes and Hepatocytes. *Digestion*.

Andersen, J. U. M. a. J. B. (2015). Liver cancer oncogenomics: opportunities and dilemmas for clinical applications. *Hepat Oncol, 2*(1), 79-93.

Angela M Zivkovic, J. B. G., and Arun J Sanyal. (2007). Comparative review of diets for the metabolic syndrome- implications for nonalcoholic fatty liver disease. *Am J Clin Nutr, 86*, 285-300.

Bortolotti, K.-A. L. a. M. (2008). Role of dietary carbohydrates and macronutrients in the pathogenesis of nonalcoholic fatty liver disease. *Current Opinion in Clinical Nutrition and Metabolic Care, 11*(4), 477-482.

Bottai G., P. B., CalinG. A. & Santarpia L. (2014). Targeting the microRNA-regulating DNA damage/repair pathways in cancer. *Expert Opin. Biol. Ther.*

C. Gaemers I., M. S. J., Kunne C., Wallner C., van Werven J., Nederveen A., H. Lamers W. (2011). Lipotoxicity and steatohepatitis in an overfed mouse model for non-alcoholic fatty liver disease. *Biochimica et Biophysica Acta*

.

C. Oliveira L.S., A. S. D., Barbosa-da-Silva S., Mandarim-de-Lacerda C.A., B. Aguila M. (2014). The inflammatory profile and liver damage of a sucrose-rich diet in mice. *Journal of Nutritional Biochemistry, 25*

.

Clavijo-Cornejo, D., Enriquez-Cortina, C., Lopez-Reyes, A., Dominguez-Perez, M., Nuno, N., Dominguez-Meraz, M., . . . Gomez-Quiroz, L. E. (2013). Biphasic regulation of the NADPH oxidase by HGF/c-Met signaling pathway in primary mouse hepatocytes. *Biochimie, 95*(6), 1177-1184.

Cortez-Pinto, M. V. M. a. H. (2016). Diet, Microbiota, Obesity, and NAFLD: A Dangerous Quartet. *Int J Mol Sci, 17*(4), 481.

Cronin K. A., L. A. J., Scott S., Sherman R. L. , Noone A. M., Howlader N., Henley S. J., Anderson R. N., Firth A. U., Ma J., Kohler B. A. and Jemal A. (2018). Annual Report to the Nation on the Status of Cancer, Part I: National Cancer Statistics. *Cancer Res*.

D. Clavijo-Cornejo, C. E.-C., A. López-Reyes, M.Domínguez-Pérez, N. Nuño, M. Domínguez-Meraz, L. Bucio, V. Souza, V. M. Factor, S. S. Thorgeirsson, M. C. Gutiérrez-Ruiz, & Gómez-Quiroz, a. L. E. (2013). Biphasic Regulation of the NADPH Oxidase by HGF/c-Met

Signaling Pathway in Primary Mouse Hepatocytes. *Biochimie*.

Domínguez-Pérez M., N. o.-L. m. N., Clavijo-Cornejo D., Luna-López A., Souza V., Bucio L., U. Miranda R., Muñoz L., Gomez-Quiroz L. E., Uribe-Carvajal S. and Gutiérrez-Ruiz M. C. (2016

). Hepatocyte Growth Factor Reduces Free Cholesterol-Mediated Lipotoxicity in Primary Hepatocytes by Countering Oxidative Stress. *Oxidative Medicine and Cellular Longevity*.

Dominique., P. (2007). Role of mitochondria in non-alcoholic fatty liver disease. *Journal of Gastroenterology and Hepatology, 22*

.

Dufour., C. M. a. J. F. (2016). The story of HCC in NAFLD: from epidemiology, across pathogenesis, to prevention and treatment. *Liver Int, 36*(3), 317-324.

E. Kuntz, H.-D. K. (2002). Hepatology. Principles and Practice. In Springer (Ed.), (Vol. 2).

Enriquez-Cortina, C., Bello-Monroy, O., Rosales-Cruz, P., Souza, V., Miranda, R. U., Toledo-Perez, R., . . . Gomez-Quiroz, L. E. (2017). Cholesterol overload in the liver aggravates oxidative stress-mediated DNA damage and accelerates hepatocarcinogenesis. *Oncotarget, 8*(61), 104136-104148.

F. M. Marcello Dallio, C. M. D. C., A. G. Gravina, G. Viscardi, C. Loguercio, F. Ciardiello and A. Federico. (2018). Carcinogenesis as a result of multiple Inflammatory and oxidative hits: a comprehensive review from tumor microenvironment to gut microbiota. *NEOPLASIA, 20*, 721-733.

Friedman S. L., N.-T. B. A., Rinella M. and Sanyal A. J. (2018). Mechanisms of NAFLD development and therapeutic strategies. *Nature Medicine*.

Garc´ıa-Ruiz C., R. V., Baulies A., and Fernández-Checa J. C. (2016). Mitochondrial Cholesterol and the Paradox in Cell Death. *Experimental Pharmacology*.

Hernandez, I., Dominguez-Perez, M., Bucio, L., Souza, V., Miranda, R. U., Clemens, D. L., . . . Gutierrez-Ruiz, M. C. (2015). Free fatty acids enhance the oxidative damage induced by ethanol metabolism in an in vitro model. *Food Chem Toxicol, 76*, 109-115.

Janie L. Baratta, A. N., Bryan Lopez, Natasha Kasabwalla, Kenneth J. Longmuir, and Richard T. Robertson. (2009). Cellular Organization of Normal Mouse Liver: A Histological, Quantitative Immunocytochemical, and Fine Structural Analysis

. *Histochem Cell Biol.*

K., C.-H. J. a. M. C. (2017). Impact of High-Carbohydrate Diet on Metabolic Parameters in Patients with Type 2 Diabetes. *Nutrients*.

K. J. Thompson, R. Z. S., D. A. Iannitti, I. H. McKillop and D. Sindram. (2013). Diet-induced obesity and ethanol impair progression of hepatocellular carcinoma in a mouse mesenteric vein injection model. *Surg Endosc*.

Kew, M. C. (2014). Hepatic iron overload and hepatocellular carcinoma. *Liver Cancer, 3*(1), 31-40.

Khairy MA Zoheir, A. A. A.-R., Gamaleldin I Harisa, Abdelkader E Ashour, Sheikh Fayaz Ahmad, Sabry M Attia, Saleh A Bakheet, Hala E Abdel-Hamied, Adel R Abd-Allah, Ashok Kumar. (2015). Gene expression of IQGAPs and Ras families in an experimental mouse model for hepatocellular carcinoma- a mechanistic study of cancer progression. *Int J Clin Exp Pathol, 8*, 8821-8831.

Kristine Pelton, M. R. F. a. K. R. S. (2012). Cholesterol and prostate cancer. *Curr Opin Pharmacol, 12*(6), 751-759.

L. Diesen D., M. a. C. K. P. (2011). Nitric Oxide and Redox Regulation in the Liver: Part II Redox biology in Pathologic Hepatocytes and Implications for intervention. *NIH Public Access*.

L. E. Gomez-Quiroz, D. S., Yun-Han Lee, M. Kitadea, T. Gaiserd, M. Gillena, Seung-Bum Lee, M. C. Gutierrez-Ruiz, E. A. Conner, V. M. Factor, Snorri S. Thorgeirssona and J. U. Marquardt. (2016). Loss of c-Met signaling sensitizes hepatocytes to lipotoxicity and induces cholestatic liver damage by aggravating oxidative stress. *Toxicology*.

L. Sahuquillo, A. L. p. (2011). Estudio de la función hepática: magnitudes bioquímicas. *SEQC*.

L. Vena, J. W. a. G. M. W. (1996). N-Nitrosodiethylamine Mechanistic Data and Risk Assessment: Bioactivation, DNA-Adduct Formation, Mutagenicity, and Tumor Initiation. *Pharmacol. Ther., 71*, 57-81.

López-Islas A., C.-H. V., Pérez-Aguilar B., Palestino-Domínguez M., Souza V., U. Miranda R., Bucio L., Gómez-Quiroz L. E. and Gutiérrez-Ruiz M. C. (2016). Cholesterol Enhances the Toxic Effect of Ethanol and Acetaldehyde in Primary Mouse Hepatocytes. *Oxidative Medicine and Cellular Longevity*.

M., G. M. a. B. K. (2014). The DNA Damage Response: Implications for Tumor Responses to Radiation

and Chemotherapy. *The Annual Review of Medicine*.

M. Marı´, F. C., A. Colell, A. Morales, J. Caballeria, A. Fernandez, C. Enrich, J. C. Fernandez-Checa and C. Garcı´a-Ruiz. (2006). Mitochondrial free cholesterol loading sensitizes to TNF- and Fas-mediated steatohepatitis. *CELL METABOLISM, 4*.

María Concepción Gutiérrez Ruiz, M. D. n. P. r., Sandra Rodríguez González,, & Natalia Nuño Lámbarri, C. L. R. y. L. E. G. m.-Q. (2012). La dieta alta en colesterol altera el proceso reparador del factor de crecimiento de hepatocitos. *Gaceta Médica de México, 148*(3), 236-242.

María Guadalupe Castro-Martínez, D. Z. B.-L., & Jesús Cenobio Ramírez-Martínez, J. E.-d. l. P. (2012). Prevalencia de hígado graso no alcohólico en individuos con síndrome metabólico. *Cirugía y Cirujanos, 80*(2), 128-133.

Mazzocca A., F. G., Misciagna G., I. Carr B. (2016). A systemic evolutionary approach to cancer: Hepatocarcinogenesis

as a paradigm. *MEDICAL HYPOTHESIS*.

Messier C., W. K., Liang J., Du L. and Puissant D. (2007

). The effects of a high-fat, high-fructose, and combination diet on learning, weight, and glucose regulation in C57BL/6 mice. *ELSEVIER, 178*

.

Montserrat Marí, F. C., Anna Colell, Albert Morales, Juan Caballeria, Anna Fernandez, Carlos Enrich, Jose´ C. Fernandez-Checa, and Carmen García-Ruiz. (2006). Mitochondrial free cholesterol loading sensitizes to TNF- and Fas-mediated steatohepatitis. *Cell Metab, 4*(3), 185-198.

Moreira, C. C. a. P. I. (2018). Oxidative Stress: A Major Player in Cerebrovascular Alterations Associated to Neurodegenerative Events. *Frontiers in Physiology*.

Murai, T. (2012). The role of lipid rafts in cancer cell adhesion and migration. *Int J Cell Biol, 2012*(2012), 763283.

N. Aravalli R., J. S. C., and N. K. Cressman E. (2008). Molecular Mechanisms of Hepatocellular Carcinoma

.

Nuño-Lámbarri N., D. n.-P. r. M., Baulies-Domenech A., Monte M. J., G. Marin, J. J., Rosales-Cruz P., Souza V., U. Miranda R., Bucio L., Montalvo-Jave E. E., Gutiérrez-Ruiz M. C., García-Ruiz C., Fernández-Checa J. C. and Gomez-Quiroz L. E. (2016). Liver Cholesterol Overload Aggravates Obstructive Cholestasis by Inducing Oxidative Stress and Premature Death in Mice. *Oxidative Medicine and Cellular Longevity*.

P. Jegatheesan, S. B., K. Freese, A.-J. Waligora-Dupriet, E. Nubret, M.-J. Butel, I. Bergheim and J.-P De Bandt. (2016). Preventive effects of citrulline on Western diet-induced non-alcoholic fatty liver disease in rats. *British Journal of Nutrition*.

R. N. Aravalli, E. N. K. C. a. C. J. S. (2012). Cellular and molecular mechanisms of hepatocellular carcinoma: an update. *Arch Toxicol*.

R. Pascale M., F. C. D., Feo F. (2016). Sulfatase 1: a new Jekyll and Hyde in hepatocellular carcinoma? *Translational Gastroenterology and Hepatology*.

Rudel, L. L., & Morris, M. D. (1973). Determination of cholesterol using o-phthalaldehyde. *J Lipid Res, 14*(3), 364-366.

S. Reuter, S. C. G., M. M. Chaturvedi and B. B. Aggarwal. (2010). Oxidative stress, inflammation, and cancer: How are they

linked? *Free Radic Biol Med*.

Sibulesky, L. (2013). Anatomía normal del hígado. *Clinical Liver Disease, 2*.

T. Teratani, K. T., T SUZUKI, T. OSHIKAWA, H. YOKOYAMA, K. SHIMAMURA, S. TOMINAGA, S. HIROI, R. IRIE, Y. OKADA,C. KURIHARA, H. EBINUMA, H. SAITO, R. HOKARI, K. SUGIYAMA, T. KANAI, S. MIURA, and T. HIBI. (2012). A High-Cholesterol Diet Exacerbates Liver Fibrosis in Mice via Accumulation of Free Cholesterol in Hepatic Stellate Cells

. *Gastroenterology*.

Wanga H., Z. X., Tenga L., Legerskib R. J. (2015). DNA damage checkpoint recovery and cancer development. *ELSEVIER*.

Xiao-Yan Duan, Q. P., Shi-Yan Yan, Wen-Jin Ding, Jian-Gao Fan and L. Qiao. (2014). High-saturate-fat diet delays initiation of diethylnitrosamine-induced hepatocellular carcinoma. *BMC Gastroenterology, 14*.

YaryginK.N., K. I. V. a. (2017). Cellular Mechanisms of Liver Regeneration and Cell-Based Therapies of Liver Diseases. *BioMed Research International*.

Zelber-Sagi, S., Ratziu, V., & Oren, R. (2011). Nutrition and physical activity in NAFLD: an overview of the epidemiological evidence. *World J Gastroenterol, 17*(29), 3377-3389.

Zhao-Shan N., X.-J. N., Wen-Hong W. (2016). Genetic alterations in hepatocellular carcinoma: An update. *World Journal of Gastroenterology*.

Zheng-Jie X., J.-G. F., Xiao-Dong D., Liang Q. and Guo-Liang W. (2010). Characterization of High-Fat, Diet-Induced, Non-alcoholic Steatohepatitis with Fibrosis in Rats. *Dig Dis Sci*.

Zhishi Yang, W. Q., Yao Chen, Bo Yuan, Xiaoling Song, Bibo Wang, Feng Shen, Jing Fu, Hongyang Wang. (2018). Cholesterol inhibits hepatocellular carcinoma invasion and metastasis by promoting CD44 localization in lipid rafts.

Cancer Letters

.

Zou Y., L. J., Lu C., Wang J., Ge J., Huang Y., Zhang L. and Wang Y. (2006). High-fat emulsion-induced rat model of nonalcoholic steatohepatitis. *ELSEVIER*.